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Thioredoxin H (TrxH) contributes to adversity adaptation and pathogenicity of *Edwardsiella piscicida*

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Abstract

Thioredoxins (Trxs) play an important role in defending against oxidative stress and keeping disulfide bonding correct to maintain protein function. *Edwardsiella piscicida*, a severe fish pathogen, has been shown to encode several thioredoxins including TrxA, TrxC, and TrxH, but their biological roles remain unknown. In this study, we characterized TrxH of *E. piscicida* (named TrxH_{Ep}) and examined its expression and function. TrxH_{Ep} is composed of 125 residues and possesses typical thioredoxin H motifs. Expression of *trxH_{Ep}* was upregulated under conditions of oxidative stress, iron starvation, low pH, and during infection of host cells. *trxH_{Ep}* expression was also regulated by ferric uptake regulator (Fur), an important global regulatory of *E. piscicida*. Compared to the wild type TX01, a markerless *trxH_{Ep}* in-frame mutant strain TX01Δ*trxH* exhibited markedly compromised tolerance of the pathogen to hydrogen peroxide, acid stress, and iron deficiency. Deletion of *trxH_{Ep}* significantly retarded bacterial biofilm growth and decreased resistance against serum killing. Pathogenicity analysis shows that the inactivation of *trxH_{Ep}* significantly impaired the ability of *E. piscicida* to invade host cells, reproduce in macrophages, and infect host tissues. Introduction of a trans-expressed *trxH* gene restored the lost virulence of TX01Δ*trxH*. There is likely to be a complex relationship of functional complementation or expression regulation between TrxH and another two thioredoxins, TrxA and TrxC, of *E. piscicida*. This is the first functional report of TrxH in fish pathogens, and the findings suggest that TrxH_{Ep} is essential for coping with adverse circumstances and contributes to host infection of *E. piscicida*.

Introduction

Edwardsiella piscicida (formerly included in *E. tarda*) [1, 2], one family member of Enterobacteriaceae, is a Gram-negative, motile, rod-shaped bacterium. It is a serious fish pathogen and has a broad host range that includes many species of economically important fish such as Japanese eel, flounder, turbot, red sea bream, tilapia, and channel catfish [3]. Edwardsiellosis, which is caused by *E. piscicida*, is one of the most severe diseases in aquaculture with freshwater as well as marine water supply, and has

led to great economic losses in the aquaculture industry worldwide [4–7]. In addition to fish, *E. piscicida* is also pathogenic to humans and can cause gastrointestinal disorders and other diseases [8]. Since *E. piscicida* is an important pathogen of aquatic animals, more and more studies about *E. piscicida* are reported. A large number of virulence factors/systems, such as type III (T3SS) and type VI (T6SS) secretion systems, a two-component regulatory system, hemolysin, LuxS/AI-2 quorum sensing system, molecular chaperons and RNA-binding protein Hfq, ferric uptake regulator, lysozyme inhibitors, and so on, are known to be involved in *E. piscicida* stress resistance or pathogenicity [9–13]. Accumulating evidence has made clear that *E. piscicida* is an intracellular pathogen with the capacity to evade host immune defense [11, 14]. Recently, a study on intracellular trafficking pathways indicates a clear preference of *E. piscicida* for certain

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endocytic pathways and an involvement of endosomes, lysosomes, and cytoskeletons in the infection process [15]. Cheng et al. reported that this bacteria uses the tri-carboxylic acid cycle to evade complement-mediated killing [16]. Another study showed that Sip2 in *E. piscicida* contributes to serum survival, acid resistance, intracellular replication, and host infection [17]. However, studies on the tolerance of *E. piscicida* to oxidative stress are limited.

Oxidative stress is a problem often encountered by most microorganisms. Reactive oxygen species (ROS) are abundantly generated in various environments including in host environments, leading microorganisms to develop multiple strategies to cope with oxidative stress [18]. One of these strategies is the thioredoxin system. The thioredoxin system is widely distributed in several species, from archaea and bacteria to humans, and is composed of thioredoxin (Trx), thioredoxin reductase (TrxR), and nicotinamide adenosine dinucleotide phosphate (NADP/NADPH) [19]. This antioxidant system transfers electrons from NADPH through TrxR to Trx and then to a large range of proteins that play critical roles in DNA synthesis and oxidative stress [20]. The major function of the Trx system is to reduce disulfide bridges in target proteins and help organisms recover from ROS stress [21, 22]. In microbes, such as *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Helicobacter pylori*, thioredoxins have been confirmed to involve in maintaining the intracellular redox homeostasis and play a key role in the protection of cells against ROS [23–25]. Moreover, the Trx system also provides the electron donor for many critical enzymes by its function of disulfide reductase and thus is related to a series of cellular functions [26].

Trx is a small (around 12 kDa) ubiquitous protein characterized by a conserved motif involving two redox-active Cys separated by a pair of amino acids (CxxC motif) [27]. Besides the oxidoreductase function, Trx has the function of regulating cell growth, inhibiting apoptosis and regulating gene transcription [28]. The *E. coli* genome encodes two Trx, Trx1 (TrxA), and Trx2 (TrxC). Trx1 is the first thioredoxin discovered in *E. coli*, and its function as a hydrogen donor in redox reactions was identified [29]. *H. pylori* also contains two genes that encode Trx, *trxA* and *trxC*. TrxA expression in *H. pylori* is induced by stress agents, and it is the electron donor for antioxidant enzymes [24, 30]. TrxC acts as a chaperone and transforms denatured arginase into its active form [31]. There are three thioredoxin in the *E. piscicida* genome, TrxA, TrxC, TrxH [32]. To our knowledge, there are no reports about the function study of TrxH in this pathogenic bacteria. In this study, we characterized TrxH in *E. piscicida* (named TrxH_{Ep}), examined its expression profiles under different conditions, and analyzed its role in adversity

and infection. Our results provide the first insights into the biological function of *E. piscicida* TrxH.

Materials and methods

Bacteria and growth conditions

Escherichia coli BL21 (DE3) was purchased from TransGen (Beijing, China). *E. coli* S17-1λpir was purchased from Biomedal (Sevilla, Spain). *E. piscicida* TX01 was isolated from diseased fish [33]. Bacteria were cultured in Luria–Bertani broth (LB) at 37 °C (for *E. coli*) or 28 °C (for *E. piscicida*). Where indicated, chloramphenicol, tetracycline, polymyxin B, and 2,2′-dipyridyl (Dp) were supplemented at the concentrations of 30 μg/mL, 15 μg/mL, 100 μg/mL, and 100 μM respectively.

Construction of the *trxH_{Ep}* mutation and its complementation

The primers used in this study are listed in Table 1. To construct the *trxH_{Ep}* knockout strain, TX01Δ*trxH*, in-frame deletion of a 261 bp segment (residues 24 to 110) of *trxH_{Ep}* was performed by overlap extension PCR as follows: the first overlap PCR was performed with the primer pair TrxHF1/R1, the second overlap PCR was performed with the primer pair TrxHF2/R2, and the fusion PCR was performed with the primer pair TrxHF1/R2. The PCR products amplified by primer pair TrxHF1/R2 were inserted into the suicide plasmid pDM4 [34] at the Bgl II site, resulting in pDMTrxH. S17-1λpir was transformed with pDMTrxH, and the transformants were conjugated with TX01 as described previously [35]. 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

Table 1 Primers used in this study

Primer	Sequences (5′ → 3′)
TrxhKOF1	GGATCCCTCAACTTGCCAATACCAT (BamHI)
TrxhKOR1	TCACCGATGCTAGCGGCTGTACGG
TrxhKOF2	CCGCTAGCATCGGTGAAACGTCGCCA
TrxhKOR1	GGATCCATGCCGATCGCCATCAGC (BamHI)
TrxhF3	GGATCCCTGAGTATTGCCGAGC (BamHI)
TrxhR3	CATATGGCGGTCAGAAAGTCAGAGA (HindIII)
TrxhRTF	AAACGGCAGGAGCGTGAA
TrxhRTR	GGGTGCTTTGCTGGCTGA
TrxARTF	GAGTATGCAGGGAAGCTGACCAT
TrxARTR	GGTTGGCATCCAGGAAGCTTTT
TrxCRTF	TCCCGCTGTACATACCACCA
TrxCRTR	GCGCCAGAAAGTCGATCAGTA
TrxhPF4	GATATCATTAGCCCGCAAAGAC (EcoRV)
TrxhPR4	GATATCATCCACCTTTTGTAAATGTTT (EcoRV)

(marker of pDM4) was analyzed by PCR, and the PCR products were subjected to DNA sequencing to confirm in-frame deletion. This strain was named TX01 Δ *trxH*. To construct the complementary strain TX01 Δ *trxHC*, *trxH_{Ep}* was amplified by PCR with primers TrxHF3/R3, and the following experimental operations were done as described previously [35].

Resistance to stressors (H₂O₂, iron deficiency and acidic pH) and to non-immune fish serum

TX01, TX01 Δ *trxH* and TX01 Δ *trxHC* were cultured in LB medium to exponential phase. To determine hydrogen peroxide and iron deficiency tolerance, LB agar plates (pH=7) supplemented with or without 1 mM H₂O₂ or 50 μ M 2,2'-dipyridyl (Dp) were streaked with 5 μ L TX01, TX01 Δ *trxH* and TX01 Δ *trxHC*, respectively. For acid resistance, LB agar plates with pH=7 or pH=5 were streaked with the three bacteria. The plate was incubated at 28 °C for 48 h, and bacterial growth was examined. For quantitative analysis, three strains were cultured in LB medium with three stress conditions for 24 h, then the populations of cultivated bacteria were counted by diluted plate. To determine hydrogen peroxide sensitivity, TX01 and TX01 Δ *trxH* were cultured in LB medium to exponential phase, then H₂O₂ was added to 5 mM, the bacterial growth was surveyed every hour. The experiment was performed three times.

TX01, TX01 Δ *trxH* and TX01 Δ *trxHC* were cultured in LB medium to exponential phase. Then the cells were washed with PBS and resuspended in PBS. Approximately 10⁵ bacterial cells were mixed with 50 μ L fish serum or PBS (control). After incubation with mild agitation at 28 °C for 60 min, the mixtures 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 survival rate was calculated as follows: [(number of serum-treated cells)/(number of control cells)] \times 100%. The experiment was performed three times.

Biofilm assay

TX01, TX01 Δ *trxH* and TX01 Δ *trxHC* were cultured in LB medium to exponential phase and diluted to 10⁵ CFU/mL. The diluted cells were transferred into a 96-well polystyrene plate (Nunc, Denmark) and incubated at 28 °C for 24 h without agitation. Then the wells were washed gently five times with PBS. The attached cells were treated with Bouin fixative for 1 h and stained with 1% crystal violet solution for 20 min. After the treatment, the unbound dye was removed by rinsing the plate several times with running water. The plate was air dried. The bound dye was eluted in ethanol, and the A₅₇₀ of eluates

was measured. The experiment was performed three times.

Invasion of eukaryotic cell lines

Interaction of bacteria with cultured fish cells

Examination of interactions between FG cells and *E. piscicida* was performed as described previously [36]. Briefly, TX01, TX01 Δ *trxH* and TX01 Δ *trxHC* were cultured in LB medium to exponential phase. FG cells were cultured in 96-well cell culture plates and 100 μ L bacteria (1 \times 10⁷ CFU/mL) were added to FG cells at a multiplicity of infection (MOI) of 10:1. After incubation at 23 °C for 0, 2, 4, 8 h, the plates were washed five times with PBS. To determine the number of bacterial cells associated with FG cells, the washed FG cells were lysed with 200 μ L of 1% (vol/vol) Triton X-100 in PBS, and the lysate was diluted serially and was plated on LB agar plates supplemented with polymyxin B. After incubation overnight at 28 °C, the numbers of colonies that appeared on the plates were counted. For microscopy observation, TX01 and TX01 Δ *trxH* were labeled with FITC (Solarbio, Beijing, China). FG cells were cultured in Glass Bottom cell Culture Dish and infected with FITC-labeled bacteria for 4 h, the plates were washed three times with PBS. The cells were fixed by polyformaldehyde, then the fluorescence of extracellular bacteria was quenched by adding trypan blue (2 mg/mL in PBS). After washing three times with PBS, the cells were observed with a confocal microscope (Olympus Fluoview FV1000).

Bacterial replication in macrophages

The experiment was performed as described by Sui et al. [15] with slight modification. RAW264.7, a murine monocyte-macrophage cell line, were cultured in Dulbecco's minimal Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C in 5% CO₂. Then 100 μ L bacteria (1 \times 10⁷ CFU/mL) were added to RAW264.7 cells at a multiplicity of infection (MOI) of 10:1, followed by incubation at 28 °C for 2 h. Extracellular *E. piscicida* was killed by adding gentamicin (100 μ g/mL) to the plate, followed by incubation at 28 °C for 1 h. The cells were washed three times with PBS and cultured in DMEM containing 10 μ g/mL gentamicin for 0, 2, 4, and 8 h. At each time point, the number of bacteria in macrophage was detected as described above. The experiment was performed three times.

Fish and experimental challenges for bacterial dissemination in vivo

Clinically healthy Japanese flounder (*Paralichthys olivaceus*) (average 14.2 g) were purchased from a commercial fish farm in Shandong. The fish were maintained at ~22 °C in aerated seawater and fed daily with commercial

dry pellets. Fish were acclimatized in the laboratory for 2 weeks before experimental manipulation. Before the experiment, fish were randomly sampled and examined for the presence of bacteria in the blood, liver, kidney, and spleen, and no bacteria were detected from the sampled fish. For tissue collection, fish were euthanized with an overdose of MS222 (tricaine methanesulfonate) (Sigma, USA) as previously described [37]. For tissue dissemination analysis, TX01, TX01 Δ *trxH*, and TX01 Δ *trxHC* were cultured in LB medium to an OD₆₀₀ of 0.8. The cells were washed with PBS and resuspended in PBS to 10⁶ CFU/mL. Japanese flounder were divided randomly into four groups and infected by intramuscular (i.m.) injection with 50 μ L TX01, TX01 Δ *trxH*, TX01 Δ *trxHC*, or PBS. The kidney and spleen were taken aseptically from the fish at 24 h and 48 h post-infection (hpi). Bacterial recovery from the tissues was determined as reported previously [35]. The rest of the infected fish were monitored daily for mortality for 20 days. The experiment was performed in triplicate.

Quantitative real-time reverse transcriptase-PCR (RT-qPCR) analysis of *trxH_{Ep}*, *trxA*, and *trxC* expression in wild TX01 strain and mutants under different environmental conditions

To examine *trxH_{Ep}* expression under in vitro conditions, TX01 was grown in LB medium with different pH (pH 5 or 7) at 28 °C, grown in LB medium with or without 2,2'-dipyridyl at 28 °C, and grown in LB medium with or without hydrogen peroxide at 28 °C. The bacteria were harvested by centrifugation and total RNA was extracted with HP Total RNA kit (Omega Bio-Tek, USA). The RNA was treated with DNase with the kit of RNase-Free DNase Set (Omega Bio-Tek, USA). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). RT-qPCR were carried out as reported previously [35]. To examine *trxH_{Ep}* expression during infection of host cells, TX01 was cultured in LB medium to an OD₆₀₀ of 0.8 and resuspended in PBS to 10⁶ CFU/mL. The resuspension was used to infect FG cells, a cell line derived from flounder gill cells, as described above [38]. The cells were harvested and used for total RNA extraction and RT-qPCR analysis of *trxH_{Ep}* expression as described above. The experiment was performed three times.

A *fur* mutant strain of *E. piscicida* was obtained in another study (data not published). The wild type *E. piscicida* TX01 and *fur* mutant strain were cultured in LB medium to the early exponential phase, then bacteria were harvested and total RNA was extracted. The expression of *trxH_{Ep}* expression in two strains were examined by RT-qPCR as described above.

To examine the expression of *trxA* and *trxC* in wild type *E. piscicida* and *trxH_{Ep}* knockout strain, TX01 and TX01 Δ *trxH* were cultured in LB medium with or without H₂O₂, cultured in LB medium with pH = 7 or pH = 5, and incubated with or without FG cells. The bacteria were harvested and total RNA was extracted. The expression of *trxA* and *trxC* expression in two strains were examined by RT-qPCR as described above.

Transcriptional regulation of the *trxH_{Ep}* promoter by Fur

The speculative promoter of *trxH_{Ep}* (the 510 bp DNA upstream of *trxH_{Ep}* operon) was cloned by primers TrxhPF4/TrxhPR4 and was inserted into the *Sma*I site of pSC11, a promoter probe plasmid [39], resulting in pSH510. pSH510 was introduced into *E. coli* DH5 α by transformation and cultured on X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) plate. DH5 α /pSH510 was then transformed with pT (control) and pTFur, which expressed the Fur and was constructed as described by Wang et al. [40], and cultured on X-gal plate. The transformants were subjected to β -galactosidase assay [40].

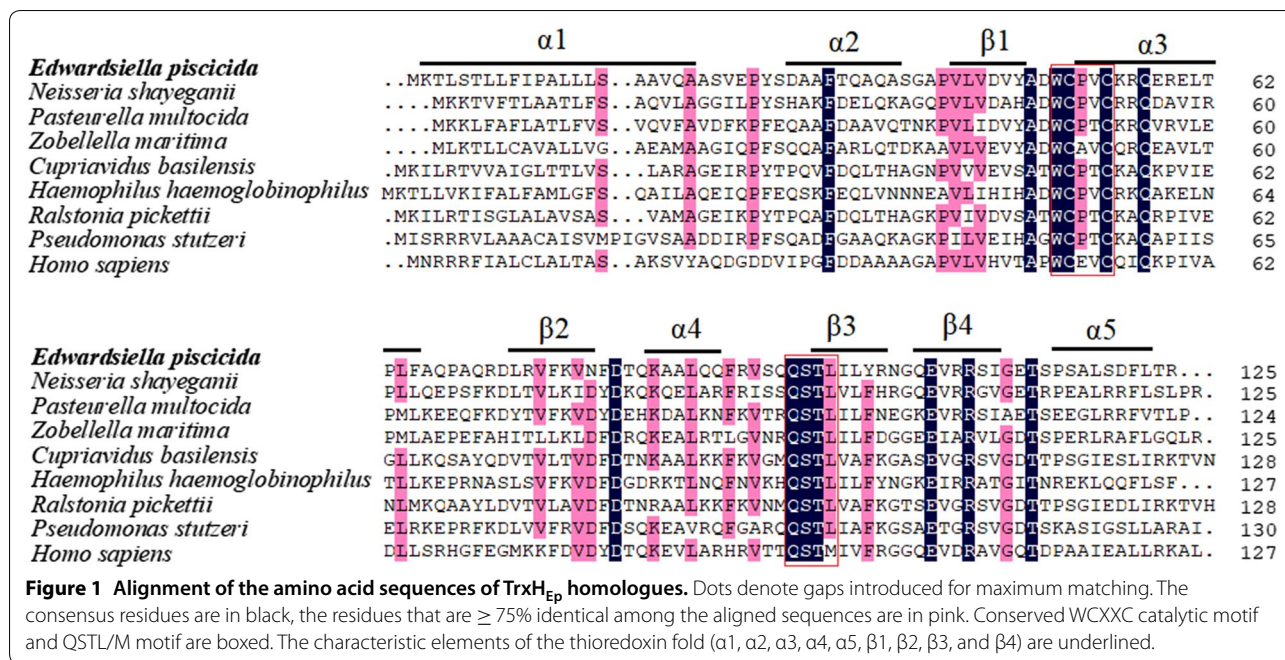
Statistical analysis

All statistical analyses were performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA), and statistical significance was defined as $P < 0.05$.

Results

Characterization of the *TrxH_{Ep}* sequence

trxH_{Ep} codes for a putative protein of 125 amino acids, which contains a thioredoxin domain. According to BLAST sequence analyses, TrxH_{Ep} shares 49.6%–38.5% overall sequence identities with the thioredoxin homology of a number of species including *Neisseria shayegani*, *Pasteurella multocida*, *Zobellella maritima*, *Cupriavidus basilensis*, *Haemophilus haemoglobinophilus*, *Ralstonia pickettii*, *Pseudomonas stutzeri*. Curiously, among the species with sequence identities of more than 30%, there are no common animal pathogenic bacteria even including *E. coli* (except *Edwardsiella*). TrxH_{Ep} also share sequence identity of 37.1% with thioredoxin of *Homo sapiens*. The WCXXC catalytic motif, the conserved active site of the TrxH family [41], exists in TrxH_{Ep}, suggesting that TrxH_{Ep} is one member of the TrxH protein family. Another conservative motif, QSTL/M, also exists in TrxH_{Ep} (Figure 1). Protein secondary structure prediction shows that the characteristic elements of the thioredoxin fold (α 1, α 2, α 3, α 4, α 5, β 1, β 2, β 3, and β 4) are found in TrxH_{Ep} (Figure 1).



Mutation of *trxH_{Ep}* has multiple effects

To examine its functional importance, the *trxH* gene of *E. piscicida* TX01 was knocked out by markerless in-frame deletion of a region encoding amino acid residues 24 to 110. The resulting mutant was named TX01Δ*trxH*. Meantime, the complementary strain TX01Δ*trxHC*, which is a genetic variant of TX01Δ*trxH* that expresses *trx* in trans from a plasmid, was constructed.

Effect on bacterial resistance against stressors

Growth analysis shows that when cultured in LB medium or LB agar medium, TX01Δ*trxH* and TX01 exhibited a comparative growth rate (Figure 2A and data not shown). This indicates that the *trxH_{Ep}* mutation had no effect on growth of *E. piscicida* in normal conditions. However, when cultured in an LB agar plate containing H₂O₂, TX01 grew normally, whereas TX01Δ*trxH* hardly grew, and the amount of TX01Δ*trxH* in H₂O₂ stress was significantly lower than TX01 (Figure 2D). Consistently, when bacteria grew to early logarithmic phase in LB medium and hydrogen peroxide was added to the medium, growth analysis shows that TX01Δ*trxH* exhibited a higher sensitivity to H₂O₂ than TX01 at a subsequent growth period (Figure 2E). When cultured under acidic condition, TX01Δ*trxH* grew poorer than TX01 and the amount of TX01Δ*trxH* was significantly lower than that of TX01 (Figure 2B). Similarly, when cultured under iron deficiency, the growth of TX01Δ*trxH* was obviously retarded, compared to TX01 (Figure 2C). In

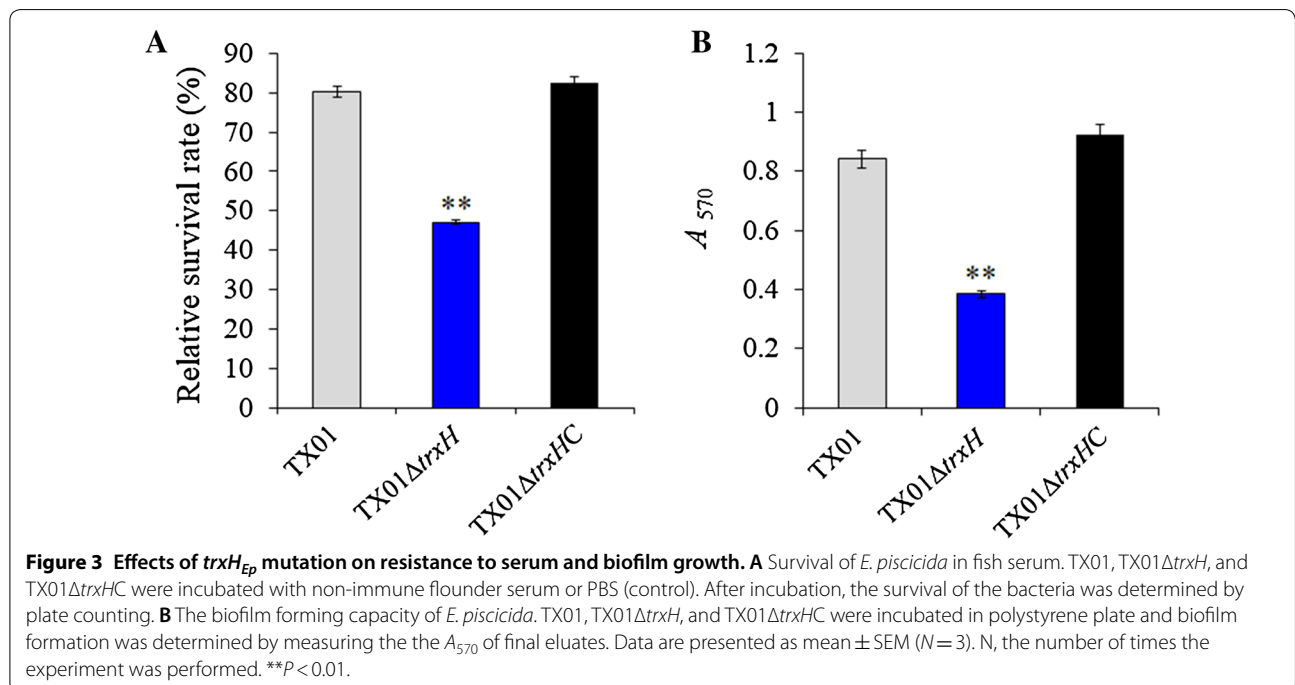
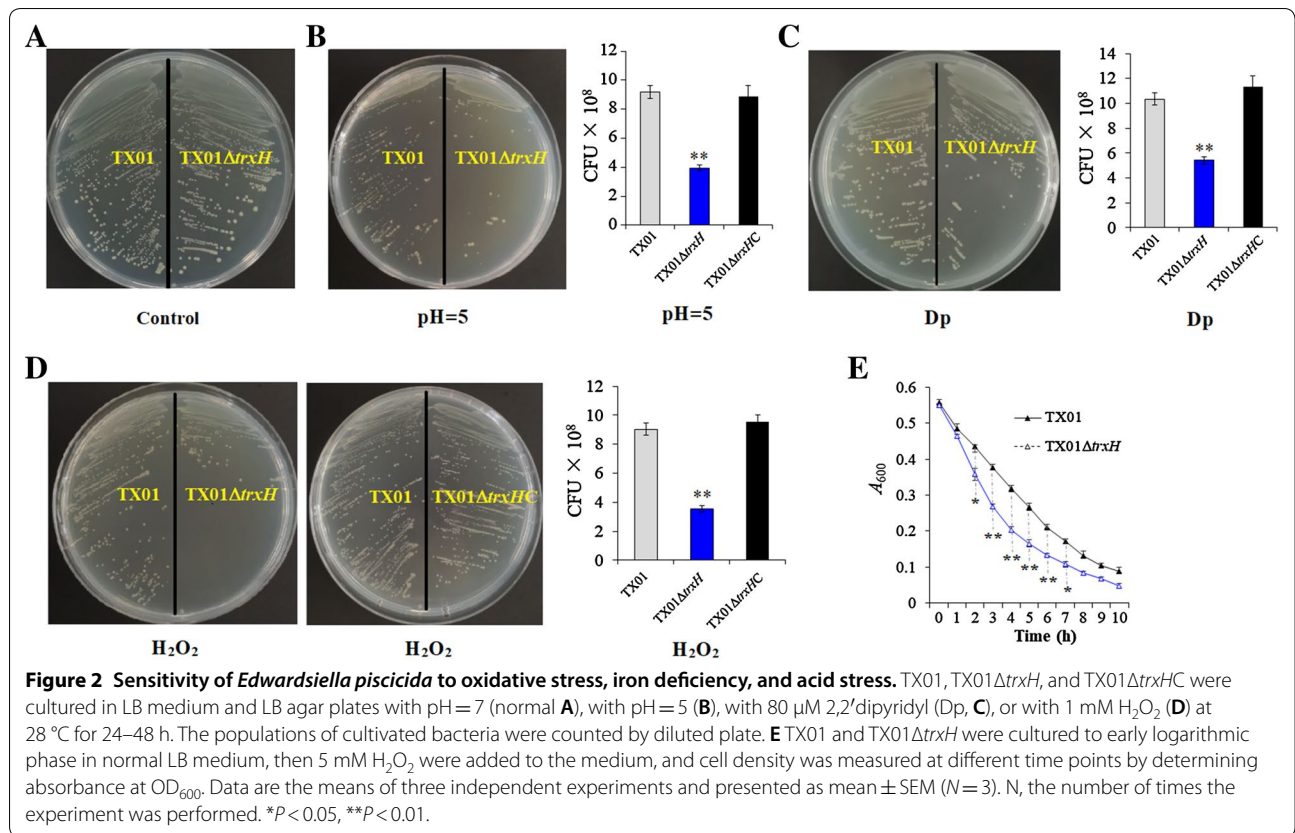
contrast to TX01Δ*trxH*, TX01Δ*trxHC* and wild TX01 exhibited comparative capability of resistance against stresses caused by H₂O₂, acid, and Dp (Figure 2 and data not shown).

Effect on bacterial resistance to non-immune fish serum

Resistance against host serum killing is an important part of the virulence mechanism in many pathogens, and *E. piscicida* is known to resist the bactericidal effect of fish serum [14]. To examine whether *trxH_{Ep}* mutation affects the ability of serum tolerance, TX01, TX01Δ*trxH*, and TX01Δ*trxHC* were incubated with non-immune flounder serum for 1 h, and the survival of bacteria was determined by plate count. The result shows that TX01 and TX01Δ*trxHC* exhibit apparent serum resistance, as 80.3% and 82.6% of the cells survived after incubation with flounder serum, respectively. However, TX01Δ*trxH* displayed only 47.1% of surviving cells after serum treatment, which is significantly lower than for TX01 (Figure 3A).

Effect on biofilm formation

Since *trxH_{Ep}* mutations have no effect on planktonic growth of *E. piscicida* in normal conditions, we wanted to know whether bacterial biofilm formation was influenced by deletion of *trxH_{Ep}*. The biofilm forming capacity of *E. piscicida* were analyzed and the result shows that biofilm growth of TX01Δ*trxH* was significantly slower (2.2-fold less) than that of TX01. And the biofilm growth of TX01Δ*trxHC* was similar to that of TX01 (Figure 3B).



Effect on cell invasion in vitro

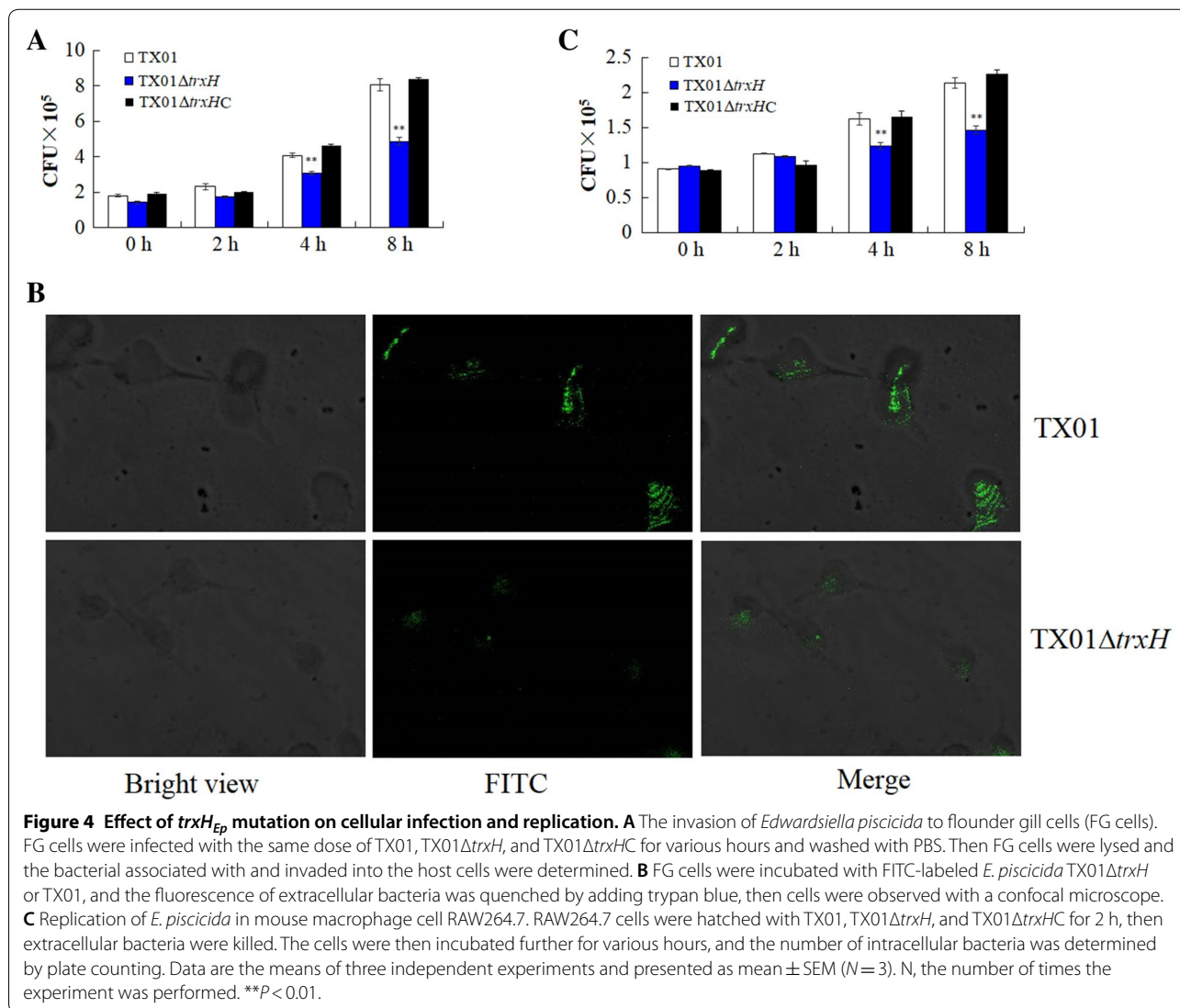
To examine whether *trxH_{Ep}* mutation played any role in the infectivity of TX01, cultured FG cells were incubated with the same dose of TX01, TX01 Δ *trxH*, or TX01 Δ *trxHC*. The bacterial cells associated with infection and those invading the host cells were enumerated. The results show that the amount of TX01 Δ *trxH* recovered from FG cells was significantly lower than that of TX01 at 4 and 8 hpi, and the amount of TX01 Δ *trxHC* was comparative to that of TX01 (Figure 4A). Consistently, microscopic analysis shows that fluorescence intensity of cells infected by FITC-labeled TX01 Δ *trxH* was significantly weaker than that of cells infected by FITC-labeled TX01 (Figure 4B), indicating that the ability to infect the host cell was declined when *trxH_{Ep}* was inactivated.

It is known that *E. piscicida* is able to survive and replicate in the mouse macrophage cell line RAW264.7 [15].

To examine whether *trxH_{Ep}* mutation played any role in the intracellular survival of TX01, cultured RAW264.7 cells were incubated with the same dose of TX01 or TX01 Δ *trxH*, and extracellular bacteria were killed. The cells were then incubated further for various hours, and the number of intracellular bacteria was determined by plate count. The results show that the number of TX01 Δ *trxH* recovered from the intracellular RAW264.7 cells was significantly lower than those of TX01 at 4 and 8 hpi, and the amount of TX01 Δ *trxHC* was comparative to that of TX01 (Figure 4C).

Effect on bacterial dissemination in the fish tissues in vivo and general virulence

To examine the effect of *trxH_{Ep}* mutation on tissue infectivity, flounder were infected with the same dose of TX01, TX01 Δ *trxH*, or TX01 Δ *trxHC*, and bacterial



recoveries from spleen and kidneys were determined at 24 and 48 hpi. The results show that bacterial recoveries from TX01Δ*trxH*-infected fish were significantly lower than those from TX01-infected fish at 24 hpi and 48 hpi, and bacterial recoveries from TX01Δ*trxHC*-infected fish were similar to those from TX01-infected fish (Figure 5A).

To examine the effect of *trxH_{Ep}* mutation on the overall bacterial virulence, Japanese flounder infected with TX01, TX01Δ*trxH*, or TX01Δ*trxHC* were used to monitor for mortality. The results show that at the end of the monitored period (20 days), the survival rate of TX01Δ*trxH*-infected fish was 43.3%, which was significantly higher than that of TX01-infected fish (13.3%) (Figure 5B).

Expression of *trxH_{Ep}*, *trxA* and *trxC* in wild TX01 strain and mutants under different environmental conditions

Expression of *trxH_{Ep}* is modulated by stress

Since thioredoxin plays a role in maintaining the intracellular redox homeostasis, and since it may participate in response to adversity, we determined the expression of *trxH_{Ep}* under normal conditions (i.e., cultured in LB medium), adverse conditions (i.e., cultured in LB medium with H₂O₂, Dp, or cultured in low pH LB medium), and infecting host cell condition by RT-qPCR. The results show that the expression of *trxH_{Ep}* was significantly enhanced when bacteria faced hydrogen peroxide pressure, acid pressure, iron deficiency, and an intracellular environment, compared to the expression of *trxH_{Ep}* in normal condition (Figures 6A and B).

Expression of *trxH_{Ep}* is regulated by Fur (Ferric uptake regulator)

In a previous study of *E. piscicida*, we obtained a *fur* mutant strain, which exhibited much higher virulence than wild type *E. piscicida* strain TX01 (data not published). Proteomic analysis shows that the expression of TrxH was significantly up-regulated in the *fur* mutant strain, compared to the wild type strain (data not shown). So we detected the expression of *trxH_{Ep}* at the mRNA level. RT-qPCR shows that the expression of *trxH_{Ep}* in the *fur* mutant strain was 2.4-fold higher than that of *trxH_{Ep}* in the wild type strain (Figure 6C). Subsequently, we detected the regulatory effect of Fur on promoter activity of *trxH_{Ep}*. The speculative promoter of *trxH_{Ep}*, H510, was cloned to a promoter probe plasmid pSC11 and introduced into DH5α by transformation, resulting in DH5α/pSH510. When DH5α/pSH510 was cultured in LB agar plate with X-gal, the bacterial colonies were blue, which indicates that H510 has promoter activity. DH5α/pSH510 was then transformed with pTFur and pT (control). On the X-gal plate, the blue of DH5α/pSH510/pTFur was obviously weak, compared with DH5α/pSH510/pT (Figure 6D). β-Galactosidase assays show that galactosidase activity produced by DH5α/pSH510/pTFur was significantly lower (3.1 fold) than that produced by DH5α/pSH510/pT. These results indicate that Fur negatively regulated transcription of *trxH_{Ep}*.

Expression of *trxA* and *trxC* in TX01Δ*trxH*

There are two other thioredoxins, TrxA and TrxC, in *E. piscicida*, and their functions are unknown. To detect

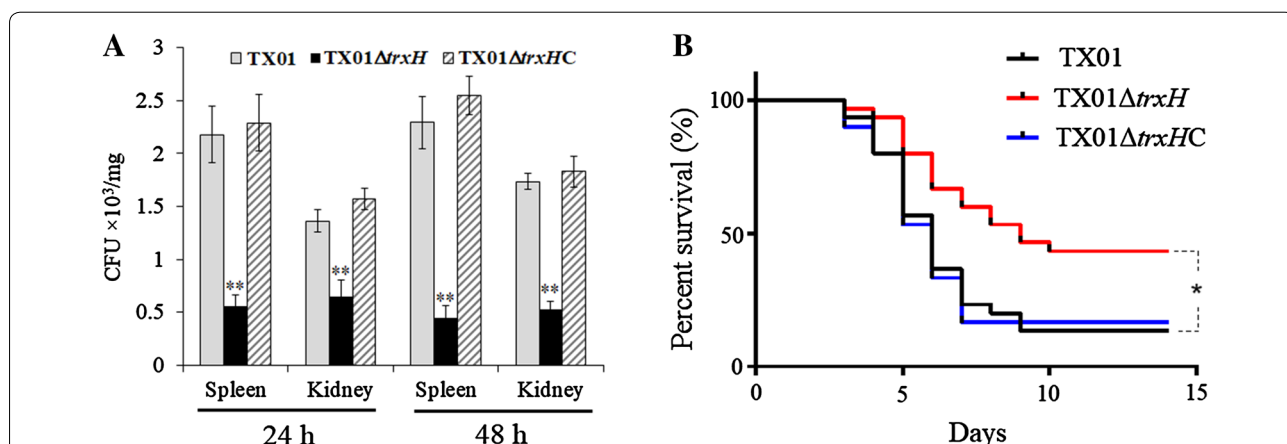


Figure 5 In vivo infection of *Edwardsiella piscicida* in Japanese flounder. **A** Bacterial dissemination in the fish tissues. Flounder were infected with the same dose of TX01, TX01Δ*trxH*, or TX01Δ*trxHC*, bacterial recoveries from spleen and kidney were determined by plate count at 24 and 48 hpi. **B** Host mortality induced by *E. piscicida*. Flounder were infected with equivalent doses of TX01, TX01Δ*trxH*, and TX01Δ*trxHC*, and accumulated mortality were monitored for a period of 20 days (only 15 days are shown since no more deaths occurred after 15 days). Significance between the survivals of wild type and mutant infected fish was determined with logrank test. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. *P < 0.05, **P < 0.01.

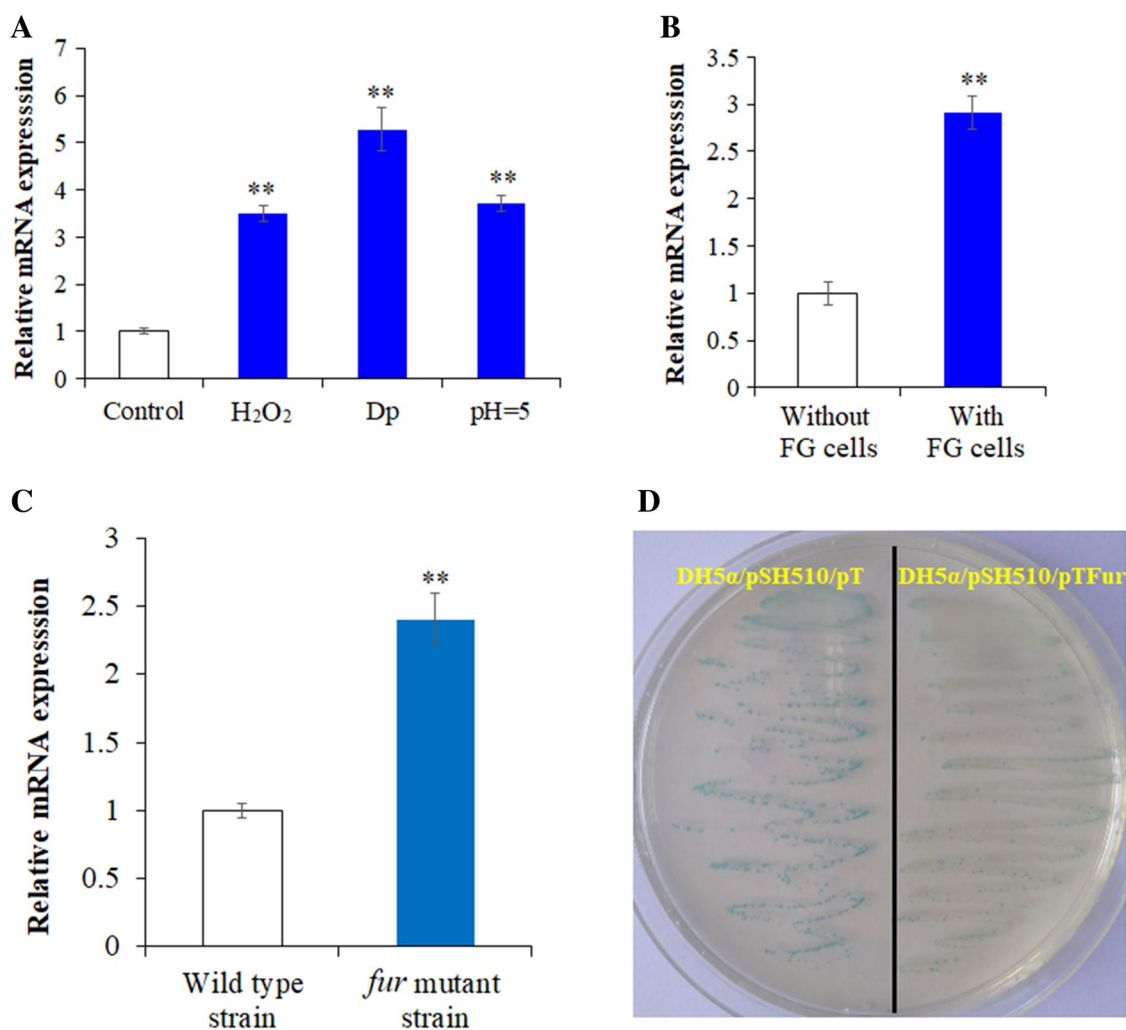


Figure 6 Expression analysis of *trxB_p*. **A** Expression of *trxB_p* under different conditions. RT-qPCR was performed with total RNA extracted from *Edwardsiella piscicida* TX01 cultured in normal LB medium (control, pH = 7), in hydrogen peroxide condition (normal LB medium with H₂O₂), in acid condition (pH = 5), in iron limitation condition (with the iron chelator 2,2'-dipyridyl, Dp), Expression level of *trxB_p* in normal LB medium was set as 1. **B** RT-qPCR was performed with total RNA extracted from TX01 incubated with or without (control) FG cells. Expression level of *trxB_p* without FG cells was set as 1. **C** RT-qPCR was performed with total RNA extracted from wild type TX01 and *fur* mutant strain cultured in normal LB medium. Expression level of *trxB_p* in wild type TX01 strain was set as 1. **D** DH5α/pSH510/pTFur and DH5α/pSH510/pT were streaked and cultured on LB plate with X-gal, kanamycin, and ampicillin. Data are presented as mean ± SEM (N = 3). N, the number of times the experiment was performed. **P < 0.01.

whether TrxA and TrxC undertake more functions when the TrxH function was deactivated, we examined the expression of *trxA* and *trxC* in TX01Δ*trxH*. The results show that the deletion of *trxH* has no effect on the expression of *trxC* in normal conditions. However, when facing oxidative stress, acid pressure, or infecting host cell, the expression of *trxC* in TX01Δ*trxH* was significantly up-regulated, compared to that in TX01. On the contrary, the deletion of *trxH* significantly induced 4.4-fold expression of *trxA* in normal conditions, but *trxA* expression exhibited no difference between TX01 and TX01Δ*trxH* in the presence of hydrogen peroxide. Under

an acidic environment and infecting host cell, the mRNA level of *trxA* in TX01Δ*trxH* was 3.1- and 4.3-fold high, compared to the level of *trxA* in TX01 (Figure 7). These findings suggest that there is likely a complex relationship of functional complementation or expression regulation between TrxH and another two thioredoxins, TrxA and TrxC, of *E. piscicida*.

Discussion

Thioredoxins play major roles in protection of cells against toxic oxygen species as well as maintaining the intracellular thio-disulfide balance in bacteria [42]. In

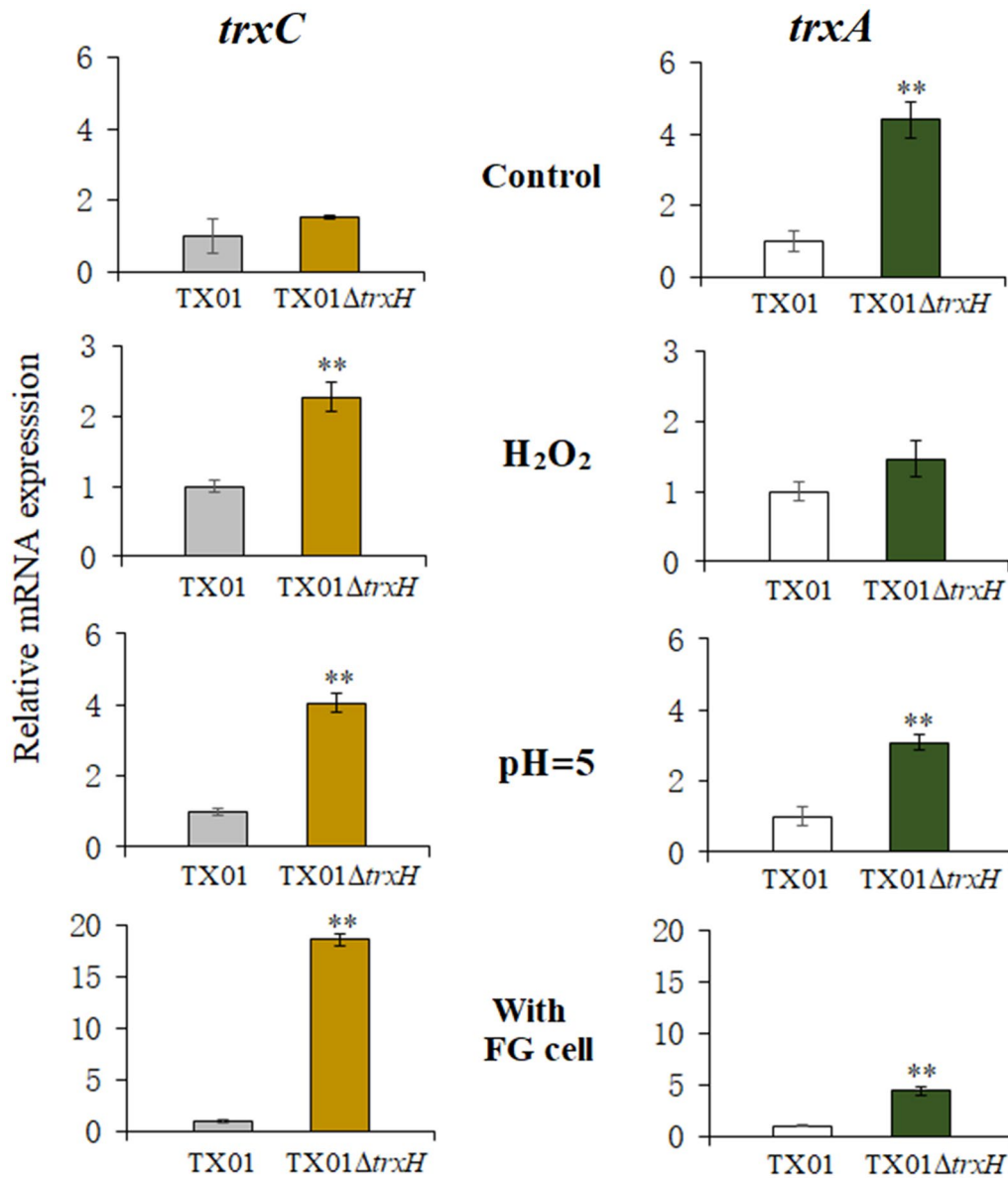


Figure 7 Expression of *trxC* and *trxA* in wild type *Edwardsiella piscicida* TX01 strain and TX01Δ*trxH* strain. Total RNA was extracted from *E. piscicida* TX01 and TX01Δ*trxH* cultured in normal LB medium (control, pH = 7), in hydrogen peroxide condition (with H₂O₂), in acid condition (pH = 5), or incubating with FG cells, respectively. Expressions of *trxC* and *trxA* were analyzed by RT-qPCR. Data are presented as mean ± SEM (N = 3). N, the number of times the experiment was performed. **P < 0.01.

E. piscicida, the function of thioredoxins is completely unknown. Moreover, we did not find any reports about the function of thioredoxin H in pathogen bacteria. In this study, a thioredoxin H homologue, TrxH, was identified and characterized from *E. piscicida*. TrxH_{Ep} contains an unusual motif (CPVC), not the typical Trx motif (CGPC), which is found in most Trx proteins. It is not known how this affects its activity or its roles.

It is reported that the expression of *trx* in many bacteria is induced by oxidative stress [20]. Similarly, in *E. piscicida*, the expression of *trxH*_{Ep} was significantly enhanced in the presence of H₂O₂. In other adverse conditions including acid stress and iron deficiency, *trxH*_{Ep} expression is upregulated. When bacteria infect host cells, the expression of *trxH*_{Ep} also increases. Under these conditions, the highest induction of *trxH*_{Ep} occurred with iron deficiency, in which Fur exists predominately as a

biologically inactive apoprotein [43]. Consistently, the expression of *trxH_{Ep}* in the *fur* mutant strain was higher than that in the wild type strain. As far as we know, this is the first report that Trx expression was regulated by Fur.

Besides TrxH, there are another two thioredoxin, TrxA and TrxC in *E. piscicida*. When *trxH_{Ep}* was inactivated, the expression of *trxC* did not change under normal conditions. However, under stress or infecting host cell conditions, *trxC* expression was significantly induced. As a chaperone, TrxC maybe undertook more roles in adverse conditions especially when *trxH* was inactivated in *E. piscicida*. On the contrary, the expression of *trxA* was directly or indirectly regulated by TrxH, which is sustained by the fact that Trx possesses the property of regulating gene expression [28]. However, in the presence of hydrogen peroxide *trxA* expression did not appear significantly different between wild TX01 and mutant TX01Δ*trxH*. These findings show that there is likely to be a complex relationship between the three *trx* genes, and further research needs to be performed in the future.

Since the expression of *trxH_{Ep}* is induced by adverse conditions, it probably participates in stress resistance. Our results show that when *trxH_{Ep}* was inactive, the mutant TX01Δ*trxH* exhibited a higher sensitivity to H₂O₂, which indicates that *trxH_{Ep}* plays an important role in tolerance to oxidative stress. Similar results were reported in other bacteria. In *H. pylori*, the Δ*trxC* mutant significantly decreased the ability of tolerance to oxygen exposure [24]. In *Listeria monocytogenes*, deletion of *trxA* markedly compromised tolerance of the pathogen to the thiol-specific oxidizing agent diamide [20]. Since Trx is involved in a series of cellular functions [26], we speculate that *trxH_{Ep}* participates in coping with other adversities, and our results confirm that *trxH_{Ep}* was involved in tolerance to acid stress and iron deficiency stress, which are similar to the adverse environment in the host.

It is generally known that Trx donates electrons to the disulfide bond system and helps isomerization of incorrectly paired disulfide bonds [20, 44]. Since many bacterial virulence factors require stable disulfide bonds for proper folding and function, Trx is closely related to infection of bacteria [45, 46]. It is reported that TrxA is essential for motility and contributes to host infection of *L. monocytogenes* [20]. In *H. pylori*, the Δ*trxC* mutant lacks mouse colonization ability, while the ability to colonize mouse stomachs is significantly reduced in the Δ*trxA* mutant [24]. In *Magnaporthe oryzae*, Trx1 and Trx2 also play important roles in bacterial pathogenesis [47]. In pathogenic fungus *Paracoccidioides lutzii*, Trx1 contributes to this organism's virulence [19]. In this study, we also examined the effect of *trxH_{Ep}* on *E. piscicida* pathogenicity. It is known that biofilm

formation and serum resistance are important parts of the virulence in multiple pathogens such as *E. piscicida* [17, 35] and *H. pylori* [48, 49]. In the latter, the inability to acquire iron from serum has been suggested to block *H. pylori* virulence by weakening its growth and survival in the serum [48]. Our results show that *trxH_{Ep}* mutation had no effect on growth of *E. piscicida*, but harmed bacterial biofilm growth. Similarly, the capability of serum resistance was significantly declined when *trxH_{Ep}* was inactivated. Consistently, upon infection with host cells, TX01Δ*trxH* compared to TX01, shows an impaired ability to infect cells and to propagate in cells, indicating that *trxH* is required for bacterial invasion of host cells and host tissues. These observations suggest that *trxH_{Ep}* may play a role during bacterial invasion.

In conclusion, this study identified and characterized thioredoxin TrxH_{Ep} from fish pathogen *E. piscicida*. Our results indicate that TrxH_{Ep} possesses conservative thioredoxin motifs and that the *trxH_{Ep}* expression seems to be up-regulated under the pressure of several stressors, including iron deficiency. Regarding this fact, our results suggest the potential role of Fur for regulating *trxH_{Ep}* expression. TrxH_{Ep} not only played an important role in coping with adverse circumstances, but functioned as a factor that is essential to bacterial infection both at the cellular level and in a live fish model. This is the first study of TrxH in fish pathogen, and the results suggest that TrxH_{Ep} exerts pleiotropic effects on the pathogenesis of *E. piscicida*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BYW performed the experiments and analyzed the data. HQH and SL were responsible for microbial cultivation and some data analysis. PT, HFD, JAX provided valuable assistance to sample preparation and research ideas. DMS and YHH designed the experiment and wrote the paper. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (41476138), the Financial Fund of the Ministry of Agriculture and Rural Affairs, P. R. of China (NFZX2018), and the Central Public-interest Scientific Institution Basal Research Fund for Chinese Academy of Tropical Agricultural Sciences (1630052018005).

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Ethics approval and consent to participate

The study was approved by the ethics committee of Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences. Efforts were taken to ensure that all research animals received good care and humane treatment.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 5 December 2018 Accepted: 26 February 2019

Published online: 15 April 2019

References

- Abayneh T, Colquhoun DJ, Sørum H (2013) *Edwardsiella piscicida* sp. nov., a novel species pathogenic to fish. *J Appl Microbiol* 114:644–654
- Liu Y, Zhao L, Yang M, Yin K, Zhou X, Leung KY, Liu Q, Zhang Y, Wang Q (2017) Transcriptomic dissection of the horizontally acquired response regulator *EsrB* reveals its global regulatory roles in the physiological adaptation and activation of T3SS and the cognate effector repertoire in *Edwardsiella piscicida* during infection toward turbot. *Virulence* 8:1355–1377
- Mohanty BR, Sahoo PK (2007) Edwardsiellosis in fish: a brief review. *J Biosci* 32:1331–1344
- Xu TT, Zhang XH (2014) *Edwardsiella tarda*: an intriguing problem in aquaculture. *Aquaculture* 431:129–135
- Katharios P, Kokkari C, Dourala N, Smyrli M (2016) First report of edwardsiellosis in cage-cultured sharpnose sea bream, *Diplodus puntazzo* from the Mediterranean. *BMC Vet Res* 11:155
- Loch TP, Hawke J, Reichley S, Faisal M, Del Piero F, Griffin MJ (2017) Outbreaks of edwardsiellosis caused by *Edwardsiella piscicida* and *Edwardsiella tarda* in farmed barramundi (*Lates calcarifer*). *Aquaculture* 481:202–210
- Shafiei S, Viljamaa-Dirks S, Sundell K, Heinikainen S, Abayneh T, Wiklund T (2016) Recovery of *Edwardsiella piscicida* from farmed whitefish *Coregonus lavaretus* (L.), in Finland. *Aquaculture* 454:19–26
- Park SB, Aoki T, Jung TS (2012) Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. *Vet Res* 43:67
- Chakraborty S, Sivaraman J, Leung KY, Mok YK (2011) The two-component PhoBPhoR regulatory system and ferric uptake regulator sense phosphate and iron to control virulence genes in type III and VI secretion systems of *Edwardsiella tarda*. *J Biol Chem* 286:39417–39430
- Leung KY, Siame BA, Tenkink BJ, Noort RJ, Mok YK (2012) *Edwardsiella tarda*—virulence mechanisms of an emerging gastroenteritis pathogen. *Microbes Infect* 14:26–34
- Li MF, Wang C, Sun L (2015) *Edwardsiella tarda* MliC, a lysozyme inhibitor that participates in pathogenesis in a manner that parallels Ivy. *Infect Immun* 83:583–590
- Yin K, Guan Y, Ma R, Wei L, Liu B, Liu X, Zhou X, Ma Y, Zhang Y, Waldor MK, Wang Q (2018) Critical role for a promoter discriminator in *RpoS* control of virulence in *Edwardsiella piscicida*. *PLoS Pathog* 14:e1007272
- Cui S, Xiao J, Wang Q, Zhang Y (2018) H-NS binding to *evpB* and *evpC* and repressing T6SS expression in fish pathogen *Edwardsiella piscicida*. *Arch Microbiol* 198:653–661
- Li MF, Sun L, Li J (2015) *Edwardsiella tarda* evades serum killing by preventing complement activation via the alternative pathway. *Fish Shellfish Immunol* 43:325–329
- Sui ZH, Xu H, Wang H, Jiang S, Chi H, Sun L (2017) Intracellular trafficking pathways of *Edwardsiella tarda*: from clathrin- and caveolin-mediated endocytosis to endosome and lysosome. *Front Cell Infect Microbiol* 7:400
- Cheng ZX, Gong QY, Wang Z, Chen ZG, Ye JZ, Li J, Wang J, Yang MJ, Ling XP, Peng B (2017) *Edwardsiella tarda* tunes tricarboxylic acid cycle to evade complement-mediated killing. *Front Immunol* 8:1706
- Li MF, Sun L (2018) *Edwardsiella tarda* Sip2: a serum-induced protein that is essential to serum survival, acid resistance, intracellular replication, and host infection. *Front Microbiol* 9:1084
- Staerck C, Gastebois A, Vandeputte P, Calenda A, Larcher G, Gillmann L, Papon N, Bouchara JP, Fleury MJJ (2017) Microbial antioxidant defense enzymes. *Microb Pathog* 110:56–65
- Cintra LC, Domingos FC, Lima YAR, Barbosa MS, Santos RS, Faria FP, Jesuino RSA (2017) Molecular cloning, expression and insulin reduction activity of a thioredoxin 1 homologue (TRX1) from the pathogenic fungus *Paracoccidioides lutzii*. *Int J Biol Macromol* 103:683–691
- Cheng C, Dong Z, Han X, Wang H, Jiang L, Sun J, Yang Y, Ma T, Shao C, Wang X, Chen Z, Fang W, Freitag NE, Huang H, Song H (2017) Thioredoxin a is essential for motility and contributes to host infection of *Listeria monocytogenes* via redox interactions. *Front Cell Infect Microbiol* 7:287
- Holmgren A (1989) Thioredoxin and glutaredoxin systems. *J Biol Chem* 264:13963–13966
- Yoshida T, Oka S, Masutani H, Nakamura H, Yodoi J (2003) The role of thioredoxin in the aging process: involvement of oxidative stress. *Antioxid Redox Signal* 5:563–570
- Peng H, Zhang Y, Trinidad JC, Giedroc DP (2018) thioredoxin profiling of multiple thioredoxin-like proteins in *Staphylococcus aureus*. *Front Microbiol* 9:2385
- Kuhns LG, Wang G, Maier RJ (2015) Comparative roles of the two *Helicobacter pylori* thioredoxins in preventing macromolecule damage. *Infect Immun* 83:2935–2943
- Liao X, Yang F, Li H, So PK, Yao Z, Xia W, Sun H (2017) Targeting the thioredoxin reductase-thioredoxin system from *Staphylococcus aureus* by silver ions. *Inorg Chem* 56:14823–14830
- Lu J, Holmgren A (2014) The thioredoxin antioxidant system. *Free Radic Biol* 66:75–87
- Meyer Y, Buchanan BB, Vignols F, Reichheld JP (2009) Thioredoxins and glutaredoxins: unifying elements in redox biology. *Annu Rev Genet* 43:335–367
- Zeller T, Klug G (2006) Thioredoxins in bacteria: functions in oxidative stress response and regulation of thioredoxin genes. *Naturwissenschaften* 93:259–266
- Laurent TC, Moore EC, Reichard P (1964) Enzymatic synthesis of deoxyribonucleotides. IV. Isolation and characterization of thioredoxin, the hydrogen donor from *Escherichia coli* B. *J Biol Chem* 239:3436–3444
- Alamuri P, Maier RJ (2006) Methionine sulfoxide reductase in *Helicobacter pylori*: interaction with methionine-rich proteins and stress induced expression. *J Bacteriol* 188:5839–5850
- McGee DJ, Kumar S, Viator RJ, Bolland JR, Ruiz J, Spadafora D, Testerman TL, Kelly DJ, Pannell LK, Windle HJ (2006) *Helicobacter pylori* thioredoxin is an arginase chaperone and guardian against oxidative and nitrosative stresses. *J Biol Chem* 281:3290–3296
- Wang Q, Yang M, Xiao J, Wu H, Wang X, Lv Y, Xu L, Zheng H, Wang S, Zhao G, Liu Q, Zhang Y (2009) Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. *PLoS ONE* 4(10):e7646
- Wang HL, Zhang M, Xiao ZZ, Sun L (2009) Genetic mechanisms multi-antimicrobial resistance in a pathogenic *Edwardsiella tarda* strain. *Aquaculture* 289:134–139
- Milton DL, O'Toole R, Horstedt P, Wolf-Watz H (1996) Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol* 178:1310–1319
- Hu YH, Zhou HZ, Jin QW, Zhang J (2016) The serine protease autotransporter Tsh contributes to the virulence of *Edwardsiella tarda*. *Vet Microbiol* 189:68–74
- Hu YH, Liu CS, Hou JH, Sun L (2009) Identification, characterization, and molecular application of a virulence-associated autotransporter from a pathogenic *Pseudomonas fluorescens*. *Appl Environ Microbiol* 75:4333–4340
- Wang GH, Wang JJ, Yue B, Du X, Du HH, Zhang M, Hu YH (2018) High mobility group box 2 of black rockfish *Sebastes schlegelii*: gene cloning, immunoregulatory properties and antibacterial effect. *Fish Shellfish Immunol* 84:719–725
- Hu YH, Dang W, Sun L (2016) A TonB-dependent outer membrane receptor of *Pseudomonas fluorescens*: virulence and vaccine potential. *Arch Microbiol* 194:795–802
- Hu Y, Wang H, Zhang M, Sun L (2009) Molecular analysis of the copper-responsive CopRSCD of a pathogenic *Pseudomonas fluorescens* strain. *J Microbiol* 47:277–286

40. Wang F, Cheng S, Sun K, Sun L (2008) Molecular analysis of the fur (ferric uptake regulator) gene of a pathogenic *Edwardsiella tarda* strain. *J Microbiol* 46:350–355
41. Chen IH, Chen HT, Huang YP, Huang HC, Shenkwen LL, Hsu YH, Tsai CH (2018) A thioredoxin NbTRXh2 from *Nicotiana benthamiana* negatively regulates the movement of Bamboo mosaic virus. *Mol Plant Pathol* 19:405–417
42. Matsuzawa A (2017) Thioredoxin and redox signaling: roles of the thioredoxin system in control of cell fate. *Arch Biochem Biophys* 617:101–105
43. Troxell B, Hassan HM (2013) Transcriptional regulation by ferric uptake regulator (Fur) in pathogenic bacteria. *Front Cell Infect Microbiol* 3:59
44. Rozhkova A, Stirnimann CU, Frei P, Grauschopf U, Brunisholz R, Grütter MG, Capitani G (2004) Structural basis and kinetics of inter- and intramolecular disulfide exchange in the redox catalyst DsbD. *EMBO J* 23:1709–1719
45. Negrea A, Bjur E, Puiaç S, Ygberg SE, Aslund F, Rhen M (2009) Thioredoxin 1 participates in the activity of the *Salmonella enterica* serovar Typhimurium pathogenicity island 2 type III secretion system. *J Bacteriol* 191:6918–6927
46. Pereira DMS, Mendes SJF, Alawi K, Thakore P, Aubdool A, Sousa NCF, da Silva JFR, Castro JA Jr, Pereira PIC, Silva LCN, Grisotto MAG, Monteiro-Neto V, Costa SKP, da Costa R, Calixto JB, Brain SD, Fernandes ES (2018) Transient receptor potential canonical channels 4 and 5 mediate *Escherichia coli*-derived thioredoxin effects in lipopolysaccharide-injected mice. *Oxid Med Cell Longev* 2018:4904696
47. Zhang S, Jiang C, Zhang Q, Qi L, Li C, Xu JR (2016) Thioredoxins are involved in the activation of the PMK1 MAP kinase pathway during appressorium penetration and invasive growth in *Magnaporthe oryzae*. *Environ Microbiol* 18:3768–3784
48. Hathroubi S, Servetas SL, Windham I, Merrell DS, Ottemann KM (2018) *Helicobacter pylori* biofilm formation and its potential role in pathogenesis. *Microbiol Mol Biol Rev* 82:e00001–18
49. Pich OQ, Merrell DS (2013) The ferric uptake regulator of *Helicobacter pylori*: a critical player in the battle for iron and colonization of the stomach. *Future Microbiol* 8:725–738

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