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Edwardsiella tarda Ivy, a Lysozyme Inhibitor That Blocks the Lytic Effect of Lysozyme and Facilitates Host Infection in a Manner That Is Dependent on the Conserved Cysteine Residue

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***Edwardsiella tarda* is a Gram-negative bacterial pathogen with a broad host range that includes fish and humans. In this study, we examined the activity and function of the lysozyme inhibitor Ivy (named Ivy_{Et}) identified in the pathogenic *E. tarda* strain TX01. Ivy_{Et} possesses the Ivy signature motif CKPHDC in the form of ^{82}CQPHNC^{87} and contains several highly conserved residues, including a tryptophan (W55). For the purpose of virulence analysis, an isogenic TX01 mutant, TXivy, was created. TXivy bears an in-frame deletion of the ivy_{Et} gene. A live infection study in a turbot (*Scophthalmus maximus*) model showed that, compared to TX01, TXivy exhibited attenuated overall virulence, reduced tissue dissemination and colonization capacity, an impaired ability to replicate in host macrophages, and decreased resistance against the bactericidal effect of host serum. To facilitate functional analysis, recombinant Ivy_{Et} (rIvy) and three mutant proteins, i.e., rIvyW55A, rIvyC82S, and rIvyH85D, which bear Ala, Ser, and Asp substitutions at W55, C82, and H85, respectively, were prepared. *In vitro* studies showed that rIvy, rIvyW55A, and rIvyH85D were able to block the lytic effect of lysozyme on a Gram-positive bacterium, whereas rIvyC82S could not do so. Likewise, rIvy, but not rIvyC82S, inhibited the serum-facilitated killing effect of lysozyme on *E. tarda*. *In vivo* analysis showed that rIvy, but not rIvyC82S, restored the lost pathogenicity of TXivy and enhanced the infectivity of TX01. Together these results indicate that Ivy_{Et} is a lysozyme inhibitor and a virulence factor that depends on the conserved C82 for biological activity.**

Lysozymes are bactericidal proteins that exist in diverse organisms ranging from animals to bacteria. Lysozymes are classified into several different types, which include chicken-type (C-type), goose-type (g-type), and invertebrate-type (i-type) lysozymes (1). Lysozymes kill bacteria by breaking the 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycans, a component of bacterial cell walls (2).

Bacteria, in particular, those of a pathogenic nature, have evolved various mechanisms to counteract the action of lysozymes. One of these mechanisms is production of lysozyme inhibitors. The first lysozyme inhibitor, Ivy (inhibitor of vertebrate lysozyme), was discovered in *Escherichia coli* in 2001 (3). Ivy is a periplasmic protein that is active mainly against C-type lysozymes. Subsequently, several different types of lysozyme inhibitors were identified exclusively in Gram-negative bacteria. These include PliC (periplasmic lysozyme inhibitor of C-type lysozyme)/MliC (membrane-associated lysozyme inhibitor of C-type lysozyme) (4), PliI (periplasmic inhibitor of I-type lysozyme) (5), and PliG (periplasmic inhibitor of G-type lysozyme) (6). Experimental evidence indicates that these inhibitors are able to confer lysozyme tolerance on Gram-negative bacteria when the outer membranes of the bacteria are permeabilized (4, 5, 7–9) and therefore contribute to evasion of the lysozyme-mediated immune response during infection of animal hosts (10, 11).

Ivy homologues have been identified in a large number of Gram-negative bacteria. Most of these proteins contain a strictly conserved CKPHDC motif, while a few contain a less conserved CKPHDC sequence (12). The structural organizations of Ivy alone and Ivy complexed with hen egg white lysozyme (HEWL), a C-type lysozyme, have been resolved. It appears that Ivy forms a homodimer, in which each monomer consists of a central β sheet made of five antiparallel β strands flanked by two short helices on one side and by an amphipathic helix on the other side (3, 12). In the Ivy-HEWL complex, the CKPHDC motif forms a loop that protrudes from Ivy and inserts into the active site of HEWL in a key-lock fashion, thus blocking the activity of the enzyme. Mutational analysis showed that of the conserved residues in CKPHDC, the His residue is essential to the activity of Ivy, whereas the disulfide linkage formed by the two cysteine residues is inessential (12).

Edwardsiella tarda is a Gram-negative bacterium and a pathogen for fish, birds, reptiles, and humans. In aquaculture, *E. tarda* is a severe fish pathogen and has caused heavy economic losses to many farmed fish species, including turbot (*Scophthalmus maximus*), flounder (*Paralichthys olivaceus*), tilapia (*Oreochromis niloticus*), and channel catfish (*Ictalurus punctatus*) (13, 14). Accumulating studies indicate that *E. tarda* possesses a large amount of virulence-associated factors/systems, notably, type III and type VI secretion systems, a quorum-sensing system, two-component systems, adhesin, invasin, and exoenzymes, which are required for

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optimal bacterial infection (15–18). As a facultative intracellular pathogen, *E. tarda* can grow extracellularly and inside fish phagocytes, though the intracellular replication mechanism is unclear (19, 20). In previous studies, we identified from *E. tarda*, via the *in vivo*-induced antigen technology (IVIAT) and a signal sequence trapping system, several *in vivo*-induced antigens and secreted proteins that are associated with pathogenicity, including the iron-cofactored superoxide dismutase, which inhibits the macrophage-mediated bactericidal effect, and the invasin Inv1 and the adhesin Eta1, both of which are involved in host infection (17–19, 21). In this report, we characterized another factor identified via IVIAT, i.e., Ivy (named Ivy_{Et} for *E. tarda* Ivy). We examined the activity of Ivy_{Et}, the potential role of Ivy_{Et} in host infection, and the dependence of Ivy_{Et} function on the conserved structural features of the protein. Our results revealed new insights into the biological properties of Ivy.

TABLE 1 Primers used in this study

Primer	Sequence (5′ → 3′)[a]
F1	GATATCGACGAGACTATTCCGCCCTCG (EcoRV)
R1	GATATCTTTCCAATCCGGCTGCGAC (EcoRV)
F2	CCGTCGGCGGTGCGCAG
R2	GCACCGCCGACGGCAGATC
F3	GCATGGTCAGCCAGCCGCACA
R3	GGCTGGCTGACCATGCCGACC
F4	GCCGGACAACTGCGGCAACC
R4	CGCAGTTGTCCGGCTGGCA
F5	GGATCCAGGGTTTTCGGTTTACT (BamHI)
R5	CTATTTCCATAACGAGGGCGGAAT
F6	CTCGTTATGGAAATAGCGCGC
R6	GGATCCGTCAGCGATTTCACGA (BamHI)

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the parentheses at the end of the sequence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* BL21(DE3) was purchased from Tiangen (Beijing, China). *E. coli* S17-1λpir was purchased from Biomedal (Seville, Spain). *E. tarda* TX01 was isolated from diseased fish (22). The Gram-positive bacterium *Micrococcus luteus* was purchased from China General Microbiological Culture Collection Center, Beijing, China. Bacteria were cultured in Luria-Bertani (LB) broth at 37°C (for *E. coli* and *M. luteus*) or 28°C (for *E. tarda*). Where indicated, chloramphenicol was supplemented at a concentration of 30 μg/ml.

Fish used for infection study. Clinically healthy turbot (*Scophthalmus maximus*) were purchased from a local fish farm and maintained at ~22°C in aerated seawater. Fish were acclimatized in the laboratory for 2 weeks before experimental manipulation. The weight and size of the fish were 15.9 ± 0.6 g and 8.0 ± 0.3 cm, respectively. Fish were fed daily with commercial dry pellets (purchased from Shandong Sheng-suo Fish Feed Research Center, Shandong, China). Before the experiments, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen, and no bacteria were detected from the examined tissues of the sampled fish. For tissue collection, fish were euthanized with an overdose of MS-222 (tricaine methanesulfonate; Sigma, St. Louis, MO) as described previously (23). Experiments involving live animals were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals promulgated by the State Science and Technology Commission of Shandong Province. The animal study was approved by the Science and Research Department of the Institute of Oceanology, Chinese Academy of Sciences.

Sequence analysis. ivy_{Et} was initially cloned with the IVIAT technology as described previously (24). The sequence of Ivy_{Et} was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. A domain search was performed with the conserved domain search program of NCBI. A signal peptide search was performed with the SignalP (v3.0) program. The theoretical molecular mass and theoretical isoelectric point were predicted using the EditSeq tool in the DNAStar software package (Madison, WI).

Plasmid and strain construction. The primers used in this study are listed in Table 1. To construct pIvy, which expresses Ivy_{Et}, ivy_{Et} was amplified by PCR with primers F1 and R1. The PCR product was ligated with the T-A cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid was digested with EcoRV. The fragment containing ivy_{Et} was retrieved and inserted into pET259 (25) at the SwaI site. The plasmid pIvyW55A, which expresses the mutant protein IvyW55A, was constructed by overlap extension PCR as follows. The first overlap PCR was performed with primers F1 and R2, the second overlap PCR was performed with primers F2 and R1, and the fusion PCR was performed with the primer pair F1/R1. The PCR product was ligated with pET259 as described above. Plasmids pIvyC82S and pIvyH85D, which express the mutant proteins IvyC82S and IvyH85D, respectively, were created by overlap extension PCR as described above. For the construction of pIvyC82S, the first and second overlap PCRs were performed with the primer pairs F1/R3 and F3/R1, respectively, and the fusion PCR was performed with the primer pair F1/R1. For the construction of pIvyH85D, the first and second overlap PCRs were performed with the primer pairs F1/R4 and F4/R1, respectively, and the fusion PCR was performed with the primer pair F1/R1. All PCR products were verified by sequence analysis.

To construct the *E. tarda* mutant TXivy, in-frame deletion of a 360-bp segment (residues 88 to 447) of ivy_{Et} was performed by overlap extension PCR as follows: the first overlap PCR was performed with primers F5 and R5, the second overlap PCR was performed with primers F6 and R6, and the fusion PCR was performed with the primer pair F5/R6. The PCR products were inserted into the suicide plasmid pDM4 (26) at the BglII site, resulting in pDMIvy. S17-1λpir was transformed with pDMIvy, and the transformants were conjugated with TX01 as described previously (22). The transconjugants were selected on LB agar plates supplemented with 10% sucrose. One of the colonies that were resistant to sucrose and sensitive to chloramphenicol (marker of pDM4) was analyzed by PCR, and the PCR products were subjected to DNA sequencing to confirm the in-frame deletion. This strain was named TXivy.

Purification of recombinant proteins. *E. coli* BL21(DE3) was transformed separately with pIvy, pIvyW55A, pIvyC82S, and pIvyH85D. The transformants were cultured in LB medium at 37°C to mid-logarithmic phase, and expression of ivy_{Et} was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After growing at 18°C for an additional 16 h, the cells were harvested by centrifugation, and His-tagged proteins were purified under native conditions using Ni-nitrilotriacetic acid agarose (Qiagen, Valencia, CA) as recommended by the manufacturer. The purified proteins were dialyzed for 24 h against phosphate-buffered saline (PBS) and treated with Triton X-114 to remove endotoxin, as reported previously (27). The proteins were concentrated with Amicon Ultra centrifugal filter devices (Millipore, Billerica, MA). The concentrated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of the proteins was determined using the Bradford method with bovine serum albumin as the standard.

Tissue dissemination and mortality analysis. For tissue dissemination analysis, strains TXivy and TX01 were cultured in LB medium to an optical density at 600 nm (OD_{600}) of 0.8. The cells were washed with PBS and resuspended in seawater. Turbot were randomly divided into four groups ($n = 40$) and immersed in seawater containing 1×10^8 CFU/ml TXivy or TX01 for 6 h. The fish were then moved to tanks containing fresh seawater and reared under normal conditions. At 0, 1, and 2 days after immersion infection, blood, kidney, liver, and spleen were aseptically taken from the fish (five per time point). The tissues were weighed and

homogenized in a glass homogenizer containing PBS. The homogenates and blood were serially diluted and plated in triplicate on LB agar plates. The plates were incubated at 28°C for 48 h, and the colonies that appeared on the plates were enumerated. The genetic identity of the colonies was verified by PCR with the primers EsrAF2/EsrAR2 and ORF26F2/ORF26R3, which amplify the genes *esrA* and *orf26*, respectively, in TX01 and TXivy (28). Selected PCR products were subjected to sequence analysis. The remaining fish were monitored for mortality for 2 weeks. The experiment was performed three times.

Bacterial replication in macrophages. Turbot head kidney (HK) macrophages were prepared as described previously (29). The macrophages were cultured in L-15 medium (Thermo Scientific HyClone, Beijing, China) in 96-well culture plates (~10^5 cells/well). TX01 and TXivy suspensions in PBS were prepared as described above and added to macrophages (10^6 CFU/well). The cells were incubated at 25°C for 0.5 h and then washed three times with PBS. Fresh L-15 medium containing 100 μg/ml gentamicin (Thermo Scientific HyClone, Beijing, China) was added to the cells, and the cells were incubated at 25°C for 1 h to kill extracellular bacteria. The plates were then washed three times with PBS and incubated at 28°C for 1 h, 3 h, 5 h, and 7 h. After incubation, the plates were washed with PBS and the cells were lysed with 100 μl 1% Triton X-100. The cell lysate was serially diluted and plated in triplicate on LB agar plates. The plates were incubated at 28°C for 48 h, and the colonies that emerged on the plates were counted. The identities of the colonies were verified as described above. The experiment was performed three times.

Serum survival analysis. Sera from three turbot were prepared as reported previously (19) and mixed together. TX01 and TXivy were cultured in LB medium to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in PBS. Approximately 10^3 bacterial cells were mixed with or without (control) 50 μl turbot serum. After incubation with mild agitation at 30°C for 60 min, the mixture was serially diluted and plated in triplicate on LB agar plates. The plates were incubated at 28°C for 48 h. The colonies that appeared on the plates were enumerated. The survival rate was calculated as follows: (number of serum-treated cells/number of untreated control cells) × 100%. The experiment was performed three times.

Lysozyme inhibitor activity of rIvy. *M. luteus* was cultured in LB medium to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in PBS to an OD_{600} of 0.7. The bacterial suspension was mixed with or without (control) 5 μg/ml HEWL (Solarbio Science & Technology, Beijing, China) in the presence or absence of 7.5 μg/ml recombinant Ivy_{Et} (rIvy) or mutant protein rIvyW55A, rIvyC82S, or rIvyH85D. The mixture was incubated at 25°C, and cell density was monitored by measuring the absorbance at 600 nm at various time points. To examine the effect of rIvy on *E. tarda* survival in the presence of HEWL, TX01 and TXivy were cultured in LB medium to an OD_{600} of 0.8 and resuspended in PBS to 10^5 CFU/ml. Turbot serum was diluted five times in PBS. HEWL was suspended in PBS to 1 mg/ml. rIvy and rIvyC82S were suspended in PBS to 1.5 mg/ml. For the assay, a bacterial suspension was added to six 0.5-ml Eppendorf tubes (10 μl/tube), and the following solutions were then added separately to each tube: 90 μl PBS (control), 50 μl diluted serum, 10 μl HEWL, 50 μl diluted serum plus 10 μl HEWL, 50 μl diluted serum plus 10 μl HEWL and 10 μl rIvy, and 50 μl diluted serum plus 10 μl HEWL and 10 μl rIvyC82S. PBS was added to each tube to make the final volume to 100 μl. The tubes were incubated at 30°C for 1 h. After incubation, the mixture in each tube was diluted in LB medium and plated in triplicate on LB agar plates. The plates were incubated at 28°C for 48 h, and the colonies that emerged on the plates were counted. The identities of the colonies were verified as described above. The assays were performed three times.

***In vivo* effect of rIvy on TXivy and TX01 infection.** TX01 and TXivy were cultured in LB medium to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in PBS to 1 × 10^7 CFU/ml. To examine the effect of rIvy on TXivy infection, turbot (average weight, 8.6 g) were randomly divided into four groups (five fish per group) and injected intraperitoneally with 100 μl TXivy, TX01, TXivy plus 50 μg rIvy, or TXivy plus 50 μg rIvyC82S. At 2 days postinfection, bacterial recoveries from blood, kidney, liver, and spleen were determined as described above. The effect of rIvy on TX01 infection was carried out in the same fashion. The experiments were performed three times.

Statistical analysis. Except for the mortality assay, in which the log-rank test was used to compare the survival distributions of the fish, all other statistical analyses were performed with analysis of variance (ANOVA) of the SPSS (v15.0) package (SPSS Inc., Chicago, IL). In all cases, the significance level was defined as a P value of <0.05.

RESULTS

Sequence characterization of Ivy_{Et}. The amino acid sequence of Ivy_{Et} is identical to the amino acid sequences of the inhibitor of the vertebrate lysozyme precursor of *E. tarda* FL6-60 and EIB202 (GenBank accession no. ADM41108.1 and ACY83906.1, respectively). Ivy_{Et} contains 151 residues and was predicted to have a molecular mass of 16.6 kDa. BLAST analysis showed that Ivy_{Et} shares the highest sequence identity (89%) with the Ivy of *Edwardsiella ictaluri* and moderate identities (25% to 46%) with the Ivy inhibitors of *Serratia marcescens* FGI94, *Serratia proteamaculans* 568, *Yersinia pseudotuberculosis* PB1/+, *Yersinia enterocolitica* subsp. *palearctica* Y11, *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966, *Burkholderia pseudomallei* MSHR346, and *Escherichia coli* K-12 (see Fig. S1 in the supplemental material). *In silico* analysis identified a putative signal peptide (residues 1 to 21) in Ivy_{Et} and a conserved Ivy domain (residues 32 to 149), with the latter containing the motif ^{82}CQPHNC^{87}. C82 and C87 are the only cysteine residues in Ivy_{Et}. In addition, Ivy_{Et} also contains several residues, i.e., A21, P53, W55, P65, and Y76, that are highly conserved among known Ivy lysozyme inhibitors.

Mutation of ivy_{Et} and its effect on bacterial pathogenicity. (i) Effect on the capacity to disseminate in host tissues and induce host mortality. To examine the biological importance of Ivy_{Et}, an ivy_{Et}-defective *E. tarda* mutant, TXivy, was constructed by markerless in-frame deletion of a 360-bp internal segment of ivy_{Et}. Compared to the wild-type strain TX01, TXivy exhibited a similar growth profile when cultured in LB medium (data not shown). To examine whether ivy_{Et} mutation affected pathogenicity, turbot were inoculated with the same dose of TXivy or TX01 via immersion, and bacterial infection of blood, kidney, liver, and spleen was analyzed by bacterial recovery analysis. The results showed that at 0 days after immersion infection, the numbers of bacteria recovered from TXivy- and TX01-infected fish were comparable, while at 1 and 2 days postinfection, the numbers of bacterial cells recovered from all examined tissues of TXivy-infected fish were significantly less than those recovered from TX01-infected fish (Fig. 1). For TX01-infected fish, mortality began to occur at 3 days postinfection, and the accumulated mortality reached 80% by 6 days postinfection. For TXivy-infected fish, mortality began to occur at 5 days postinfection and stopped at 7 days postinfection, with an accumulated mortality of 30%. The survival rate of TXivy-infected fish (70%) was significantly ($P < 0.01$) higher than that (20%) of TX01-infected fish (Fig. 2).

(ii) Effect on intracellular multiplication. Replication in host phagocytes is a prominent virulence feature of *E. tarda*. To examine the potential effect of ivy_{Et} mutation on this capacity of *E. tarda*, turbot HK macrophages were infected with TXivy or TX01, and intracellular bacterial recovery was determined at 1 h, 3 h, 5 h, and 7 h postinfection. The results showed that while the numbers

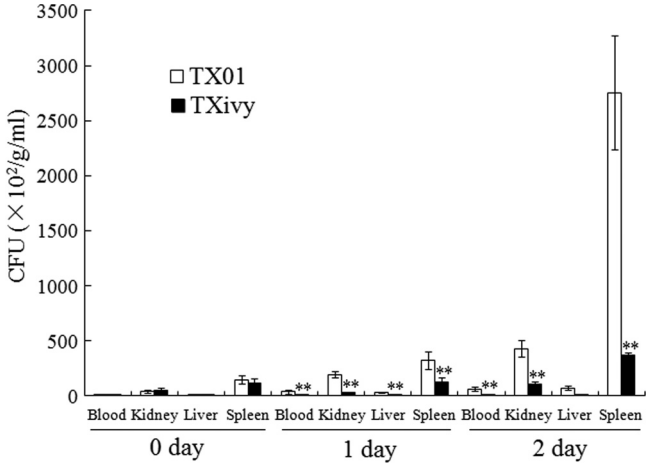

FIG 1 Dissemination and colonization of wild-type and mutant *Edwardsiella tarda* in fish tissues. Turbot were infected with *E. tarda* TX01 or TXivy via immersion. Bacterial recovery from blood, kidney, liver, and spleen was determined at 0, 1, and 2 days postinfection and is presented as the number of CFU per gram of tissue (kidney/liver/spleen) or per milliliter of blood. Data are the means of three independent experiments and presented as means ± SEMs. **, $P < 0.01$.

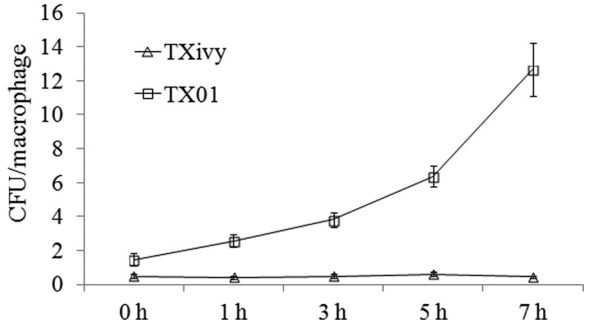

FIG 3 Replication of wild-type and mutant *Edwardsiella tarda* in HK macrophages. Turbot HK macrophages were infected with *E. tarda* TX01 or TXivy for 0.5 h. After removing the extracellular bacteria, the cells were incubated at 28°C, and intracellular bacterial recovery was determined at different time points. Data are the means of three independent experiments and presented as means ± SEMs.

of intracellular TX01 cells increased with time, the numbers of intracellular TXivy cells essentially remained unchanged (Fig. 3).

(iii) Effect on survival in host serum. *E. tarda* is known to resist the bactericidal effect of fish serum (17). To examine whether ivy_{Et} mutation had any effect on serum resistance, TXivy and TX01 were incubated with turbot serum for 1 h, and survival of the bacteria was determined by plate count. The results showed that TXivy exhibited a relative survival rate of 47%, which was significantly ($P < 0.01$) lower than that (80%) of TX01.

***In vitro* activity of rIvy and its dependence on conserved residues. (i) Inhibition of lytic effect of lysozyme against a Gram-positive bacterium.** To examine the activity of Ivy_{Et}, rIvy was purified from *E. coli* as a His-tagged protein (see Fig. S2 in the supplemental material). Since, as shown above, W55, C82, and H85 are highly conserved among known Ivy lysozyme inhibitors (see Fig. S1 in the supplemental material), we also examined the functional importance of these residues by site-directed mutagenesis, which resulted in three mutants, rIvyW55A, rIvyC82S, and rIvyH85D, that bear Ala, Ser, and Asp substitutions at W55, C82, and H85, respectively. In the case of rIvyC82S, since serine is structurally the closest replacement to cysteine, the C82S mutation may not affect the structural feature of the mutant protein, and therefore, any alteration in the biological property of rIvyC82S would most likely be associated with the role of C82 in disulfide bond formation. Bacterial survival analysis showed that when the Gram-positive bacterium *M. luteus* was incubated with HEWL, the density of bacterial cells was severely reduced in a manner that depended on the incubation time; however, when rIvy was copresent with HEWL, no apparent reduction in bacterial density was observed at most of the time points examined (Fig. 4). Similarly, when rIvyW55A and rIvyH85D were copresent with HEWL, the bacterial density remained largely comparable to the densities of the control cells incubated in the absence of HEWL. In contrast, when rIvyC82S was copresent with HEWL, the density of bacterial cells was reduced in a fashion similar to that when the cells were incubated with HEWL alone.

(ii) Inhibition of lytic effect of lysozyme against *E. tarda*. Since Gram-negative bacteria are, in general, resistant to lysozyme,

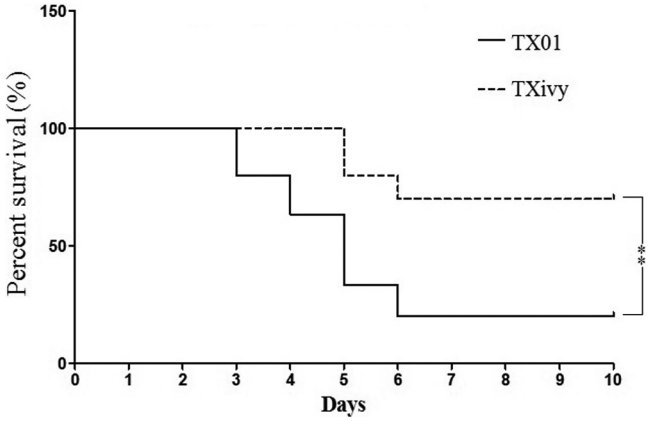

FIG 2 Survival distribution of infected fish. Turbot were infected with *Edwardsiella tarda* TX01 or TXivy via immersion. The fish were monitored daily for mortality and survival. Significance between the rates of survival of the two groups of fish was determined with the log-rank test. **, $P < 0.01$.

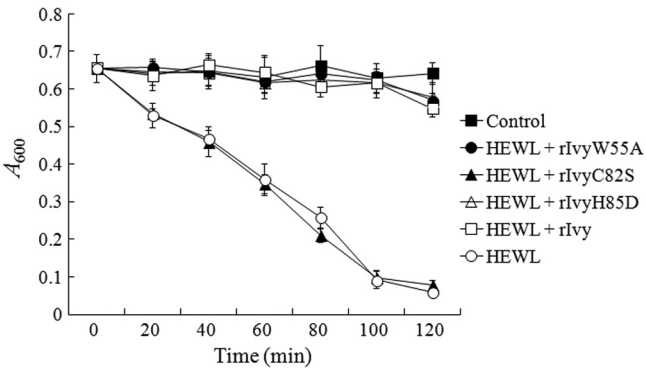

FIG 4 Lysozyme-inhibitory effect of wild-type and mutant Ivy. *Micrococcus luteus* was incubated in PBS alone (control) or in PBS containing HEWL in the presence or absence of rIvy, rIvyW55A, rIvyC82S, and rIvyH85D. Cell density was monitored by measuring the absorbance at 600 nm at various time points. Data are the means of three independent experiments and presented as means ± SEMs.

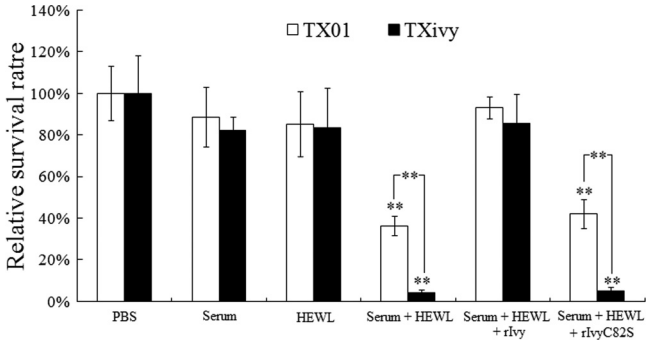

FIG 5 Effect of rIvy on serum-facilitated lysozyme killing of *Edwardsiella tarda*. *E. tarda* TX01 and TXivy were incubated with HEWL in the presence or absence of diluted fish serum plus rIvy or rIvyC82S. The control cells were incubated with PBS. The numbers of bacterial cells that survived were determined after the incubation. Data are the means of three independent experiments and presented as means ± SEMs. **, $P < 0.01$.

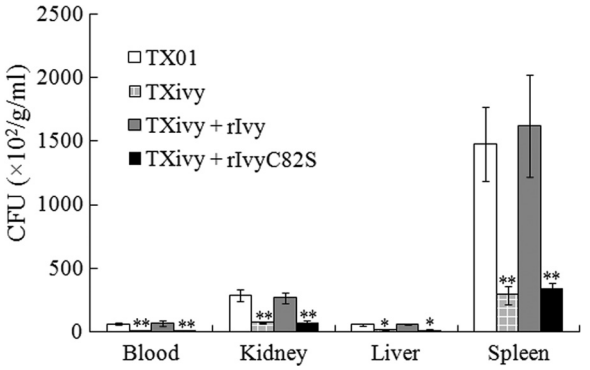

FIG 6 Effect of rIvy on the infectivity of *Edwardsiella tarda* TXivy. Turbot were infected with *E. tarda* TX01, TXivy, TXivy plus rIvy, or TXivy plus rIvyC82S. Bacterial recovery from blood, kidney, liver, and spleen was determined at 2 days postinfection and is presented as the number of CFU per gram of tissue (kidney/liver/spleen) or per milliliter of blood. Data are the means of three independent experiments and presented as means ± SEMs. **, $P < 0.01$; *, $P < 0.05$.

we examined the effect of serum-facilitated lysozyme hydrolysis of *E. tarda*, because serum complement can sensitize Gram-negative bacteria to lysozyme. For this purpose, *E. tarda* TX01 and TXivy were incubated with HEWL in the presence of diluted turbot serum. The results showed that under this condition, HEWL significantly reduced the survival rates of both TX01 and TXivy and that the survival rate of TXivy was significantly lower than that of TX01 (Fig. 5). When rIvy was copresent with HEWL, the survival rates of both TX01 and TXivy were comparable to those of the control cells incubated in the absence of HEWL. In contrast, the copresence of rIvyC82S with HEWL had no apparent effect on the survival of TX01 and TXivy.

***In vivo* effect of rIvy. (i) Rescuing effect on infectivity of *E. tarda* TXivy.** Since rIvy exhibited an apparent lysozyme-inhibitory effect under *in vitro* conditions, we examined whether it could restore the lost infectivity of *E. tarda* TXivy. For this purpose, turbot were infected with the same dose of TX01, TXivy, TXivy plus rIvy, or TXivy plus rIvyC82S, and bacterial recoveries from tissues were determined at 2 days postinfection. The results showed that the presence of rIvy increased the bacterial recoveries of TXivy from blood, kidney, liver, and spleen to levels similar to those of TX01, whereas the presence of rIvyC82S had no significant effect on the levels of recovery of TXivy (Fig. 6). The experiment was performed three times with comparable results, and the numbers of CFU of one representative experiment are presented in Table S1 in the supplemental material.

(ii) Augmenting effect on infectivity of *E. tarda* TX01. With the results presented above, we further examined whether rIvy could enhance the infectivity of wild-type *E. tarda* TX01. For this purpose, turbot were infected with TX01 or TX01 plus rIvy. Subsequent bacterial recovery analysis showed that bacterial recoveries from the blood, kidney, liver, and spleen of the fish infected with TX01 plus rIvy were significantly higher than those from the fish infected with TX01 alone (Fig. 7).

DISCUSSION

In this study, we examined the biological activity and function of *E. tarda* Ivy, Ivy_{Et}. Ivy_{Et} shares low to moderate sequence identities with known Ivy inhibitors. Unlike the majority of Ivy inhibitors, which exhibit strict sequence conservation in the CKPHDC motif, Ivy_{Et} has a variant version of CKPHDC, in which the lysine and the aspartate residues are replaced by glutamine and asparagine, respectively. The same replacement occurs in the Ivy of another species of *Edwardsiella*, *E. ictaluri*. Hence, CQPHNC may be a specific feature of the *Edwardsiella* genus. Similar sequence variations at CKPHDC have been observed in the Ivy of *Gluconobacter oxydans* (· CRPHDC) and *Burkholderia* sp. strain 383 (CKPHNC). Mutational analysis in the context of the three-dimensional structure of the *E. coli* Ivy-HEWL complex suggests that both CRPHDC and CKPHNC should still be able to have a proper interaction with lysozyme and cause lysozyme inhibition (12). In the case of Ivy_{Et}, we found that when *M. luteus* was incubated with HEWL in the presence of rIvy, bacterial survival was comparable to that in the absence of HEWL, suggesting that Ivy_{Et} is a functional lysozyme inhibitor that blocked the bactericidal activity of HEWL. Similar lysozyme inhibition effects were also observed with the mutants rIvy W55A and rIvyH85D, whereas no lysozyme inhibition was observed with the mutant rIvy C82S. These results indicate that C82, but not W55 and H85, is essential to the activity of Ivy_{Et}. These observations are in contrast to those reported for *E.*

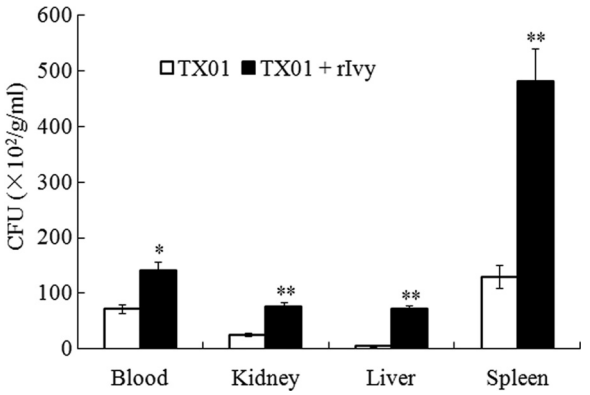

FIG 7 Effect of rIvy on the infectivity of *Edwardsiella tarda* TX01. Turbot were infected with *E. tarda* TX01 or TX01 plus rIvy. Bacterial recovery from blood, kidney, liver, and spleen was determined at 4 h postinfection and is presented as the number of CFU per gram of tissue (kidney/liver/spleen) or per milliliter of blood. Data are the means of three independent experiments and presented as means ± SEMs. **, $P < 0.01$; *, $P < 0.05$.

coli Ivy, which showed that H60 (equivalent to H85 of Ivy_{Et}) makes hydrogen bonds with D52 and E53 in the active site of HEWL, and as a result, mutation of H60 to a negatively charged amino acid dramatically reduces the activity of Ivy, whereas mutation of one of the Cys residues in CKPHDC has no significant impact (12). The difference in these observations favors the hypothesis that Ivy_{Et}, which shares low (25%) sequence identity with *E. coli* Ivy and has a variant CKPHDC motif, may utilize a lysozyme inhibition mechanism not exactly like that employed by *E. coli* Ivy. Since C82S mutation impairs the activity of Ivy_{Et}, the disulfide linkage between C82 and the only other cysteine residue (i.e., C87) is probably vital to the proper conformation of Ivy_{Et}. It is possible that the CQPHNC motif in Ivy_{Et} may still adopt a loop structure, which, however, differs from that assumed by the canonical CKPHDC sequence in the specific interactions between the residues of the loop and those in the active site of lysozyme.

Ivy, as well as other types of lysozyme inhibitors, is known to protect Gram-negative bacteria from lysozyme in the presence of the outer membrane permeabilizer lactoferrin (4–7). Other host factors, such as antimicrobial peptides, also enhance the lytic effect of lysozymes (30, 31). In our study, we found that when TX01 and TXivy were treated with HEWL in the presence of diluted serum, the survival rates of both strains were dramatically reduced, suggesting that serum components, probably complements and other innate immune factors, facilitate the bactericidal effect of HEWL. Compared to TX01, the survival rate of TXivy was significantly lower, which is consistent with the fact that TXivy is defective in the production of Ivy_{Et} and therefore more sensitive to lysozyme. When rIvy was copresent with HEWL, the survival rates of TX01 and TXivy were restored to those of the control cells, suggesting that rIvy inhibited the activity of HEWL. The observation that rIvyC82S had no apparent effect on HEWL confirmed the conclusion presented above that C82 is indispensable for the fundamental activity of Ivy_{Et}.

Recent reports showed that an *mliC* deletion in an *E. coli* mutant caused a strong reduction in serum resistance and in *in vivo* virulence, whereas *ivy* deletion had no effect on serum resistance and virulence (10); in contrast, for *Yersinia pestis*, which causes plague, *mliC* mutation did not affect lysozyme resistance and the development of plague, whereas *ivy* mutation impaired bacterial virulence (11). In our study, we found that, compared to the wild type, TXivy exhibited reduced dissemination and colonization in the tissues of turbot and caused significantly lower mortality in the host, suggesting that deletion of ivy_{Et} attenuates the overall virulence of TXivy. Previous studies showed that *E. tarda* is an intracellular pathogen that can survive in host phagocytes (19, 20). In our study, we observed markedly reduced abilities of TXivy to replicate in turbot macrophages and serum, suggesting that Ivy_{Et} is likely to function against the lysozyme-mediated defense of both cellular and humoral innate immunity. In line with these observations, *in vivo* analysis showed that when rIvy was cointroduced into turbot with TXivy, it increased the tissue infection capacity of the mutant bacterium to a level comparable to that of the wild type, while when rIvy was cointroduced into turbot with TX01, it enhanced the tissue infection capacity of the bacterium to a significant extent. Taken together, these results indicate that Ivy_{Et} is a virulence factor that is involved in multiple aspects, including tissue dissemination and intracellular replication, of *E. tarda* infection.

In conclusion, we demonstrate in this study that Ivy_{Et} is a lysozyme inhibitor that possesses a noncanonical CKPHDC motif and that the biological activity of Ivy_{Et} is dependent on the conserved cysteine residues, rather than the histidine residue, of the CKPHDC motif. Ivy_{Et} confers protection on *E. tarda* against lysozyme lysis in the presence of serum and is required for optimal infection of the host. These results add new insights into the function of the Ivy type of lysozyme inhibitors.

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