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Edwardsiella tarda Eta1, an *In Vivo*-Induced Antigen That Is Involved in Host Infection

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Edwardsiella tarda*, a Gram-negative bacterium, is a severe fish pathogen that can also infect humans. In this study, we identified, via *in vivo*-induced antigen technology, an *E. tarda* antigen, Eta1, and analyzed its function in a Japanese flounder (*Paralichthys olivaceus*) model. Eta1 is composed of 226 residues and shares homology with putative bacterial adhesins. Quantitative real-time reverse transcriptase (RT)-PCR analysis indicated that when cultured *in vitro*, *eta1* expression was growth phase dependent and reached maximum at mid-logarithmic phase. During infection of flounder lymphocytes, *eta1* expression was drastically increased at the early stage of infection. Compared to the wild type, the *eta1*-defective mutant, TXeta1, was unaffected in growth but exhibited attenuated overall virulence, reduced tissue dissemination and colonization capacity, and impaired ability to invade flounder lymphocytes and to block the immune response of host cells. The lost virulence of TXeta1 was restored when a functional *eta1* gene was reintroduced into the strain. Western blot and immunodetection analyses showed that Eta1 is localized to the outer membrane and exposed on the surface of *E. tarda* and that recombinant Eta1 (rEta1) was able to interact with flounder lymphocytes. Consistent with these observations, antibody blocking of Eta1 inhibited *E. tarda* infection at the cellular level. Furthermore, when used as a subunit vaccine, rEta1 induced strong protective immunity in flounder against lethal *E. tarda* challenge. Taken together, these results indicate that Eta1 is an *in vivo*-induced antigen that mediates pathogen-host interaction and, as a result, is required for optimal bacterial infection.

Edwardsiella tarda is a Gram-negative, motile, rod-shaped bacterium of the family *Enterobacteriaceae*. It is a pathogen with a broad host range that includes fish, birds, reptiles, and humans. *E. tarda* infection of humans can cause gastrointestinal symptoms and other disorders, such as myonecrosis and wound infections (9, 21). In aquaculture, *E. tarda* is considered a severe pathogen because of its ability to infect a wide range of marine and freshwater fish, including Japanese flounder (*Paralichthys olivaceus*), turbot (*Scophtalmus maximus*), tilapia (*Oreochromis niloticus*), eels (*Anguilla japonica*), and channel catfish (*Ictalurus punctatus*) (17, 37). *E. tarda* infection of fish may lead to the development of a systemic disease called edwardsiellosis, which in Japanese flounder is often manifested in hemorrhage, septicemia, skin lesions, and necrosis of liver, gut, and kidney (19, 26).

Functional studies have identified a number of *E. tarda* systems and factors that are involved in pathogenesis (14, 24). It is known that *E. tarda* enters host fish through the gastrointestinal tract, the gills, and the body surface and is able to resist the immune defense mediated by host complements and phagocytes (16, 23). Studies in fish models, such as blue gourami and flounder, have indicated that effective *E. tarda* infection requires two virulence-associated systems, i.e., type III (T3SS) and type VI (T6SS) secretion systems, which are vital to *E. tarda* invasion and intracellular replication (22, 35, 42, 46). Other factors known to be involved in *E. tarda* pathogenicity are hemolysin (8, 29, 40, 41), catalase (25), superoxide dismutase (4), heat shock proteins (5, 6), Dps (47), and the LuxS/AI-2 quorum-sensing system (43, 44). A recent study based on genomic subtractive hybridization identified an autotransporter adhesin, AIDA, in atypical strains of fish-pathogenic *E. tarda* (27). However, the precise functions of these virulence factors during *E. tarda* infection and the mechanism of disease occurrence are unclear.

In previous studies, we have utilized the approach of *in vivo*-induced antigen technology (IVIAT) to discover potential virulence factors associated with *E. tarda* infection (10, 11). In the current study, we examined the biological properties and function of a putative adhesin, Eta1, identified via IVIAT. We found that expression of *eta1* was drastically enhanced during infection of host cells and that mutation of *eta1* attenuates *E. tarda* virulence at the cellular and tissue levels. In addition, we also observed interaction between recombinant Eta1 and host lymphocytes, and blocking of this interaction inhibits the infectivity of *E. tarda*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* BL21, BL21(DE3), and DH5α were purchased from Tiangen (Beijing, China). *E. coli* S17-1λpir was purchased from Biomedal (Sevilla, Spain). *E. tarda* TX01 was isolated from the kidneys of diseased flounder (31) and is naturally resistant to rifampin. All strains were grown in Luria-Bertani broth (LB) (28) at 37°C (for *E. coli*) or 28°C (for *E. tarda*). Where indicated, appropriate antibiotics were supplemented as follows: chloramphenicol, 30 μg/ml; polymyxin B, 100 μg/ml; and rifampin, 20 μg/ml. All antibiotics were purchased from Sangon, Shanghai, China.

Fish used for infection study. Japanese flounder (average weight, 12.1 g) were purchased from a local fish farm and maintained at 22°C in aerated seawater. The fish were acclimatized in the laboratory for 2 weeks

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before experimental manipulation and were maintained and euthanized as reported previously (11).

Cloning and sequence analysis of *eta1*. *eta1* was cloned with the IVIAT technology, as described previously (11). The putative amino acid sequence of Eta1 was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the conserved domain search program of NCBI. The signal peptide search was performed with SignalP 3.0. The theoretical molecular mass and theoretical isoelectric point were predicted using EditSeq in the DNASTAR software package (Madison, WI).

Preparation of flounder HK lymphocytes. To prepare flounder head kidney (HK) lymphocytes, HK was removed from three flounder (average weight, 796 g) under aseptic conditions and washed 3 times with phosphate-buffered saline (PBS) containing 100 U of penicillin and streptomycin (Solarbio, Beijing, China). The tissue was passed through a metal mesh, and the cell suspension was overlaid on 1.070 and 1.077 discontinuous density of Percoll solution (Solarbio, Beijing, China). After centrifugation at 300 × *g* for 30 min at 4°C, the interface fraction containing lymphocytes was collected and washed 3 times with PBS. The lymphocytes were resuspended in L-15 medium (Thermo Scientific HyClone, Beijing, China), and the viability of the cells was examined by the trypan blue dye exclusion method. The cells were adjusted to 2×10^5 viable cells/ml in L-15, distributed into 96-well tissue culture plates, and cultured at 22°C.

qRT-PCR analysis of *eta1* expression. To examine *eta1* expression in LB medium, *E. tarda* TX01 was grown in LB medium at 28°C to optical densities at 600 nm (OD_{600}) of 0.25, 0.5, 0.9, 1.25, and 1.5. The cells were harvested by centrifugation and used for total RNA extraction with an HP Total RNA kit (Omega Bio-Tek). One microgram of total RNA was used for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was carried out as described previously (48) in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (TaKaRa, Dalian, China) with 16S rRNA as a reference, as described previously (43). The PCR primers for *eta1* were RT-F1 (5′-GTCAGGAAAGTGATTGGTGGC-3′) and RT-R1 (5′-TCCAATACTCCTTCTCGGTGC-3′). The PCR primers for the 16S rRNA gene were 16S933F (5′-GCACAAGCGGTGGAGCAT-3′) and 16SRTR2 (5′-GCCATGCAGCACCTGTCTC-3′). The PCR analysis was performed four times. All data are given in terms of relative mRNA expressed as means plus or minus standard errors of the means (SE).

To examine *eta1* expression during infection of host cells, TX01 was cultured in LB medium to an OD_{600} of 0.8; the cells were collected by centrifugation and resuspended in PBS to 10^7 CFU/ml. For cellular infection, TX01 (10^6 CFU/well) was added to HK lymphocytes cultured in 96-well culture plates (10^5 cells/well) or, as controls, to 96-well culture plates containing L-15 medium without lymphocytes. The plates were incubated at 25°C for 0 h, 0.5 h, 1 h, 2 h, 3 h, and 5 h at 25°C. After the incubation, the plates containing lymphocytes were washed 3 times with PBS to remove unattached bacteria, and the cells were then used for total RNA extraction and qRT-PCR analysis of *eta1* expression as described above. The TX01 in the control plates was collected by centrifugation and used for total RNA extraction and qRT-PCR analysis as described above.

Plasmid and strain construction. To construct pEtEta1, which expresses *eta1*, *eta1* was amplified by PCR with primers 81F1 (5′-CGCATATGGAGAATAATTTACTCGGCGA-3′ [underlined sequence, NdeI site]) and 81R1 (5′-CTCGAGTTCCCCCACTTCCTTATTTCT-3′ [underlined sequence, XhoI site]); the PCR products were ligated with the T-A cloning vector pBS-T (Tiangen, Beijing, China), resulting in pBSEta1, which was digested with NdeI/XhoI, and the fragment containing *eta1* was retrieved and inserted into pET258 (32) between NdeI and XhoI sites. To construct the *eta1*-defective strain TXeta1, an internal fragment of *eta1* (positions 67 to 303) was generated by PCR with primers KOF1 (5′-GGATCCGCACAATCCTAGAATCAGTA-3′ [underlined sequence, BamHI site]) and KOR1 (5′-GGATCCTAACTTCCATTGGCTATCT-3′ [underlined sequence, BamHI site]). The PCR products were inserted into the suicide plasmid pDM4 (18) at the compatible BglII site, resulting in pDMEta1. S17-1λpir was transformed with pDMEta1, and the transformants were conjugated with TX01 as described previously (43). The transconjugants were selected on LB agar plates supplemented with polymyxin B and chloramphenicol. One of the selected mutants was named TXeta1. Mutation of *eta1* in TXeta1 was confirmed by PCR analysis and subsequent sequencing of the PCR products and by Western blot analysis. To construct TXeta1C, pBSEta1 was digested with NdeI/XhoI, and the fragment containing *eta1* was retrieved and inserted into the expression plasmid pBT3 (45) between NdeI and XhoI sites, resulting in pBT3Eta1. TXeta1 was transformed with pBT3Eta1 via electroporation, and the transformants were selected on LB agar plates supplemented with ampicillin (resistance marker of pBT3Eta1). One of the transformants was named TXeta1C.

Purification of recombinant protein and preparation of antiserum. Recombinant Eta1 (rEta1) was purified as follows. *E. coli* BL21(DE3) was transformed with pEtEta1; the transformants were cultured in LB medium at 37°C to mid-logarithmic phase, and expression of *eta1* was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After growing at 37°C for an additional 5 h, the cells were harvested by centrifugation, and His-tagged rEta1 was purified under native conditions using nickel-nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ) as recommended by the manufacturer. The purified protein was dialyzed in PBS and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA). The concentrated protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of the protein was determined using the Bradford method (3) with bovine serum albumin as the standard. Preparation of rat anti-rEta1 serum and determination of the titers and specificities of anti-rEta1 antibodies were performed as described previously (32).

Determination of the subcellular location of Eta1. (i) Western blotting. TX01 and TXeta1 were grown in LB medium to an OD_{600} of 0.5 and harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. Extracellular proteins were prepared by concentrating the supernatant 100 times using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA). Cytoplasmic and periplasmic proteins were prepared as reported previously (45). To prepare outer membrane proteins, the cell pellet was washed 3 times with PBS and resuspended in ice-cold buffer I (50 mM Tris · Cl, 1 mM EDTA, pH 7.5). The cells were broken by sonication on ice, followed by centrifugation as described above. The supernatant was collected and centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA, 10 mM dithiothreitol (DTT), and 2% Sarkosyl and incubated at 37°C for 1 h with mixing every 5 min. The suspension was centrifuged at 100,000 × *g* for 1 h at 4°C, and the pellet was resuspended in buffer I. The concentration of the purified proteins was determined using the Bradford method (3). Western blotting was performed as follows. The proteins were subjected to electrophoresis in 0.1% SDS-12% polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were blocked for 3 h with 2% (wt/vol) bovine serum albumin (BSA)-0.1% (wt/vol) azide-PBS containing 0.05% (vol/vol) Tween 20 (PBS-T), followed by washing with PBS-T three times. The blocked membranes were incubated for 2 h with antiserum against rEta1 or antiserum against recombinant LuxS (rLuxS), an intracellular protein of *E. tarda* (43). The antisera were diluted in PBS-T containing 2% (wt/vol) BSA to 1/500. The membranes were washed three times with PBS-T. Primary antibodies were detected by incubating the membranes with anti-rat IgG-horseradish peroxidase (HRP) antibody (Tiangen, Beijing, China) (1/1,000 dilution in PBS-T) for 1 h in PBS-T–BSA. The membranes were washed three times with PBS-T, and the antibody was detected with the HRP-DAB kit (Tiangen, Beijing, China). The color reaction was stopped after 30 min by several washes with water, and the membranes were air dried.

(ii) immunofluorescence study. TX01 and TXeta1 were cultured in LB medium to an OD_{600} of 0.5. The cells were washed and diluted in PBS to 10^8 CFU/ml. Three hundred microliters of bacterial suspension was dropped onto a glass slide, and the slide was incubated at room temperature for 4 h. The cells were fixed with 4% paraformaldehyde for 10 min, followed by washing 3 times with PBS. Rat anti-rEta1 serum, preimmune serum, and rat anti-rLuxS serum were diluted 500 times in PBS, and 300 μl diluted serum was added to the bacterial cells on the slide. The slide was incubated at 30°C for 2 h and washed 3 times with PBS. Fluorescein isothiocyanate (FITC)-labeled goat anti-rat IgG (Bioss, Beijing, China) was diluted 1,000 times, and 300 μl of the diluted antibody was added to the slide. The slide was incubated and washed as described above and observed with a fluorescence microscope (Nikon E800; Japan).

Tissue dissemination and mortality analysis. For tissue dissemination analysis, TXeta1, TXeta1C, and TX01 were cultured in LB medium to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in seawater. Japanese flounder were divided randomly into four groups ($n = 35$) and immersed in seawater containing or not containing (control) 10^8 CFU/ml of TXeta1, TXeta1C, or TX01. The blood, liver, kidney, and spleen were taken aseptically from the fish (five from each group) at 12 h postinfection. Bacterial recovery from the tissues was determined as reported previously (43). The rest of the infected fish were monitored daily for mortality for 20 days.

***E. tarda* infection of host cells and bacterial-recovery analysis.** To examine *E. tarda* infection, TXeta1 and TX01 were cultured in LB medium to an OD_{600} of 0.8. The cells were washed in PBS and resuspended in L-15 to 10^7 CFU/ml. HK lymphocytes cultured in 96-well cell culture plates were supplemented with TXeta1 or TX01 (10^6 CFU/well). The plates were incubated at 30°C for 1 h, 2 h, 3 h, and 5 h, followed by washing 3 times with PBS. The cells were incubated with penicillin and streptomycin (100 U) for 2 h at 25°C to kill extracellular bacteria. Bacterial recovery was determined as reported previously (43). The experiment was performed four times.

qRT-PCR analysis of the expression of immune genes in *E. tarda*-infected flounder. Flounder were infected with TXeta1 or TX01 as described above for tissue dissemination analysis. Total RNA was extracted from HK at 1 h and 4 h postinfection with an HP Total RNA kit (Omega Bio-Tek) and used for qRT-PCR as described above with 18S rRNA and ubiquitin-conjugating enzyme (UBCE) as internal references (48). The primers used to amplify the genes of interleukin 1β (IL-1β), IL-6, IL-8, gamma interferon (IFN-γ), tumor necrosis factor-alpha (TNF-α), interferon-induced Mx protein (Mx), major histocompatibility complex (MHC) class Iα, CD40, and CD8α have been reported previously (12).

Interaction between rEta1 and flounder lymphocytes. Flounder HK lymphocytes ($\sim 10^5$) were placed onto a glass slide, and the slide was incubated at room temperature for 4 h. The cells were fixed with 4% paraformaldehyde for 10 min, followed by washing 3 times with PBS. Three hundred microliters of 10 μM rEta1 or recombinant EthR, an intracellular protein of *E. tarda* (40), was added to the lymphocytes, followed by incubation at 30°C for 1 h. After the incubation, the slide was washed 3 times with PBS and supplemented with 300 μl of 1/1,000 mouse anti-His antibody (Bioss, Beijing, China) or serum from an unvaccinated mouse (negative-control serum). The slide was incubated at 30°C for 2 h and washed as described above, followed by adding 300 μl of 1/1,000 FITC-labeled goat anti-mouse IgG (Bioss, Beijing, China). The slide was incubated and washed as described above and observed with a fluorescence microscope (Nikon E800, Japan). The ability of anti-His antibody to react with rEthR was demonstrated by Western immunoblot analysis (see Fig. S1 in the supplemental material).

Effect of anti-Eta1 antibodies on *E. tarda* infection. TX01 was diluted in PBS to 10^8 CFU/ml. Anti-Eta1 serum and preimmune serum were diluted 1,000 times, 500 times, and 100 times in PBS. TX01 suspension was mixed with each of the diluted sera at an equal volume, followed by incubation at room temperature for 0.5 h. As a control, TX01 was mixed and incubated in the same manner with an equal volume of PBS. After the incubation, the TX01-containing mixture was added to flounder HK lymphocytes cultured in 96-well cell culture plates as described above (5×10^5 CFU bacterial cells/well). The plates were incubated at 30°C for 4 h, followed by washing 3 times with PBS. The number of bacterial cells associated with lymphocytes was determined as described above by plate count. The assay was performed four times.

Vaccination. rEta1 was resuspended in PBS to a concentration of 200 μg/ml and mixed at an equal volume with aluminum hydroxide as described previously (12). As a control, PBS was also mixed similarly with aluminum hydroxide without rEta1 (PBS-aluminum hydroxide). Flounder (as described above) were divided randomly into two groups ($n = 35$) and injected intraperitoneally (i.p.) with 100 μl of rEta1 suspension or PBS-aluminum hydroxide (control). At 1 month postvaccination, 25 fish were taken from each group and challenged via i.p. injection with 100 μl TX01 that had been cultured as described above to an OD_{600} of 0.8, washed with PBS, and resuspended in PBS to 2×10^6 CFU/ml. The fish were monitored for mortality for a period of 20 days. Dying fish (5 from the PBS-injected group and 3 from the rEta1-vaccinated group) were examined for bacterial recovery from liver, kidney, and spleen as described above (see "Tissue dissemination and mortality analysis"). The relative percent survival (RPS) was calculated according to the following formula: RPS = [1 − (percent mortality in vaccinated fish/percent mortality in control fish)] × 100 (1). The vaccination trial was conducted in duplicate.

ELISA. Sera were taken from vaccinated fish and control fish at 4 weeks postvaccination and diluted 20-fold in PBS. Serum antibody against rEta1 was determined by enzyme-linked immunosorbent assay (ELISA) analysis as described previously (34).

Statistical analysis. Statistical analysis was performed by analysis of variance (ANOVA) of the SPSS 15.0 package (SPSS Inc., Chicago, IL). In all cases, the significance level was defined as a P value of < 0.05.

Nucleotide sequence accession number. The nucleotide sequence of *eta1* has been deposited in the GenBank database under accession number JN802138.

RESULTS

Characterization of the sequence of Eta1. The deduced amino acid sequence of Eta1 contains 226 amino acid residues and is predicted to have a molecular mass of 23.6 kDa and an isoelectric point of 4.7. *In silico* analysis identified no apparent conserved domain in Eta1. No putative signal peptide sequence was found in Eta1. BLAST analysis showed that Eta1 shares the highest sequence identities (60% and 53%, respectively) with the C-terminal 217-residue region of the putative adhesins of *Erwinia* sp. strain Ejp617 (GenBank accession no. ADP11523) and *Escherichia coli* strain CL3 serovar O113:H21 pathogenicity island I (GenBank accession no. AAQ19127). These data suggest the possibility that Eta1 is a novel type of adhesin.

***eta1* expression under *in vitro* conditions and during infection.** To examine *eta1* expression under *in vitro* conditions, *E. tarda* TX01 was cultured in LB medium to OD_{600} of 0.25, 0.5, 0.9, 1.25, and 1.5, and *eta1* expression was determined by qRT-PCR. The results showed that *eta1* expression peaked at an OD_{600} of 0.5 and dropped afterward, reaching the lowest level at OD_{600} of 1.25 and 1.5 (Fig. 1A). Compared to the expression level at an OD_{600} of 0.25, which, for convenience of comparison, was set as 1, the expression levels at OD_{600} of 0.5, 0.9, 1.25, and 1.5 were 2.3-, 0.7-, 0.5-, and 0.5-fold, respectively. To examine *eta1* expression during infection, flounder HK lymphocytes were infected with TX01, and *eta1* expression was determined by qRT-PCR at 0 h, 1 h, 2 h, 3 h, and 5 h postinfection. The results showed that, compared to *eta1* expression before infection (0 h), *eta1* expression after infection was significantly induced and reacheda maximum (69-fold) at 1 h

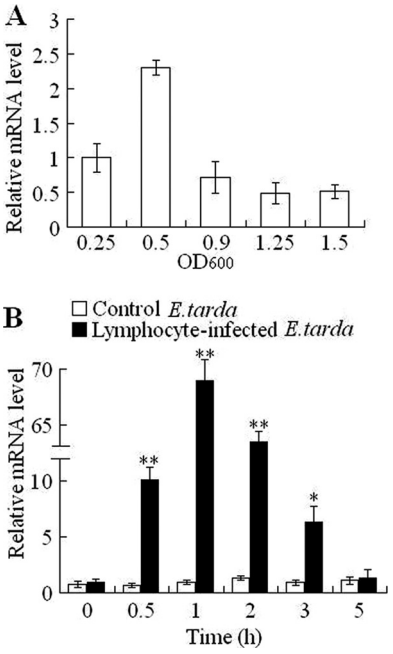

FIG 1 Expression of *eta1* under different conditions. (A) *E. tarda* TX01 was cultured in LB medium to OD_{600} of 0.25, 0.5, 0.9, 1.25, and 1.5, and expression of *eta1* was determined by qRT-PCR. The expression level at an OD_{600} of 0.25 was set as 1. (B) TX01 was incubated with or without (control) flounder head kidney lymphocytes for 0 h, 0.5 h, 1 h, 2 h, 3 h, and 5 h, and *eta1* expression in lymphocyte-associated TX01 and control TX01 was determined by qRT-PCR. The expression level of *eta1* at 0 h of infection was set as 1. The data are presented as means ± SE (*n* [the number of replicated experiments] = 4). *, $P < 0.05$; **, $P < 0.01$.

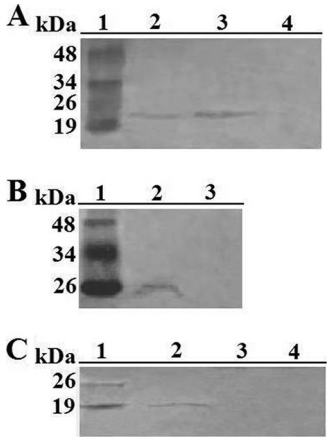

FIG 2 Western blot analysis to determine the subcellular localization of Eta1. (A) Proteins were prepared from the cytoplasm, periplasm, and extracellular (lanes 2, 3, and 4, respectively) fractions of *E. tarda* TX01 and subjected to Western blot analysis using anti-rEta1 antibodies. (B) Outer membrane proteins of TX01 (lane 2) and TXeta1 (lane 3) were blotted with anti-rEta1 antibodies. (C) Cytoplasmic (lane 2) and outer membrane (lane 3) proteins of TX01 were blotted with anti-LuxS antibodies. Lane 1 of all panels, protein marker.

postinfection (Fig. 1B). The *eta1* expression level dropped as infection progressed but remained significantly higher at 2 h and 3 h postinfection than before infection. In contrast, in TX01 incubated under the same conditions but without host cells, no apparent alteration in *eta1* expression was observed at all the examined time points.

Subcellular localization of Eta1. To examine the subcellular localization of Eta1, TX01 was cultured in LB medium to an OD_{600} of 0.5, which, as shown above, is the growth point at which a relatively high level of *eta1* expression was observed. Proteins were prepared from the cytoplasm, periplasm, outer membrane, and extracellular fractions of the culture and subjected to Western blot analysis using polyclonal antibodies raised against recombinant Eta1 (rEta1), which was prepared as a His-tagged protein from *E. coli* (see Fig. S2 in the supplemental material). The results showed that Eta1 was found in the preparations of the cytoplasm, periplasm, and outer membrane proteins but not in that of the extracellular proteins (Fig. 2A and B). To examine whether the outer membrane preparation had been contaminated with cytoplasmic proteins, the preparations of the outer membrane and cytoplasmic proteins were blotted with antibodies against recombinant LuxS (rLuxS), a cytoplasmic protein of *E. tarda* (43). The results showed that LuxS was detected in the cytoplasmic preparation but not in the outer membrane preparation (Fig. 2C), suggesting that the outer membrane faction was not contaminated with cytoplasmic proteins. These results indicated that the Eta1 observed in the outer membrane preparation was most likely due to natural outer membrane localization. To further examine whether Eta1 was exposed on the cell surface, TX01 was incubated with rat anti-rEta1 serum or preimmune serum, and the bound antibodies were detected with FITC-labeled goat anti-rat IgG. Subsequent microscopic examination showed that fluorescence was detected in TX01 treated with anti-rEta1 serum, but not in TX01 treated with preimmune serum (Fig. 3). In contrast, TX01 treated with antibodies against rLuxS failed to show fluorescence. These results suggest that Eta1 was exposed on the surface of *E. tarda* and able to interact with anti-rEta1 antibodies.

Effect of *eta1* mutation. (i) Effect on tissue dissemination and general bacterial virulence. To examine the biological importance of Eta1, a genetic variant of TX01, TXeta1, which bears an insertion mutation at *eta1*, was constructed. Western blot analysis showed that, unlike the wild type, TXeta1 was unable to produce Eta1 (Fig. 2B). Compared to the wild-type strain TX01, TXeta1 was unaffected in growth when cultured in LB medium. To examine whether the *eta81* mutation affected pathogenicity, flounder were infected via immersion with or without (control) TXeta1 or TX01, and bacterial infection of host tissues was analyzed by bacterial-recovery analysis, which showed that at 12 h postinfection, the numbers of bacterial cells recovered from the blood, liver, kidney, and spleen of TXeta1-infected fish were significantly less than those recovered from TX01-infected fish (Fig. 4A). No bacteria were recovered from the control fish. These results suggest that TXeta1 was impaired in its ability to disseminate into and colonize host tissues. Furthermore, in TX01-infected fish, mortality began to occur at 3 days postinfection, and 90% accumulated mortality was reached before the fish became stabilized, whereas in TXeta1-infected fish, no mortality was observed (Fig. 4B). In contrast to TXeta1, TXeta1C, a variant of TXeta1 that expresses *eta1* in *trans* from a plasmid, was able to disseminate into and colonize flounder tissues and to cause mortality in the infected fish in a manner similar to that of TX01 (Fig. 4), suggesting that the defect in the virulence of TXeta1 could be rescued by *eta1* complementation.

(ii) Effect on infection of host cells and on cellular immune response. To examine the effect of *eta1* mutation on the infectivity

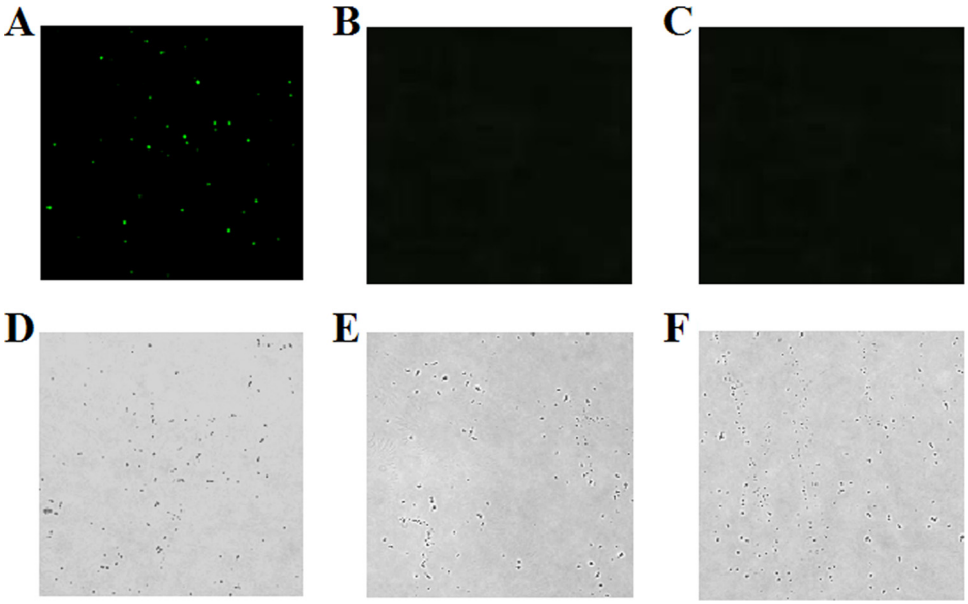

FIG 3 Exposure of Eta1 on the surface of *E. tarda*. *E. tarda* TX01 was incubated with anti-rEta1 serum (A and D), preimmune serum (B and E), or anti-rLuxS serum (C and F) and then treated with FITC-labeled goat anti-rat IgG. The cells were observed under a fluorescence microscope with (A, B, and C) or without (D, E, and F) fluorescence-exciting light. Magnification, ×200.

of *E. tarda* at the cellular level, flounder HK lymphocytes were infected with TX01 or TXeta1, and cellular infection was determined at 1 h, 2 h, 3 h, and 5 h postinfection by bacterial-recovery analysis. The results showed that bacterial recovery rates from TXeta1-infected cells at all the examined time points were significantly lower than those from TX01-infected cells (Fig. 5). qRT-PCR analysis of immune gene expression in flounder HK at 1 h and 4 h after TXeta1 and TX01 infection showed that, compared to TX01, which is known to inhibit the host immune response, TXeta1 infection induced a significant increase in the expression of IL-1β, IL-6, IL-8, TNF-α, IFN-γ, Mx, MHCIα, CD8α, and CD40 in a manner that was dependent on the infection time (Fig. 6).

Binding of recombinant rEta1 to flounder lymphocytes. Since Eta1 is a surface-exposed protein and its production is required for effective infection of host cells, we wondered whether Eta1 was capable of direct interaction with host cells. To investigate this question, the His-tagged rEta1 or rEthR, an intracellular *E. tarda* protein prepared under the same conditions as those under which rEta1 was prepared, was incubated with flounder HK lymphocytes, and the cells were subsequently subjected to fluorescence immunodetection with anti-His antibody or serum from an unvaccinated mouse (control serum). The results showed that for cells treated with anti-His antibody, fluorescence was detected in cells preincubated with rEta1, but not in cells preincubated with rEthR (Fig. 7). In contrast, for cells treated with the control serum,

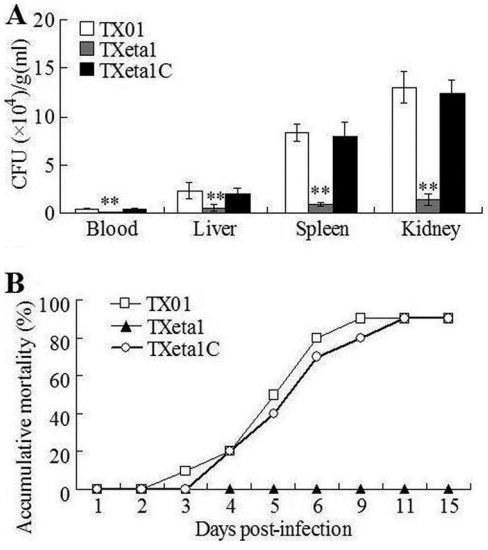

FIG 4 Virulence of TX01, TXeta1, and TXeta1C. (A) Flounder were infected via immersion with TX01, TXeta1, and TXeta1C. Bacterial recovery from blood, liver, kidney, and spleen was determined at 12 h postinfection. The data are presented as means ± SE ($n = 4$). **, $P < 0.01$. (B) Flounder were infected as for panel A and monitored daily for mortality.

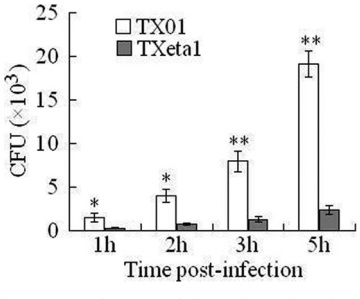

FIG 5 TX01 and TXeta1 infection of flounder cells. Flounder head kidney lymphocytes were infected with TX01 or TXeta1 for 1 h, 2 h, 3 h, and 5 h. Bacterial recovery from the infected cells at each time point was determined by plate count. At each time point, the significance in bacterial recovery between TX01- and TXeta1-infected cells is indicated by the asterisks. The data are presented as means ± SE ($n = 4$). *, $P < 0.05$; **, $P < 0.01$.

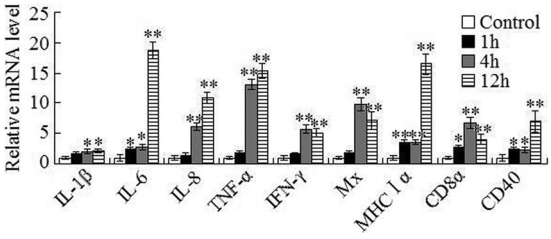

FIG 6 Effect of TXeta1 and TX01 infection on the expression of immune genes. Flounder were infected with TXeta1 or TX01, and the expression of IL-1β, IL-6, IL-8, TNF-α, IFN-γ, Mx, MHC class Iα, CD40, and CD8α in head kidney was determined at 1 h and 4 h postinfection by quantitative real-time RT-PCR. For each gene, the expression level induced by TX01 was set as 1 (control). The data are presented as means ± SE ($n = 4$). *, $P < 0.05$; **, $P < 0.01$.

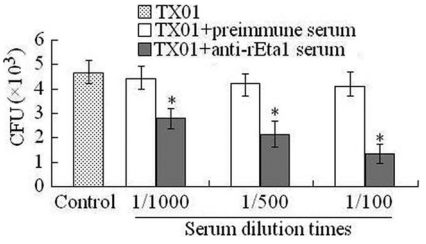

FIG 8 Effect of anti-rEta1 antibodies on *E. tarda* infection. Flounder head kidney lymphocytes were infected with *E. tarda* TX01 in the presence or absence (control) of different dilutions of anti-rEta1 serum or preimmune serum. Bacterial recovery from the infected cells was determined by plate count. The data are presented as means ± SE ($n = 4$). *, $P < 0.05$.

no fluorescence was detected in cells preincubated with rEta1 or rEthR (data not shown).

Effect of anti-Eta1 antibodies on *E. tarda* infection. To further examine whether the interaction between Eta1 and host cells was required for bacterial infection, flounder HK lymphocytes were infected with TX01 in the presence or absence of anti-rEta1 serum or preimmune serum in different dilutions. The results showed that the presence of anti-rEta1 serum significantly reduced the number of bacterial cells that invaded lymphocytes in a manner that was dependent on the dose of the antiserum (Fig. 8). In contrast, the presence of preimmune serum had no effect on cellular infection.

Immunoprotective potential of rEta1. The above results, i.e., that Eta1 is a surface-exposed protein that is highly induced during infection and interacts directly with host cells, led us to wonder whether rEta1 could induce protective immunity if used as a vaccine. To examine this possibility, flounder were vaccinated with rEta1 and challenged with a lethal dose of TX01 at 4 weeks postvaccination. The fish were subsequently monitored for mortality for 20 days, and the results showed that the accumulated mortalities of rEta1-vaccinated fish and the control fish were 12% and 72%, respectively. Hence, the protective efficacy, in terms of RPS, of rEta1 was 83.3%. Comparable results were obtained in the duplicate vaccination trial. Microbiological examination of dying fish indicated that TX01 was the only type of bacterium recovered from the liver, spleen, and blood of the fish, suggesting that mortality was caused by TX01 infection. ELISA analysis showed that specific serum antibodies were produced in rEta1-vaccinated fish (see Fig. S3 in the supplemental material). These results indicate that rEta1 is a protective antigen.

DISCUSSION

In this study, we analyzed the function of an *in vivo*-induced antigen, Eta1, from the pathogenic *E. tarda* strain TX01 isolated from diseased flounder. Expression analysis showed that when TX01 was cultured under *in vitro* conditions in the rich LB medium, the *eta1* expression level changed with the growth phase. However, the magnitude of the change was relatively small, as the peak expression level was only 4.6-fold that of the bottom level. In contrast, expression of *eta1* was significantly induced during infection of host lymphocytes, particularly at the early stage of in-

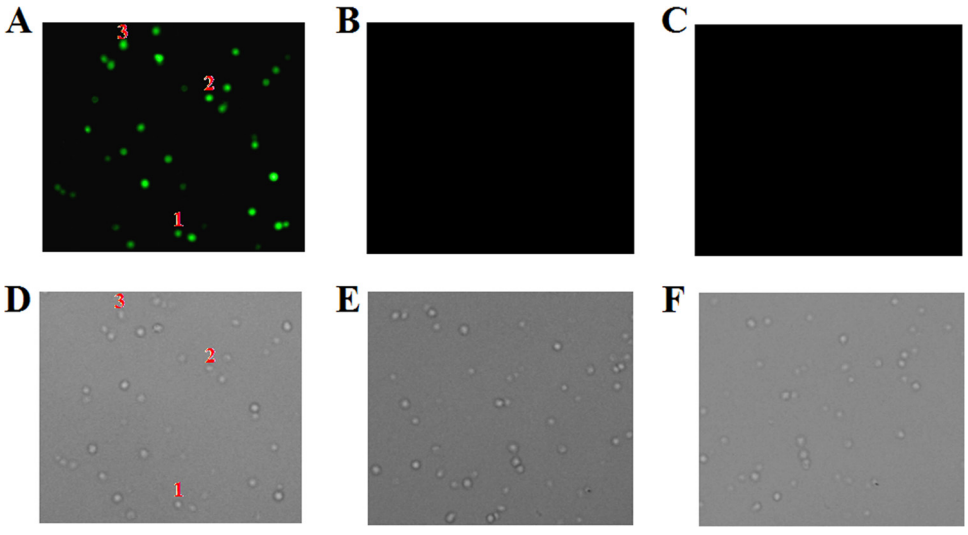

FIG 7 Binding of rEta1 to flounder lymphocytes. Flounder head kidney lymphocytes were incubated with His-tagged rEta1 (A, B, D, and E) or rEthR (C and F), and the bound protein was detected by immunodetection with mouse anti-His antibody (A, C, D, and F) or preimmune serum (B and E) and then treated with FITC-labeled goat anti-mouse IgG. The cells were observed under a fluorescence microscope with (A, B, and C) or without (D, E, and F) fluorescence-exciting light. Magnification, ×200. The numbers in panels A and D indicate examples of corresponding cells.

fection. These results indicate that *eta1* expression is strongly induced during the process of host cell invasion, which is consistent with the identification of Eta1 as an *in vivo*-induced antigen in the first place, and they suggest a potential involvement of Eta1 in host infection.

To examine whether Eta1 was required for *E. tarda* pathogenesis, an Eta1-deficient mutant, TXeta1, was constructed. TXeta1 was comparable to the wild type in growth when cultured in LB medium, suggesting that Eta1 is not an essential protein during *in vitro* growth. It is known that *E. tarda* possesses a strong tissue invasion and dissemination capacity that enables the pathogen to overcome the innate immunity barrier and eventually reach the internal organs of the host (16, 33). In this study, we found that following immersion infection of flounder, both TXeta1 and TX01 were able to spread to the liver and spleen; however, the numbers of TXeta1 bacteria recovered from these tissues were more than 10-fold less than those of recovered TX01, suggesting that mutation of *eta1* impairs the ability of TXeta1 to disseminate into and colonize host tissues. Consistent with these observations, TX01 infection induced 90% accumulated mortality, while no mortality was observed in TXeta1-infected fish. These results indicate that TXeta1 was attenuated in overall virulence. The observation that introduction of a functional *eta1* gene into TXeta1 restored the virulence of TXeta1 suggests that the virulence attenuation of TXeta1 was indeed due to *eta1* mutation. In agreement with these observations made in a live-fish model, cellular-infection analysis showed that, compared to TX01, TXeta1 exhibited significantly reduced ability to invade host lymphocytes.

Previous studies have shown that one of the prominent virulence features of *E. tarda* is its capacity to interfere with the normal immune response of host phagocytes, and this capacity is essential to the intracellular survival of *E. tarda* (23, 47). In this study, we found that the expression profiles of the immune genes in TX01- and TXeta1-infected flounder HK differed remarkably. Compared to TX01, TXeta1 stimulated the expression of a wide spectrum of immune genes that are involved in proinflammatory and specific immune responses, which probably accounts for the resistance of the host to TXeta1 infection. Hence, it appears that unlike the wild-type TX01, the *eta1*-defective mutant TXeta1 is no longer able to inhibit the general immune response of the host.

Accumulating studies have indicated that for many human pathogens, adhesins play a vital role in infection by mediating host cell adhesion and invasion (7, 15, 20, 30, 36). These proteins bind to receptors on the surfaces of host cells, thereby facilitating bacterial translocation into the targeted cells (13). In our study, we found that recombinant Eta1 was localized to the cell surface. Since no apparent signal peptide was identified in Eta1, it is possible that translocation of Eta1 may be through a nonclassical pathway. Eta1 was able to interact with flounder lymphocytes, which, together with the observation that Eta1 expression was upregulated during lymphocyte infection, suggests a possibility for Eta1 to act as an adhesin. Consistent with this hypothesis, the presence of anti-rEta1 antibodies reduced TX01 invasion into lymphocytes. Since preimmune serum had no effect on TX01 infection, it is likely that the reduced infectivity of TX01 observed in the presence of anti-rEta1 antibodies is due to specific binding of the antibodies to Eta1 on the pathogen, which may have masked the region of Eta1 that is essential to host cell interaction.

It is known that in some bacterial pathogens, surface proteins, such as invasins, that are involved in direct interactions with the host system possess vaccine properties (2, 38, 39). In our study, we found that rEta1 induced a protection rate of 83.3% upon lethal *E. tarda* challenge, suggesting that rEta1 is a strong protective immunogen. The immunoprotective property of rEta1 is in line with its virulence role played during infection. It is likely that, as shown by ELISA analysis, vaccination of rEta1 induced production of specific antibodies, which upon *E. tarda* infection bind to the infected pathogen via Eta1, which is highly expressed *in vivo* and localized on the cell surface. Since Eta1 is essential to effective host cell infection, binding of Eta1 by its antibodies very likely interferes with the normal function of Eta1, as was observed under *in vitro* conditions with flounder lymphocytes, resulting in a marked elevation in the survival rate of the vaccinated fish.

In conclusion, the results of this study indicate that Eta1 is an *in vivo*-induced antigen that is highly expressed upon encountering the host system during infection. With flounder as a model, it was found that mutation of *eta1* reduces the ability of *E. tarda* to infect host cells, disseminate into host tissues, and cause mortality of the infected fish. Purified recombinant Eta1 is able to interact with flounder lymphocytes, and blocking this interaction inhibits cellular infection. Consistent with these observations, Eta1 induces strong immunoprotection in flounder when used as a subunit vaccine. These results suggest that Eta1 possibly functions as an adhesin during *E. tarda* infection and, as a result, is required for optimal bacterial invasion.

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