Edwardsiella tarda MliC: a lysozyme inhibitor that participates in pathogenesis in a manner that parallels Ivy

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Running title: Activity and function of E. tarda MliC
Edwardsiella tarda, a bacterial pathogen to farmed fish as well as humans, possesses the genes of two lysozyme inhibitors, i.e. IvyEt and mliCEt. We have recently studied IvyEt and found it implicated in E. tarda virulence. In the present study, we characterized MliCEt in comparison with IvyEt in a turbot model. MliCEt contains the FWSKG motif and two cysteines (C33 and C98) that are highly conserved in subgroup I MliC but unknown in functional importance. To examine the essentialness of these conserved structural features, recombinant MliC (rMliC) and its mutants bearing C33S and W79A (of the FWSKG motif) substitutions were prepared. Subsequent analysis showed that rMliC (i) inhibited lysozyme-induced lysis of a Gram-positive bacterium, (ii) reduced serum-facilitated lysozyme killing of E. tarda, and (iii), when introduced into turbot, promoted bacterial dissemination in fish tissues. C33S mutation had no influence on the activity of rMliC, while W79A mutation slightly but significantly enhanced the activity of rMliC. Both the mliC (TXΔmliC) and the ivy knockout TXΔivyEt knockouts were severely attenuated in the ability of tissue invasion, host lethality, serum surviving, and intracellular replication. The lost virulence of TXΔmliC was restored by complementation with an introduced mliC gene. Compared to ivy or mliC single knockout, mliC-ivy double knockout was significantly impaired in most of the virulence features. Together these results provide the first evidence that the conserved cysteine is functionally dispensable to a subgroup 1 MliC, and that as a virulence factor, MliC most likely works in a concerted and parallel manner with Ivy.

Keywords: Edwardsiella tarda; lysozyme inhibitor; virulence; infection
Edwardsiella tarda is a rod-shaped Gram-negative bacterium of the family Enterobacteriaceae. It is a zoonotic pathogen that can infect fish, birds, reptiles, and humans (1). In aquaculture, E. tarda is known to affect a large number of freshwater and marine fish and have caused heavy economic losses (2). As a human pathogen, E. tarda is the etiological agent of gastroenteritis and extraintestinal diseases such as peritonitis, meningitis, and myonecrosis (3). Recent studies with different fish models revealed that E. tarda infection requires the participation of a wide range of factors, including exoenzymes, adhesin, invasin, type III and type VI secretion systems, quorum sensing system, two-component systems, and iron assimilation and utilization systems (4,5).

Lysozymes play a key role in innate immunity in all animals. They catalyze the hydrolysis of the β-1, 4-glycosidic bond between the N-acetylmuramic acid and N-acetylglucosamine of bacterial peptidoglycan, resulting in peptidoglycan degradation and cell lysis (6,7). To date three major classes of animal lysozymes have been identified, of which, chicken (C-) and goose (G-) type lysozymes are present in all vertebrates, while I-type lysozyme is found only in invertebrates. The three types of lysozymes differ considerably in amino acid sequence but are similar in three-dimensional structure (8). In mammals, lysozymes are involved in modulation of inflammation and immune response and constitute part of defensive bactericidal systems (9,10).

As a strategy to protect themselves from the action of lysozymes, bacteria have developed specific evasion mechanisms by producing a group of proteins called lysozyme inhibitors, which interact with and block the activity of different lysozymes (11,12). To date, inhibitors against all three major types of lysozymes have been identified in Gram-negative bacteria. These inhibitors are known as inhibitor of
vertebrate lysozyme (Ivy) targeting at C-type lysozyme, periplasmic lysozyme inhibitor of C-type lysozyme (PliC), membrane-associated lysozyme inhibitor of C-type lysozyme (MliC), periplasmic inhibitor of I-type lysozyme (PliI), and periplasmic inhibitor of G-type lysozyme (PliG) (13-17). Of these inhibitors, MliC/PliC have been found in various bacterial species, and high-resolution structures of the MliC/PliC of Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, and Brucella abortus have been reported (18-21). It seems that MliC proteins fall into two subgroups, which differ in dimer formation. Subgroup 1 is represented by E. coli MliC (MliC\textsubscript{Ec}), which exists as a monomer in solution (18), while subgroup 2, which include P. aeruginosa MliC (MliC\textsubscript{Pa}) and B. abortus PliC, appear to be dimeric proteins in crystal structure (19,21).

In a previous study, we identified and characterized the Ivy of E. tarda (Ivy\textsubscript{Et}). We found that Ivy\textsubscript{Et} is able to inhibit the activity of C-type lysozyme and involved in the pathogenesis of E. tarda (22). In the present study, we aimed to examine the biological activity of MliC of E. tarda and assess the role of MliC in bacterial infection in comparison with that of Ivy\textsubscript{Et}. Our results provide new insights to the functional properties of these two types of lysozyme inhibitors.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** E. tarda TX01 was originally isolated from diseased turbot (23) and can cause lethal infection in a number of fish species including turbot, flounder, tongue sole, and zebrafish. Escherichia coli BL21(DE3) was purchased from Tiangen (Beijing, China). E. coli S17-1::pir was purchased from Biomedal (Sevilla, Spain). The Gram-positive bacterium Micrococcus luteus was purchased from China General Microbiological Culture Collection Center, Beijing, China.
Bacteria were cultured in Luria-Bertani broth (LB) at 37°C (for *E. coli* and *M. luteus*) or 28°C (for *E. tarda*). Where indicated, chloramphenicol, tetracycline, and polymyxin B were supplemented at the concentration of 30 µg/ml, 20 µg/ml, and 100 µg/ml respectively.

**Fish.** Clinically healthy turbot (*Scophthalmus maximus*) (9.7 ± 1.2 g) were purchased from a local fish farm and maintained at ~20°C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Before experiment, 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 MS222 (tricaine methanesulfonate) (Sigma, St. Louis, MO, USA) as described previously (24).

**Sequence analysis.** The sequence of *mliC*<sub>E</sub> 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 theoretical molecular mass and theoretical isoelectric point were predicted using EditSeq in the DNASTAR software package (Madison, WI, USA). Multiple sequence alignment was created with DNAMAN.

**Plasmid construction.** The primers used in plasmid construction are listed in Table 1. To construct pEtMliC, which expresses recombinant MliC<sub>E</sub> (rMliC), *mliC*<sub>E</sub> 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 *mliC*<sub>E</sub> was retrieved and inserted into pET32a (Novagen, San Diego, CA, USA) at the EcoRV site. The plasmid pEtMliCC33S, which expresses the mutant protein rMliCC33S, was constructed by overlap extension PCR as follows. The first overlap PCR was performed with the primers F1 and R2, the second overlap PCR was performed
with the primers F2 and R1, and the fusion PCR was performed with the primer pair F1/R1. The PCR product was ligated with pET32a as above. The plasmid pETMliCW79A, which expresses the mutant protein rMliCW79A, was created by overlap extension PCR as above, in which 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. To construct the low-copy plasmid pJTMliC that expresses mliC_Et, mliC_Et was amplified by PCR with primers F1/R1; the PCR product was ligated with the TA cloning vector pBS-T, and the recombinant plasmid was digested with EcoRV. The fragment containing mliC_Et was retrieved and inserted into plasmid pBT3 (25) at the EcoRV site, resulting in pBT3MliC. pBT3MliC was digested with Swal, and the fragment carrying mliC_Et was inserted into plasmid pJT (26) at the Swal site, resulting in pJTMliC. All PCR products were verified by sequence analysis.

Construction mliC_Et and ivy_Et knockouts. To construct _E. tarda_ TXΔmliC, in-frame deletion of a 237 bp segment (residues 31 to 267) of mliC_Et was performed by overlap extension PCR as follows: the first overlap PCR was performed with primers F4 and R4, the second overlap PCR was performed with primers F5 and R5, and the fusion PCR was performed with the primer pair F4/R5. The PCR product was inserted into the suicide plasmid pDM4 (27) at the BglII site, resulting in pDMMliC. S17-1 λpir was transformed with pDMMliC, and the transformants were conjugated with TX01 as follows. The donor and recipient strains were cultured in LB medium to OD_600 of 0.8 and mixed at a ratio of 3:1. The mixture was dropped onto a LB agar plate, and the plate was incubated at 28°C for 24 h. After incubation, the bacteria on the plate were resuspended in 2 ml LB and plated on a LB agar plate containing polymixin B and chloramphenicol. The colonies that appeared were selected on LB agar plate containing 10% sucrose. One of the colonies that were resistant to sucrose and sensitive to chloramphenicol was analyzed by PCR, and the PCR product was sequenced to confirm in-frame deletion of mliC_Et. This strain was named TXΔmliC.
TXΔivy has been reported previously (previously named TXivy) (22). To construct mliCEt-ivyEt double knockout, S17-12pir was transformed with pDMMliC; the transformants were conjugated with TXΔivy, and the transconjugants were selected as described above. One of the transconjugants was named TXΔmliCivy. All mliCEt and ivyEt single and double deletions in the respective knockout strains were confirmed by PCR. To construct the mliCEt complement strain TXΔmliC+mliC, S17-12pir was transformed with pJTMliC, and the transformants were conjugated with TXΔmliC. The transconjugants were selected on LB agar plates supplemented with tetracycline (marker of pJ) and polymyxin B (marker of TX01 and its derivatives). One of the transformants was named TXΔmliC+mliC.

**Purification of recombinant proteins.** *E. coli* BL21(DE3) was transformed separately with pEtMliC, pEtMliCC33S, pEtMliCW79A, and pET32a for purification of the His-tagged rMliC, rMliCC33S, rMliCW79A, and rTrx tag respectively. rTrx was used as a control protein in the functional study of rMliC, rMliCC33S, and rMliCW79A, since, as indicated above, these latter three proteins were expressed from pET32a and consequently bear a Trx tag as well as a His tag. The transformants were cultured in LB medium at 37°C to mid-logarithmic phase, and expression of recombinant proteins were induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After growth at 18°C for an additional 12 h, the cells were harvested by centrifugation, and the proteins were purified using Ni-NTA agarose (QIAGEN, Valencia, CA, USA) as recommended by the manufacturer. The purified proteins were reconstituted by dialyzing against reconstitution buffer (50 mM Tris–HCl, 50 mM NaCl, 5 mM β-mercaptoethanol, 5 mM EDTA, 20% glycerol, 2 mM reduced glutathione, 0.2 mM oxide glutathione, and a gradient of 6 M, 4 M, 2 M, 1 M, 0.5 M, and 0 M urea, pH 8.5) at 4°C, with 12 h of dialysis at each gradient. The proteins were then dialyzed in PBS for 24 h. The proteins were treated with Triton X-114 to remove endotoxin as reported previously (28). The proteins were concentrated using
PEG20000 (Solarbio, Beijing, China). 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.

**Immunoblot.** TX01 was cultured in LB medium to an OD_{600} of 0.8. Extracellular and whole-cell proteins were prepared and subjected to immunoblot as reported previously (29) with rat antibodies against recombinant MliC and LuxS (30). The antibodies were prepared as reported previously (31).

**Lysozyme inhibitor activity assay – (i) with *M. luteus* as a target bacterium.** *M. luteus* was cultured in LB medium to an OD_{600} of 0.8 and washed with PBS. Hen egg white lysozyme (HEWL) (Solarbio, Beijing, China) was added to the bacterial suspension to the final concentration of 5 µg/ml. The same volume of PBS was added to the control sample. The cells were then mixed with different concentrations (10 µM, 40 µM, or 80 µM) of rMliC or rTrx. The mixture was incubated at 37°C. After 30 min, 60 min, 90 min, and 120 min incubation, an aliquot of cells was diluted serially and plated on LB agar plates. The plates were incubated at 37°C for 24 h, and the colonies that appeared on the plates were counted. The genetic identity of the colonies was verified by PCR with specific primers and sequence analysis of the PCR products.

**(ii) With *E. tarda* as a target bacterium.** *E. tarda* TX01 was cultured in LB medium to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in PBS to 10^7 CFU/ml. Turbot serum was diluted three times in PBS. Ten microliters of bacterial cells was mixed with or without (control) 50 µl diluted serum. HEWL (final concentration 100 µg/ml) or HEWL plus rMliC (final concentration 80 µM) was then added to the cells. The control cells were added with PBS. The cells were incubated at 28°C for 2 h. After incubation, the cells were serially diluted and plated on LB agar plates. The plates were incubated at 28°C.
for 48 h, and the colonies that appeared on the plates were enumerated.

In vivo effect of rMliC on E. tarda infection. TX01 and TXΔmliC were cultured as above and resuspended in PBS to $5 \times 10^5$ CFU/ml. Turbot were randomly divided into three groups and injected intraperitoneally with 100 µl 80 µM rMliC or rTrx or PBS. At 4 h post-injection, turbot in each group were infected via intramuscular injection with 100 µl TX01 or TXΔmliC. At 8 h post-infection, kidney and spleen were aseptically taken from the fish (five/time point) and homogenized in PBS. The homogenates 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 E. tarda-specific primers and sequence analysis of the PCR products.

Tissue dissemination and mortality analysis. For tissue dissemination analysis, E. tarda TX01, TXΔmliC+mliC, TXΔmliC, TXΔivy, and TXΔmliCivy were cultured in LB medium to an OD$_{600}$ of 0.8. The cells were washed with PBS and resuspended in PBS to $5 \times 10^5$ CFU/ml. Turbot were randomly divided into five groups (N = 35) and infected via intramuscular injection with 100 µl TX01, TXΔmliC+mliC, TXΔmliC, TXΔivy, or TXΔmliCivy. At 12 h, 24 h, and 48 h post-infection, kidney and spleen were aseptically taken from the fish (five/time point) and homogenized in PBS. The homogenates 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 appeared on the plates were enumerated. For mortality analysis, five groups (N=20) of turbot were infected as above with TX01, TXΔmliC+mliC, TXΔmliC, TXΔivy, or TXΔmliCivy; the fish were monitored daily for mortality for 15 days.

Serum survival assay. E. tarda TX01, TXΔmliC+mliC, TXΔmliC, TXΔivy, and TXΔmliCivy were cultured in LB medium to an OD$_{600}$ of 0.8. The cells were washed with PBS and resuspended in PBS. Approximately $10^5$ bacterial cells were mixed with 50 µl untreated turbot serum or heat-inactivated turbot
serum (control). After incubation with mild agitation at 28°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, and the colonies that appeared on the plates were enumerated. The genetic identity of the colonies was verified as above. The survival rate was calculated as follows: (number of serum-treated cells/number of heat-inactivated serum-treated cells) × 100%.

Bacterial replication in head kidney monocytes (HKM). Turbot HKM was prepared as follows. Head kidney was taken from turbot (~812 g) under aseptic conditions. The tissues were ground and passed through a sterile metal mesh (75 mm) with PBS containing 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Carlsbad, USA). The cell suspension was centrifuged at 300 g for 5 min, and the cell pellet was washed and resuspended in PBS containing 10% FBS. HKM was extracted from the cell suspension with Fish Monocyte Separation Kit (Hao Yang Biological Manufacture Co., Tianjin, China) (Figure S1). The cells were cultured in L-15 medium (Thermo Scientific HyClone, Beijing, China) in 96-well culture plates (10^5 cells/well). TX01, TXΔmliC+mliC, TXΔmliC, TXΔivy, and TXΔmliCivy were prepared as described above and added to HKM (10^5 CFU/well). The cells were incubated at 28°C for 1 h and 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 28°C for 1 h to kill extracellular bacteria. The plates were then washed three times with PBS and incubated at 28°C for 0 h, 2 h, 4 h, and 8 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 identity of the colonies was verified as described above.

Production of reactive oxygen species (ROS) and acid phosphatase activity assay. ROS
production was determined as reported previously (32). For acid phosphatase activity analysis, *E. tarda* TX01, TXΔmliC+ΔmliC, TXΔmliC, TXΔivy, and TXΔmliCivy were prepared as described above and added to HKM cultured as above (10^5 CFU/well). The same volume of PBS was added to the control cells. The cells were incubated at 28°C for 2 h or 4 h, followed by washing three times with PBS. After incubation, the cells were determined for acid phosphatase activity as follows: the cells were lysed by adding 100 μl of 1% Triton X-100 to each well, followed by incubation at 4°C for 20 min. After incubation, acid phosphatase activity was determined using Acid Phosphatase Assay Kit (Beyotime, Beijing, China) according to manufacturer's instruction.

**Statistical analysis.** All experiments were performed three times as indicated in figure legends. Except for mortality assay, in which Logrank 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 15.0 package (SPSS Inc., Chicago, IL, USA). In all cases, the significance level was defined as *P* < 0.05.

**RESULTS**

**Sequence of MliC_Et.** A search of the genomes of several *E. tarda* isolates for lysozyme inhibitor genes revealed that, in addition to *ivy*, *E. tarda* possesses a mliC homologue (named mliC_Et). Sequence analysis showed that MliC_Et is composed of 102 amino acid residues, with a predicted molecular mass of 11.55 kDa and a pI of 9.24. It is most closely related to the MliC of *Edwardsiella ictaluri* (88% overall identity), moderately related to the MliC of a number of bacteria, including *Pantoea stewartii*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, and *E. coli* (40.4%-56.2% overall identity), and distantly related to the MliC of *P. aeruginosa* (23.6% overall identity) (Supplementary data, Figure S2). The overall sequence...
identity between MliC<sub>Et</sub> and Ivy<sub>Et</sub> is 15.9%. In silico analysis showed that MliC<sub>Et</sub> contains the characteristic MliC domain with the highly conserved residues including Y31, C33, T39, V40, D54, G68, Y71, Y76, and C98 that are preserved among subgroup I MliC. C33 and C98 are the equivalent cysteine residues that in MliC<sub>Ec</sub> are known to form an intracellular disulfide bond (18). In addition, MliC<sub>Et</sub> contains the sequence motif "FWSKG" that is universally present in subgroup I MliC.

**Lysozyme-inhibitory activity of rMliC.** Since lysozyme inhibitors in their natural forms are usually difficult to obtain, especially with relatively high purity, these proteins have been studied in the form of recombinant proteins (13,17,20,21,33). In our study, to examine the biological activity of MliC<sub>Et</sub>, rMliC was purified from *E. coli* as a Trx-tagged protein. To examine the anti-lysozyme effect of rMliC, *M. luteus* was incubated with HEWL in the presence or absence of different concentrations of rMliC, and bacterial survival was then determined. The results showed that the presence of rMliC, but not rTrx, which was purified under the same condition as rMliC, significantly increased bacterial survival in a manner that depended on the dose of rMliC (Fig. 1).

**Effect of rMliC on the survival of *E. tarda.* (i) **In vitro effect.** Serum-facilitated lysozyme killing analysis showed that when *E. tarda* TX01 was incubated with HEWL in the presence of diluted turbot serum, which served to sensitize the bacterium to lysozyme, bacterial survival was significantly reduced; however, when *E. tarda* TX01 was incubated with HEWL in the presence of turbot serum plus rMliC, bacterial survival was increased to the level comparable to that of the control cells treated with PBS (Fig. 2).

(ii) **In vivo effect on *E. tarda* infection.** To examine whether rMliC could affect the infectivity of *E. tarda*, turbot were inoculated with *E. tarda* TX01 in the presence or absence of rMliC or rTrx, and bacterial dissemination into and colonization of the spleen and kidney of the fish were determined. The
results showed that in the presence of rMliC, bacterial recoveries from both tissues were significantly increased, whereas in the presence of rTrx, bacterial loads in the tissues were comparable to those of the control fish (Fig. 3).

**Extracellular production of MliC**. With the above results, which showed that rMliC as an exogenously added protein functioned *in vivo*, we wondered whether TX01 could naturally produce MliC into the extracellular milieu. To investigate this question, immunoblot was conducted to examine potential existence of MliC in the extracellular as well as the whole-cell proteins of TX01. The results showed that anti-rMliC antibodies detected MliC in both fractions (Figure S3). When the antibodies against LuxS, a cytoplasmic protein of *E. tarda* (30), were used in the immunoblot, LuxS was detected in the fraction of whole-cell proteins but not in the fraction of extracellular proteins, which ruled out the possibility that the MliC detected in the extracellular fraction was due to contamination of cytoplasmic proteins.

**Functional importance of the conserved residues of rMliC**. Since the residues C33 and W79 are highly conserved among known MliC, we examined the functional importance of these residues to the activity of rMliC. For this purpose, the mutant proteins rMliCC33S and rMliCW79A, which bear C33S and W79A substitutions respectively, were prepared and examined for lysozyme-inhibitory activity. The results showed that the survival rates of *M. luteus* after incubation with HEWL plus rMliCC33S were similar to those after incubation with HEWL plus rMliC (Fig. 4). Compared to *M. luteus* incubated with HEWL plus rMliC, *M. luteus* incubated with HEWL plus rMliCW79A exhibited similar survival rates after 30 min to 90 min incubation; however, when the incubation was extended to 120 min, the survival rate of rMliCW79A-treated cells was slightly but significantly (*P* < 0.05) increased compared to that of rMliC-treated cells (Fig. 4).
Construction of mliC<sub>Et</sub> single knockout and mliC<sub>Et</sub>-ivy<sub>Et</sub> double knockout. Since, as said above, E. <i>tarda</i> possesses two lysozyme inhibitor genes, i.e. ivy<sub>Et</sub> and mliC<sub>Et</sub>, of which ivy<sub>Et</sub> is known to be involved in bacterial pathogenesis (22), we wanted to examine the biological significance of MliC<sub>Et</sub> in comparison with that of Ivy<sub>Et</sub>. For this purpose, two genetic variants of TX01 were created, i.e., TX<sub>Δ</sub>mliC and TX<sub>Δ</sub>mliCivy, which are mliC<sub>Et</sub> knockout and mliC<sub>Et</sub>-ivy<sub>Et</sub> double knockout respectively. Growth analysis showed that like TXΔivy, which exhibited no growth defect compared to the wild type strain TX01 (22), TXΔmliC and TXΔmliCivy displayed growth profiles similar to that of TX01 when cultured in LB medium (data not shown).

Comparison of the infectivity of mutant and wild type <i>E. tarda</i> in different aspects. (i) The capacity to disseminate in host tissues and to cause host mortality. To compare their <i>in vivo</i> infectivity, the mutant and wild type <i>E. tarda</i> were inoculated into turbot via muscle injection. In addition, in order to examine whether any effect caused by mliC<sub>Et</sub> mutation could be rescued by an exogenously introduced mliC<sub>Et</sub> allele, the strain TXΔmliC+mliC, which is TXΔmliC harboring a low-copy plasmid that expresses mliC<sub>Et</sub>, was similarly inoculated into turbot. At 12 h, 24 h, and 48 h post-infection, bacterial dissemination and colonization in kidney and spleen were determined by plate count. The results showed that in both tissues and at all examined time points, the numbers of bacteria recovered from TXΔmliC- and TXΔivy-infected fish were comparable and significantly lower than those from TX01-infected fish, while the bacterial recoveries from TXΔmliCivy-infected fish were significantly lower than those from either TXΔmliC- or TXΔivy-infected fish (Fig. 5). In contrast, bacterial recoveries from fish infected with TXΔmliC+mliC were similar to those from TX01-infected fish. To examine whether rMliC had any effect on TXΔmliC infection, turbot were infected with TXΔmliC in the presence of rMliC or rTrx, and bacterial recoveries from kidney and spleen were determined at 8 h post-infection. The results showed that for both
tissues, the bacterial loads in the fish treated with rMliC were significantly higher than those in the untreated control fish or in the fish treated with rTrx (Fig. 3).

Mortality analysis showed that mortality began to occur at 3 d post-infection in fish infected with TX01 and TX\(\Delta\)mliC+\(\Delta\)mliC, and at 4 d post-infection in fish infected with TX\(\Delta\)mliC and TX\(\Delta\)ivy. At the end of the monitored period, the survival rates of TX\(\Delta\)mliCivy-, TX\(\Delta\)mliC-, and TX\(\Delta\)ivy-infected fish were 100%, 55% and 65% respectively, which were significantly higher than those of the fish infected with TX01 and TX\(\Delta\)mliC+\(\Delta\)mliC (0%) (Fig. 6).

(ii) **The capacity to resist the bactericidal effect of host serum.** The ability to survive in host serum is a key virulence property of *E. tarda* (30,31). When incubated with turbot serum, TX\(\Delta\)mliC was found to exhibit a significantly lower survival rate than the wild type TX01 (Fig. 7). The survival rate of TX\(\Delta\)mliC was comparable to that of TX\(\Delta\)ivy but was significantly higher than that of TX\(\Delta\)mliCivy (Fig. 7).

In contrast to the mutants, TX\(\Delta\)mliC+\(\Delta\)mliC was comparable to TX01 in survival rate.

(iii) **The capacity to replicate in host HKM and to inhibit HKM activation.** Another feature that is vital to *E. tarda* infection is the ability to replicate in host phagocytes and to block activation of these cells. Intracellular infection study showed that, when the mutants and wild type *E. tarda* were incubated with turbot HKM, the intracellular bacterial recoveries of TX01 and TX\(\Delta\)mliC+\(\Delta\)mliC increased steadily with time during the monitored period, whereas no apparent increase in the intracellular number of TX\(\Delta\)mliC, TX\(\Delta\)ivy, or TX\(\Delta\)mliCivy was detected (Fig. 8). Compared to TX01-infected HKM, HKM infected with TX\(\Delta\)mliC, TX\(\Delta\)ivy, and TX\(\Delta\)mliCivy exhibited no apparent difference in ROS production (data not shown); however, HKM infected with the three mutants, in particular TX\(\Delta\)mliCivy, exhibited significantly higher levels of acid phosphatase activity (Fig. 9).
In this study, we examined the biological activity and function of *E. tarda* MliC, MliC<sub>Et</sub>. We found that MliC<sub>Et</sub> in the primary structure is closer to subgroup 1 MliC represented by MliC<sub>Ec</sub> than to subgroup 2 MliC represented by MliC<sub>Pa</sub>. It is known that subgroup 2 MliC contain conserved residues, such as the L34, L36, L62, L69, and V81 in MliC<sub>Pa</sub> that are involved in hydrophobic interactions at the dimeric interface, whereas in subgroup 1 MliC this feature is lacking (18). In MliC<sub>Et</sub>, none of the hydrophobic residues conserved in subgroup 2 MliC is present. In contrast, MliC<sub>Pa</sub> possesses the FWSKG motif that is universally preserved in subgroup I MliC and likely participates in substrate binding (18). These structural characteristics indicate that MliC<sub>Et</sub> is a member of subgroup I MliC.

Lysozyme-inhibiting activity has been reported for MliC<sub>Ec</sub> and MliC<sub>Pa</sub> (14). In the case of MliC<sub>Et</sub>, we observed a dose-dependent inhibitory effect of rMliC against HEWL-induced lysis of *M. luteus*, suggesting that MliC<sub>Et</sub> is an active lysozyme inhibitor. In documented studies of recombinant lysozyme inhibitors, the recombinant proteins were used at the dose of 10 μM to 200 μM for different analyses (13,17); in our study, the concentration of rMliC (80 μM) used was relatively high based on the consideration that the protein had been undergone reconstitution. Previous studies showed that in the presence of outer membrane permeabilizers or antimicrobials that destabilize the outer membrane of Gram-negative bacteria, lysozyme can effectively kill Gram-negative bacteria (14,16,17,36-38). In our study, we found that in the presence of diluted fish serum, which facilitates the action of lysozyme on Gram-negative bacteria, rMliC reduced the killing effect of HEWL against *E. tarda* to a significant extent. Since the serum is from turbot, the susceptible host of *E. tarda*, this in vitro observation suggested that rMliC may function in vivo as well. Consistently, the presence of rMliC significantly enhanced the ability
of TX01 and TXΔmliC to disseminate into and colonize host tissues. It is likely that although TX01 can produce MliC<sub>E</sub> during infection, the mount of naturally produced MliC<sub>E</sub> may not be enough to combat host lysozymes in the sense that some of the inoculated bacterial are inevitably killed by lysozyme-induced lysis. However, when the infection is conducted in the presence of rMliC, the exogenously added protein will inactivate a certain amount of host lysozymes and thus enable more bacterial cells to escape from lysozyme killing, which leads to faster and more effective tissue dissemination and colonization. In line with the in vivo observations, immunoblot detected MliC<sub>E</sub> in the culture supernatant of TX01, suggesting that MliC<sub>E</sub> is produced extracellularly by the pathogen. These results suggest the possibility that MliC<sub>E</sub> likely functions, at least in part, as an extracellular lysozyme inhibitor.

A NMR structural study showed that MliC<sub>E</sub> folds into an eight-stranded antiparallel β-barrel that is stabilized by an intramolecular disulfide bond formed by the only two cysteine residues in the protein, and that a conserved patch on the open end of the barrel composed of the sequences SASGARY and FWSKG represents a possible site for substrate binding or protein-protein interaction (18). However, the real functional importance of these conserved structures in subgroup 1 MliC has not been investigated. In our study, to ascertain the essentialness of the conserved residues, we compared the activity of wild type MliC and mutant MliC<sub>E</sub> bearing substitutions at the W residue of the FWSKG motif and at one of the highly conserved cysteine residues. We found that rMliCC33S was comparable to rMliC in activity, suggesting that C33, and consequently, the intracellular disulfide linkage, is functionally dispensable. In contrast to rMliCC33S, rMliCW79A exhibited slightly but significantly stronger lysozyme-inhibitory activity than rMliC. Considering that subgroup 1 MliC exists as a monomer, FWSKG is more likely involved in substrate binding than in protein dimerization. As such, it is possible that W79 may participate...
in the hydrophobic interaction with key residues of lysozyme, and that substitution of W79 with alanine, which, like tryptophan, has a hydrophobic side chain but much smaller in size, may result in better substrate interaction and hence augmented inhibitory effect on lysozyme.

Several reports have shown that lysozyme inhibitors are implicated in bacterial pathogenesis, however, variations exist among different bacterial species. For example, in *E. coli*, Ivy is known to enhance bacterial survival in human saliva (39), and knockout of *mliC*, but not *ivy*, decreased the serum survival and lethality of the bacterium (12); in plague-causing *Yersinia pestis*, deletion of *mliC* had no effect on lysozyme resistance or the development of plague, while deletion of *ivy* attenuated bacterial virulence (40). In *E. tarda*, our recent study showed that knockout of *ivy* reduced bacterial virulence in various aspects (22). Similarly, in the present study, we found that compared to the wild type, TX\textDelta mliC was impaired in the ability of tissue dissemination, inducing mortality in the host, and resistance against the bactericidal activity of host serum, most likely due to the reduced capacity of TX\textDelta mliC to combat the lysozymes present in host tissues and serum. Previous studies showed that in *S. typhi*, *mliC* expression was expressed during growth in human macrophages, and that *mliC* disruption led to decreased survival of the bacteria in macrophages (41,42). In *E. tarda*, which is known to be capable of replicating in phagocytes and inhibiting host immune response (43-45), we found that, unlike the wild type, TX\textDelta mliC was unable to multiplicate in turbot HKM, suggesting that MliC\textsubscript{Et} is required for intracellular replication. Consistent with this observation, the acid phosphatase activity of TX\textDelta mliC-infected cells was significantly higher than that of TX01-infected cells, suggesting that TX\textDelta mliC was defective in blocking HKM activation in some aspects. The observation that TX\textDelta mliC+mliC exhibited virulence features comparable to that of the wild type strain indicates that the genetic defect of TX\textDelta mliC can be complemented by the introduced *mliC*\textsubscript{Et} in TX\textDelta mliC+mliC, which supports the conclusion that the
alterations in the virulence properties of TXΔmliC were indeed due to mliCETO knockout.

In a recent study of the lysozyme inhibitors of an avian pathogenic E. coli, it was observed that mliC knockout reduced serum resistance, but ivy knockout and ivy-mliC double knockout had no effect on serum resistance, and that while mliC knockout was attenuated in virulence, the ivy-mliC double knockout exhibited full virulence capacity almost like that of the wild type (12). In contrast, in E. tarda we found that TXΔmliC was similar to TXΔivy in virulence properties, and that in the abilities of tissue dissemination, serum resistance, and host lethality, the mliCETO-ivyETO double knockout strain TXΔmliCivy was significantly weakened compared to either mliCETO or ivyETO single knockout. These results indicate that MliCETO and IvyETO are not functionally substitutable; rather, it is more likely that these two proteins may act in a concerted manner that produces an additive effect on bacterial infectivity. This lack of functional redundancy is in line with the fact that mliCETO and ivyETO are the only lysozyme inhibitor genes possessed by E. tarda.

In conclusion, in this study we demonstrate that MliCETO is a lysozyme inhibitor implicated in various aspects of bacterial virulence and required for host infection. We observed for the first time that the biological activity of a subgroup 1 MliC is independent of the highly conserved cysteine residue involved in intracellular disulfide bond formation, and that MliCETO as a virulence factor probably works in a parallel, un-redundant manner with IvyETO. These results add new insights to the function as well as the function-structure relationship of MliC.
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FIGURE LEGENDS

Figure 1. Effect of rMliC on the growth and survival of Micrococcus luteus in the presence of lysozyme. M. luteus was incubated in PBS containing hen egg white lysozyme (HEWL) or HEWL plus different concentrations of rMliC or rTrx. The control cells were incubated in PBS alone. At various time points, the number of viable cells was determined by plate count. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. Significance between survivals against HEWL in the presence and absence of rMliC is indicated with asterisk. **P < 0.01; *P < 0.05.

Figure 2. Effect of rMliC on serum-facilitated lysozyme killing of Edwardsiella tarda. E. tarda TX01 was incubated with hen egg white lysozyme (HEWL) in the presence or absence of diluted fish serum plus rMliC_Et. The control cells were incubated with PBS. The numbers of survived bacterial cells (presented as colony forming unit, CFU) were determined after the incubation. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. **P < 0.01.

Figure 3. Effect of rMliC on the infection of wild type and mutant Edwardsiella tarda. Turbot were infected with E. tarda TX01 or TXΔmliC in the presence or absence (control) of rMliC or rTrx. At 8 h post-infection, bacterial recovery from spleen and kidney was determined and presented as colony forming unit (CFU) per milligram of tissues. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. **P < 0.01.

Figure 4. Effect of C33S and W79A mutation on the activity of MliC. Micrococcus luteus was incubated...
in PBS containing hen egg white lysozyme (HEWL) or HEWL plus rMliCC33S, rMliCW79A, rMliC, or rTrx. The control cells were incubated with PBS alone. At various time points, the number of viable cells was determined by plate count. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. Significance between survivals against HEWL in the presence of rMliC and the mutant proteins is indicated with asterisk. *P < 0.05.

Figure 5. Infection of wild type and mutant *Edwardsiella tarda* in fish tissues. Turbot were inoculated with *E. tarda* TX01, TXΔmliC+mliC, TXΔmliC, TXΔivy, or TXΔmliCivy. Bacterial recovery from kidney (A) and spleen (B) was determined at different time points and presented as colony forming unit (CFU) per milligram of tissues. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. **P < 0.01; *P < 0.05.

Figure 6. Survival distribution of infected fish. Turbot were infected with *Edwardsiella tarda* TX01, TXΔmliC+mliC, TXΔmliC, TXΔivy, or TXΔmliCivy. The fish were monitored daily for mortality and survival. Significances between the survivals of TX01-infected fish and other groups of fish were determined with logrank test. The experiment was performed three times, and the mean survival rates were shown. **P < 0.01.

Figure 7. Survival of mutant and wild type *Edwardsiella tarda* in host serum. *E. tarda* TX01, TXΔmliC, TXΔivy, TXΔmliCivy, and TXΔmliC+mliC were incubated with turbot serum, and the numbers of survived bacterial cells were determined after the incubation. Data are the means of three independent experiments and are presented as means ± SEM. **P < 0.01.
Figure 8. Replication of wild type and mutant *Edwardsiella tarda* in head kidney monocytes (HKM).

Turbot HKM was infected with *E. tarda* TX01, TXΔmliC, TXΔivy, TXΔmliCivy, and TXΔmliC+mliC for 1 h. After removing extracellular bacteria, the cells were incubated at 28°C for different hours, and intracellular bacterial recovery was determined. CFU, colony forming unit. Data are the means of three independent experiments and are presented as means±SEM.

Figure 9. Acid phosphatase activity of turbot head kidney monocytes (HKM) infected with wild type and mutant *Edwardsiella tarda*. Turbot HKM were infected with *E. tarda* TX01, TXΔmliC, TXΔivy, TXΔmliCivy, and TXΔmliC+mliC for 2 h or 4 h, and acid phosphatase activity of the cells was determined. Data are the means of three independent experiments and are presented as means±SEM. **P < 0.01.
| Primer | Sequence (5’-3’)
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*Underlined nucleotides are restriction sites of the enzymes indicated in the parentheses at the end of the sequence.*