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Universal stress proteins contribute *Edwardsiella piscicida* adversity resistance and pathogenicity and promote blocking host immune response

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ABSTRACT

Universal stress proteins (Usps) exist ubiquitously in bacteria and other organisms. Usps play an important role in adaptation of bacteria to a variety of environmental stresses. There is increasing evidence that Usps facilitate pathogens to adapt host environment and are involved in pathogenicity. *Edwardsiella piscicida* (formerly included in *E. tarda*) is a severe fish pathogen and infects various important economic fish including tilapia (*Oreochromis niloticus*). In *E. piscicida*, a number of systems and factors that are involved in stress resistance and pathogenesis were identified. However, the function of Usps in *E. piscicida* is totally unknown. In this study, we examined the expressions of 13 *usp* genes in *E. piscicida* and found that most of these *usp* genes were up-regulated expression under high temperature, oxidative stress, acid stress, and host serum stress. Particularly, among these *usp* genes, *usp13*, exhibited dramatically high expression level upon several stress conditions. To investigate the biological role of *usp13*, a markerless *usp13* in-frame mutant strain, TX01Δ*usp13*, was constructed. Compared to the wild type TX01, TX01Δ*usp13* exhibited markedly compromised tolerance to high temperature, hydrogen peroxide, and low pH. Deletion of *usp13* significantly retarded bacterial biofilm growth and decreased resistance against serum killing. Pathogenicity analysis showed that the inactivation of *usp13* significantly impaired the ability of *E. piscicida* to invade into host cell and infect host tissue. Introduction of a *trans*-expressed *usp13* gene restored the lost virulence of TX01Δ*usp13*. In support of these results, host immune response induced by TX01 and TX01Δ*usp13* was examined, and the results showed reactive oxygen species (ROS) levels in TX01Δ*usp13*-infected macrophages were significantly higher than those in TX01-infected cells. The expression level of several cytokines (IL-6, IL-8, IL-10, TNF-α, and CC2) in TX01Δ*usp13*-infected fish was significantly higher than that in TX01-infected fish. These results suggested that the deletion of *usp13* attenuated the ability of bacteria to overcome the host immune response to pathogen infection. Taken together, our study indicated Usp13 of *E. piscicida* was not only important participant in adversity resistance, but also was essential for *E. piscicida* pathogenicity and contributed to block host immune response to pathogen infection.

1. Introduction

Tilapia (*Oreochromis niloticus*) is an important economic fish farmed worldwide [1]. Currently, tilapia has suffered serious diseases, and the frequent occurrence of edwardsiellasis has seriously restricted the healthy development of tilapia aquaculture industry. It has been confirmed that *Edwardsiella piscicida* is one of the main pathogens of tilapia [2,3]. *E. piscicida* (formerly included in E. tarda) [4,5], a Gram-negative pathogen, belongs to Enterobacteriaceae family, which have a board range of host including fish, birds, even human [6,7]. *E. piscicida* is recognized to cause edwardsiellosis which is a severe systemic disease to many bred fish, and result in important economic losses in aquaculture [8–12]. Functional studies in *E. piscicida* have identified a number of systems and factors that are involved in stress resistance and pathogenesis [13]. Type III (T3SS) and type VI (T6SS) secretion systems are considered to be vital to *E. piscicida* invasion and intracellular replication [14–16]. A large number of other virulence factors/systems, such as two-component regulatory system [17], LuxS/AI-2 quorum

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sensing system [18], RNA-binding protein Hfq [19], serum-induced protein Sip2 [20], heat shock proteins [21], and so on, are known to be involved in *E. piscicida* stress resistance or pathogenicity. Recently, small RNA and thioredoxins were found to be essential for coping with adverse circumstance and contribute to host infection of *E. piscicida* [22,23]. There are a series of proteins called universal stress proteins (Usps) in *E. piscicida* [24]. However, the function of these Usps in *E. piscicida* is totally unknown.

Usps are a class of stress-related conserved proteins, which are present in archaea, bacteria, fungi, plants, and even some invertebrates [25–27]. Under environmental stress, most cells express Usps for their survival and Usps play an important role in response various adversity. In stress conditions such as heat shock, the presence of oxidants, acid environment, high salinity environment, starvation for nutrients, macromolecular damage, or other stress which may arrest cell growth, Usps are overproduced and aid the organism in surviving in such uncomfortable conditions through a variety of mechanisms [27–33]. By and large, the host environment can be a source of numerous stresses to a pathogen. There is increasing evidence that Usps facilitate the adaption of bacterial pathogens to the human host environment, thereby facilitating colonization and pathogenicity [34]. *Mycobacterium tuberculosis* was one of the first pathogens associated with a role for Usps in the infection process [35]. It was reported that Usp Rv2623 was upregulated in intracellular *M. tuberculosis* cells [36] and was required for the entry of the tubercle bacillus into the chronic phase of infection in the host [37]. In *Acinetobacter baumannii*, UspA plays a pivotal role in stress response and is essential for pneumonia and sepsis pathogenesis [38]. In *Salmonella*, Usps are contribute to growth arrest, stress and virulence [39]. In *Edwardsiella ictaluri*, Usps are important factors contributing virulence in catfish [40].

In this study, we examined the expressions of 13 *usp* genes in *E. piscicida* and found that most of these *usp* genes expression were upregulated under high temperature, oxidative stress, acid stress, and host serum stress. One of *usp* genes, *usp13*, was dramatically upregulated upon several stress conditions. Usp13 is homologue with CpxP, which is a small periplasmic protein. The *cpxP* gene was originally identified as its expression was upregulated upon stress response in *Escherichia coli* [41]. The envelope stress response system of Gram-negative bacteria is an important part of bacterial resistance to environmental stresses. CpxP is thought to interact with the two-component sensor kinase, CpxA, to inhibit induction of the Cpx envelope stress response in the absence of protein misfolding [42]. Except functioning as universal stress protein, CpxP was reported as a virulence factor. CpxP was crucial for virulence of uropathogenic *E. coli* [43]. In *E. ictaluri*, CpxP is contribution to regulate cell membrane stress and host infection [40]. In current study, we investigated role of Usp13 in adversity adaptation and infection. Our results provide the first insights into the biological function of the *E. piscicida* Usps.

2. Materials and methods

2.1. Bacteria and growth conditions

E. 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 [18]. Bacteria were cultured in Luria-Bertani broth (LB) at 37 °C (for *E. coli*) or 28 °C (for *E. piscicida*). Where indicated, chloramphenicol, tetracycline, and polymyxin B, were supplemented at the concentration of 30 μg/mL, 15 μg/mL, and 100 μg/mL.

2.2. Quantitative real time reverse transcription-PCR (RT-qPCR) analysis of usp genes expression under different conditions

To examine *usp* genes expression under in vitro conditions, TX01 was grown in LB medium with different temperature (28 °C and 42 °C), with different pH (pH 5 or 7) at 28 °C, with or without hydrogen peroxide at 28 °C, and with or without host serum. 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 was carried out using the SYBR ExScript RT-qPCR Kit (Takara, Dalian, China) as described previously [23]. The experiment was performed three times.

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)
Usp1-RTF	GTTCATCCCTCGGTTCCC
Usp1-RTR	CCTCCAGCCGTTTCTCG
Usp2-RTF	CCCGCAGACCAATCAGTTC
Usp2-RTR	GCCCAGCAGATAGGTTTTCAT
Usp3-RTF	CGATGAGGTGCTGAAGGAG
Usp3-RTR	ACCAAGACGCTGGTGGTC
Usp4-RTF	CATGCCTGTCAGGTTGCC
Usp4-RTR	AGGCTCTGCTGCTCCATC
Usp5-RTF	GGCGGAAACTCACCCTACT
Usp5-RTR	GTGCCCCAAAATCACCAA
Usp6-RTF	CTCCGATGAAACGCACCA
Usp6-RTR	GGCATCCACCAGCACCT
Usp7-RTF	GCGTTATTTTGGGCGTTG
Usp7-RTR	TTTGCTGGGCTGACCGT
Usp8-RTF	TGACGCTGTGCAATGAGC
Usp8-RTR	GGTGTGGGCGATAGGGTAAT
Usp9-RTF	TCCTATGAGATGACGACCCTG
Usp9-RTR	CACCTCCTGGATGATGGC
Usp10-RTF	GTGGCTGCCCAGTTCAAT
Usp10-RTR	CCCGAGCAGGTAGGTGGT
Usp11-RTF	GCGTTTGTCACCGCCTAC
Usp11-RTR	ATGCGAGCCAACCACGAT
Usp12-RTF	GGCTCTGGCTATCACCCTAT
Usp12-RTR	CTCTGTGCGGCATACTGG
Usp13-RTF	TTGGCTGAGAAGATGGCG
Usp13-RTR	TGCTGATAGCGTGCGTTT
16S-RTF	GCACAAGCGGTGGAGCAT
16S-RTR	ACGAGCTGACGACAGCCAT
CC2-RTF	TGGGTTCGTGCCAAGATTGTTGCA
CC2-RTR	TGAAGGAGAGGCGGTGGATGTTAT
CXCL10-RTF	CTATCCATGGAGCCTCAGGT
CXCL10-RTR	CTTCTTGAGCGTGGCAATAA
HSP70-RTF	TGCCTTTGTCCAGACCGTAG
HSP70-RTR	GTGTCCAACGCTGTCATCAC
IFN-γ-RTF	GAGCTGCAAATGGATGGATGA
IFN-γ-RTR	GGCGGTACAAGTGATTAGGGA
IL6-RTF	ACAGAGGAGGCGGAGATG
IL6-RTR	GCAGTGCTTCGGGATAGAG
IL8-RTF	GGAGACAGAGATCATTGCC
IL8-RTR	AGTCATCTCGTGAAAGGAAC
IL10-RTF	CCCTGA AGAGAGATGTCA
IL10-RTR	GTGTCGTTTAGAAGCCAG
TNF-α-RTF	CTTCCCATAGACTCTGAGTAGCG
TNF-α-RTR	GAGGCCAACAAAATCATCATCCC
Act-β-RTF	GCTACTCCTTCACCACCACAG
Act-β-RTR	CGTCAGGCAGCTCGTAACTC
Usp13F1	GGATCCCAAACGCCGTAACCCA (*Bam*HI)
Usp13R1	ACCTCCGTGGATCAGAAGTCCCAGTTA
Usp13F2	TCTGATCCACGGAGGTCATCATAAGC
Usp13R2	GGATCCAGGGTTTCGGCAGGTAG (*Bam*HI)
Usp13F3	CCAGTATAGCGAATTACGGAC
Usp13R3	GAGCGACGATAACCTCAAA
Usp13F4	GGATCCATGCATAAAACTGCAACC (*Bam*HI)
Usp13R4	CATATGCTTCTGGGCAGAAGTATTC (*Nde*I)

2.3. Construction of the usp13 mutation and its complementation

The primers used in this study were listed in Table 1. To construct the *usp13* knockout strain, TX01Δ*usp13*, in-frame deletion of a 330 bp

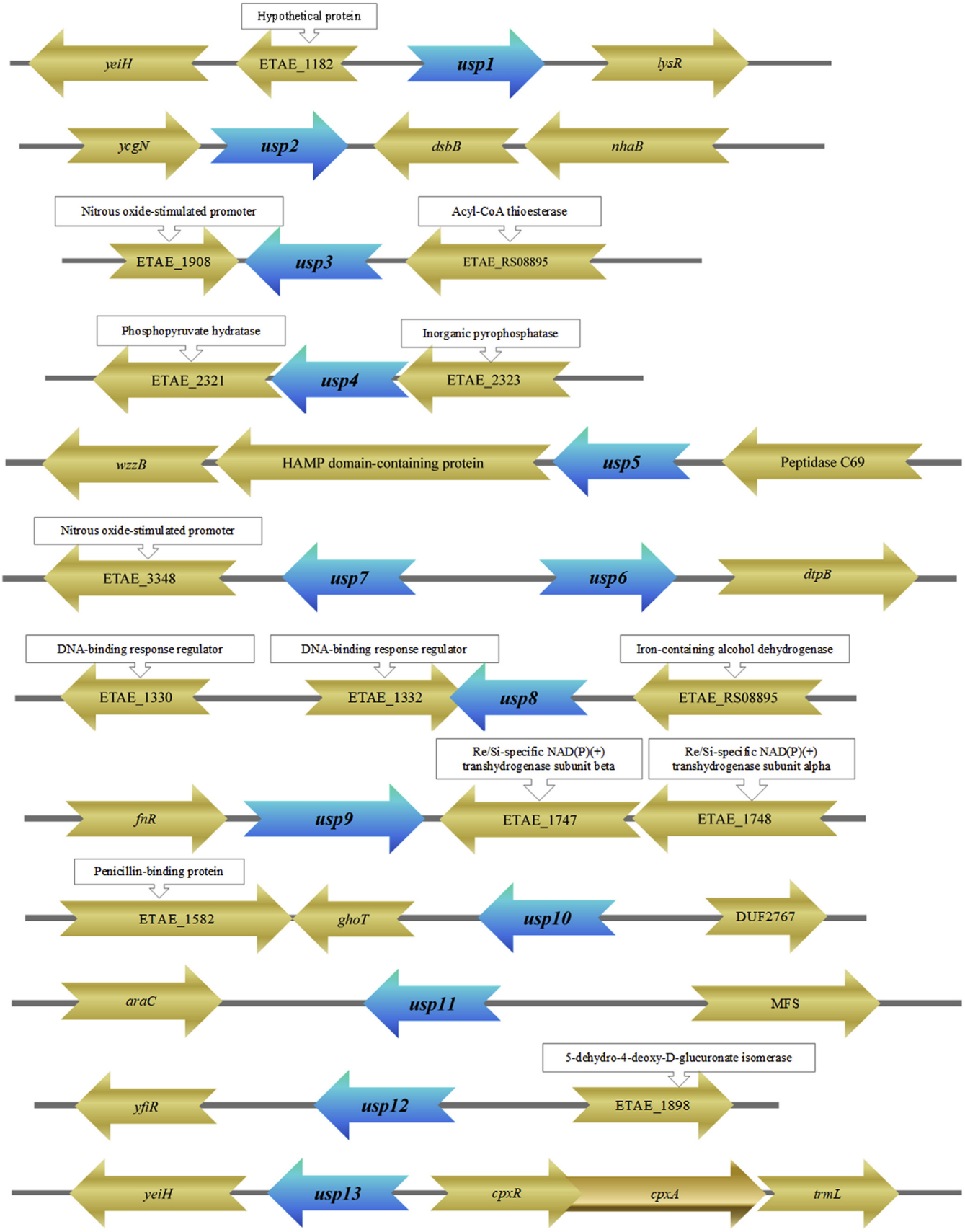

Fig. 1. Genetic organization of *usp* genes in *Edwardsiella piscicida*.

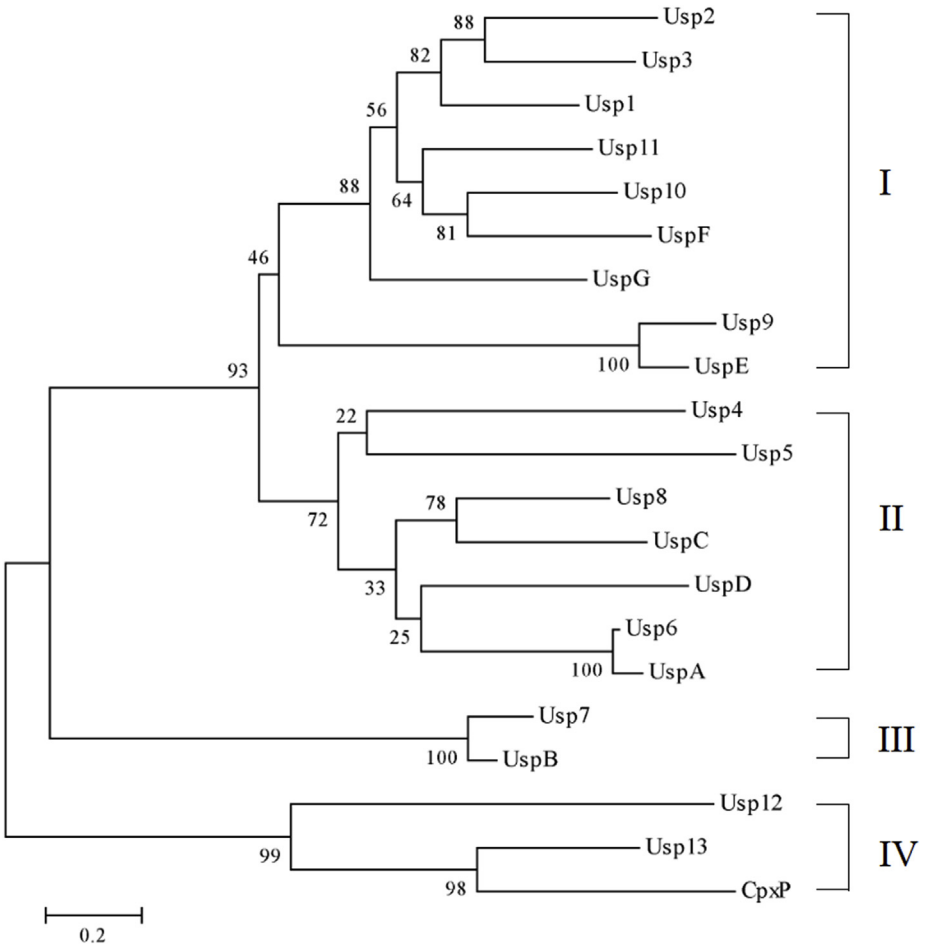

Fig. 2. Phylogenetic relationships among Usps of *Edwardsiella piscicida* using amino acid sequences. The phylogenetic tree calculation was performed by using the MEGA5 program, based on a sequence distance method that utilizes the neighbor-joining algorithm. Values at branches indicate the percent bootstrap support for 1000 replicates. The Gen Bank accession numbers of the aligned sequences are as follows: UspA: AAC76520.1; UspB: AAC76519.1; UspC: AAC74965.1; UspD: AAC76905.1; UspE: AAC74415.1; UspF: AAC74458.2; UspG: AAC73708.1; CpxP: AAT48235.1; Usp1: WP_012848044.1; Usp2: WP_012848320.1; Usp3: WP_012848746.1; Usp4: WP_012849152.1; Usp5: WP_012848569.1; Usp6: WP_012850148.1; Usp7: WP_012850146.1; Usp8: WP_015461302.1; Usp9: WP_012848585.1; Usp10: WP_012848435.1; Usp11: WP_012850200.1; Usp12: WP_012848734.1; Usp13: WP_012850246.1.

segment (residues 33 to 142) of *usp13* was performed by overlap extension PCR as follows: the first overlap PCR was performed with the primer pair Usp13F1/R1, the second overlap PCR was performed with the primer pair Usp13F2/R2, and the fusion PCR was performed with the primer pair Usp13F1/R2. The PCR products amplified by primer pair Usp13F1/R2 were inserted into the suicide plasmid pDM4 at the *Bgl*II site, resulting in pDMUsp13. S17-1λpir was transformed with pDMUsp13, and the transformants were conjugated with TX01 as described previously [19]. The transconjugants were selected on LB agar plates supplemented with 10% sucrose. One of the colonies that was resistant to sucrose and sensitive to chloramphenicol was analyzed by PCR, and the PCR products were subjected to DNA sequencing to confirm in-frame deletion. This strain was named TX01Δ*usp13*. To constructed the complementary strain TX01Δ*usp13*C, *usp13* was amplified by PCR with primers Usp13F4/R4, and the following experimental operations were done as described previously [19].

2.4. Resistance to high temperature, oxidative stress, and acidic stress and to non-immune fish serum

TX01, TX01Δ*usp13*, and TX01Δ*usp13*C were cultured in LB medium to exponential phase. TX01 and TX01Δ*usp13* were transferred to fresh LB medium and cultured at 28 °C and 42 °C, and cell density was measured at different time points by determining absorbance at OD_{600}. To determine acid tolerance, TX01 and TX01Δ*usp13* were transferred to fresh LB medium with different pH (pH 5 or 7) at 28 °C, and cell density was measured at different time points. H_2O_2 sensitivity was examined as reported as previously [23].

TX01, TX01Δ*usp13*, and TX01Δ*usp13*C were cultured in LB medium to exponential phase. Then the cells were washed with PBS and resuspended in PBS. Approximately 10^5 bacterial cells were mixed with 50 μL fish serum or PBS (control). After incubation with mild agitation at 25 °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)] × 100%. The experiment was performed three times.

2.5. Biofilm assay and motility assay

TX01, TX01Δ*usp13* and TX01Δ*usp13*C were cultured in LB medium to exponential phase and diluted to 10^5 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 A_{570} of eluates was measured. The experiment was performed three times.

To measure motility, TX01 and TX01Δ*usp13* were cultured in LB medium to an OD_{600} of 1.0, and 5 μL cell suspension were spotted onto the center of fresh swimming plates, which contained LB medium plus 0.3% (w/v) agar. The plates were then incubated at 28 °C. After 48 h, the motility of the bacteria was assessed by examining the diameter of the motility halo on the soft agar. The experiment was performed three times.

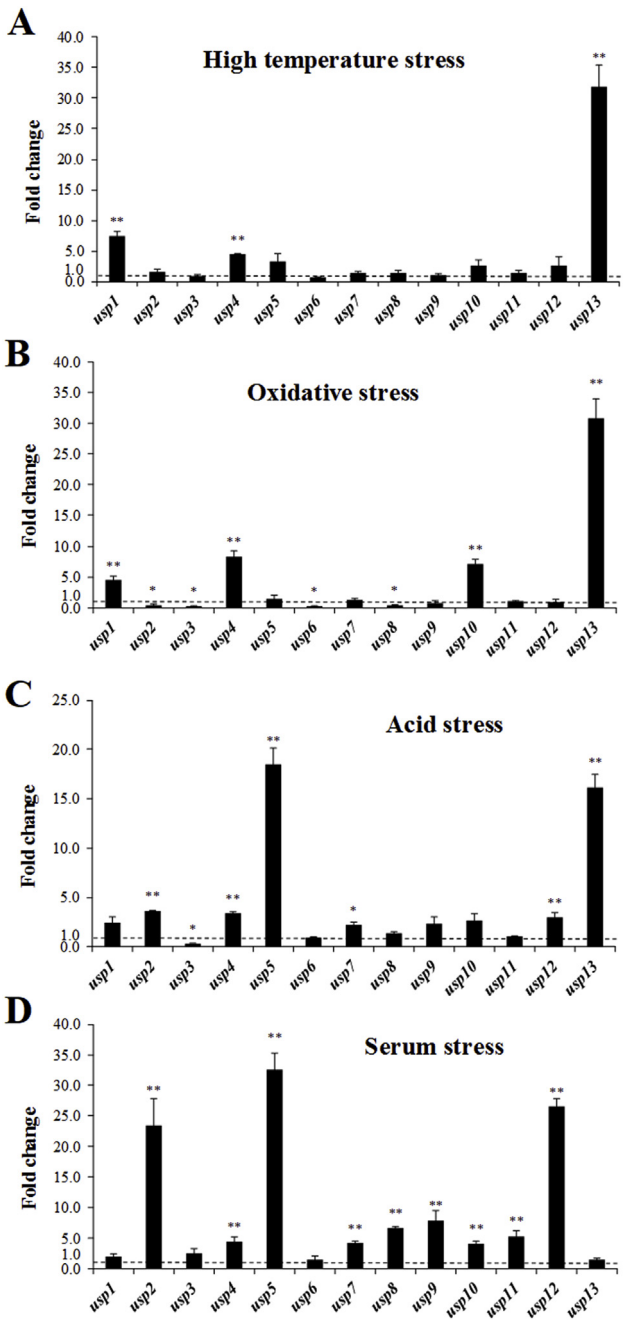

Fig. 3. Expression analysis of *usp* genes in *Edwardsiella piscicida* under different stress conditions. Expression of 13 *usp* genes under high temperature stress (A), oxidative stress (B), acid stress (C), and serum stress (D). RT-qPCR was performed with total RNA extracted from *Edwardsiella piscicida* TX01 cultured in normal LB medium (control, pH = 7, 28 °C), in high temperature (normal LB medium at 42 °C), in hydrogen peroxide condition (normal LB medium with H_2O_2), in acid condition (pH = 5), in serum stress (incubated with the host serum). Expression level of *usp* genes in normal LB medium was set as 1. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. *, $P < 0.05$; **, $P < 0.01$.

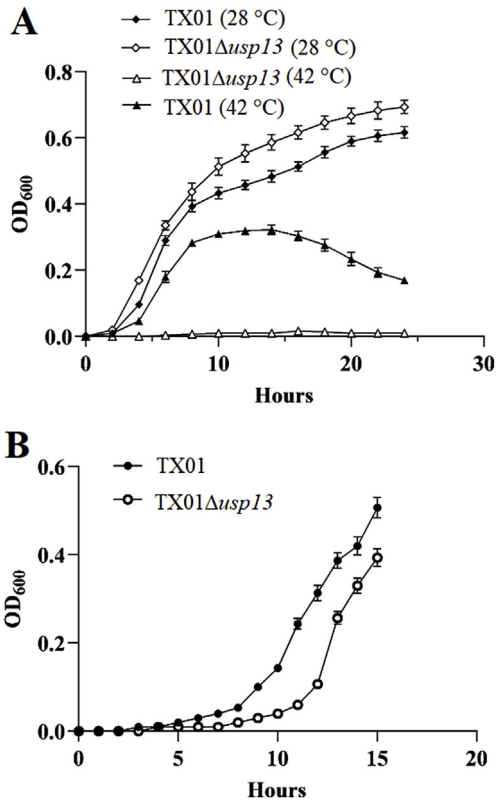

Fig. 4. Growth analysis of *Edwardsiella piscicida*. A, TX01 and TX01Δ*usp13* were cultured in LB medium at normal temperature (28 °C) or high temperature (42 °C), and cell density was measured at different time points by determining absorbance at OD_{600}. B, TX01 and TX01Δ*usp13* were cultured in LB medium of pH 5.0 and cell density was measured at different time points by determining absorbance at OD_{600}. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed.

2.6. Invasion of eukaryotic cell lines

(i) Interaction of bacteria with cultured fish cells. Examination of interactions between FG cells [44] and *E. piscicida* was performed as described previously [23]. Briefly, FG cells were cultured in 96-well cell culture plates to monolayer and mixed with strain TX01, TX01Δ*usp13*, or TX01Δ*usp13* C at a multiplicity of infection (MOI) of 10:1. After incubation at 25 °C for 1 h and 2 h, the plates were washed three times with PBS. The washed FG cells were lysed with 200 μL of 1% (vol/vol) Triton X-100 in PBS, then the number of bacteria were counted by diluted plate. The experiment was performed three times.

(ii) Bacterial replication in macrophages. The experiment was performed as described by Wang et al. [23]. RAW264.7, a murine monocyte-macrophage cell line [45], 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% CO_2. Then 100 μL bacteria (1×10^6 CFU/mL) were added to RAW264.7 cells, followed by incubation at 28 °C for 2 h. Extracellular *E. piscicida* was killed by adding gentamicin (100 μg/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 μg/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.

2.7. Fish and experimental challenges for bacterial dissemination in vivo

Clinically healthy tilapia (average 13.3 g) were purchased from a commercial fish farm of Hainan. The fish were maintained at ~25 °C in aerated water and fed daily with commercial dry pellets. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Before experiment, fish were randomly sampled and examined for the presence of bacteria in 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

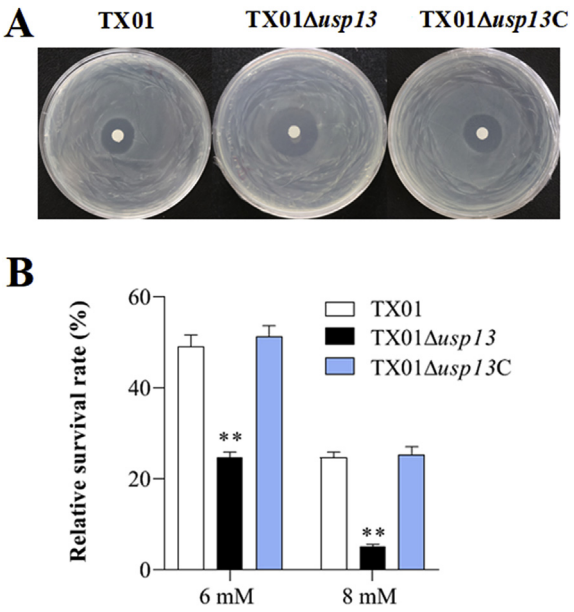

Fig. 5. Sensitivity of *Edwardsiella piscicida* to oxidative stress. A, TX01, TX01Δ*usp13* and TX01Δ*usp13*C were spread on LB agar plates placed with a sterile filter disk containing 1 M H_2O_2, and the plates were incubated at 28 °C for 24 h. B, TX01, TX01Δ*usp13* and TX01Δ*usp13*C were cultured to early logarithmic phase in normal LB medium and bacteria were transferred equally to two tubes. Then H_2O_2 of different concentration or PBS (control) were added to the medium and bacteria were cultured for 1 h. The number of the bacteria was determined by plate counting. Data are the means of three independent experiments and presented as means ± SEM (N = 3). N, the number of times the experiment was performed. **, $P < 0.01$.

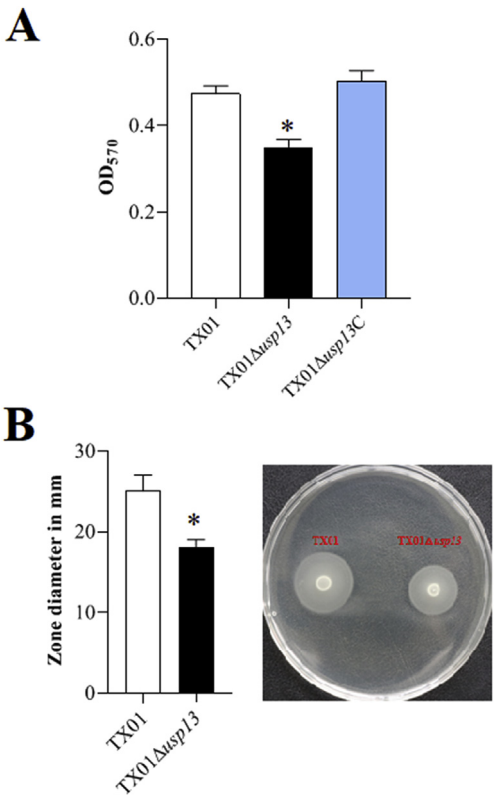

Fig. 6. Effects of *usp13* mutation on biofilm growth and motility. A, the biofilm forming capacity of *E. piscicida*. TX01, or TX01Δ*usp13* were incubated in polystyrene plate and biofilm formation was determined by measuring the A_{570} of final eluates. B, the motility of *E. piscicida*. TX01 and TX01Δ*usp13* were cultured in LB medium to an OD_{600} of 1.0, and 5 μL cell suspensions were spotted onto the center of swimming plates containing LB medium plus 0.3% (w/v) agar. The plates were incubated at 28 °C for 2 days and the motility zone diameter was measured. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. *, $P < 0.05$.

methanesulfonate) (Sigma, USA) as described previously [46]. For tissue dissemination analysis, TX01, TX01Δ*usp13*, and TX01Δ*usp13*C were cultured in LB medium to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in PBS to 10^6 CFU/mL. Tilapia were divided randomly into four groups and infected by intramuscular (i.m.) injection with 50 μl of TX01, TX01Δ*usp13*, and TX01Δ*usp13*C, or PBS. The kidney, liver, and spleen were taken aseptically from the fish at 24 h and 48 h post-infection. Bacterial recovery from the tissues was determined as reported previously [19]. The rest of the infected fish were monitored daily for mortality for 20 days. The experiment was performed in triplicate.

2.8. Reactive oxygen species (ROS) production

Tilapia head kidney (HK) macrophages were prepared as described previously [47]. ROS production was determined as described as previously [19]. HK macrophages in a 96-well microplate (~10^5 cells/well) were incubated with TX01, TX01Δ*usp13*, and TX01Δ*usp13*C (10^6 CFU/well) for 2 h. The cells were washed with PBS for three times. One hundred microliters of 1 mg/mL nitroblue tetrazolium (Sangon, Shanghai, China) in L-15 was added to the cells. After incubation at 25 °C for 2 h, the reaction was stopped by adding 100% methanol. The plate was washed with 70% methanol, and the reduced formazan was solubilized in 100 μL of 2 M KOH and 120 μL of dimethyl sulfoxide. The plate was read at 630 nm with a microplate reader. The experiment was performed three times.

2.9. RT-qPCR analysis of immune-related genes expression

Tilapia were infected with TX01Δ*usp13* and TX01Δ*usp13*C, or PBS for 24 h as above. Spleen was taken aseptically from the fish and used for total RNA extraction with E.Z.N.A Total RNA Kit (Omega Bio-Tek Inc., USA). cDNA synthesis was performed as above. RT-qPCR was

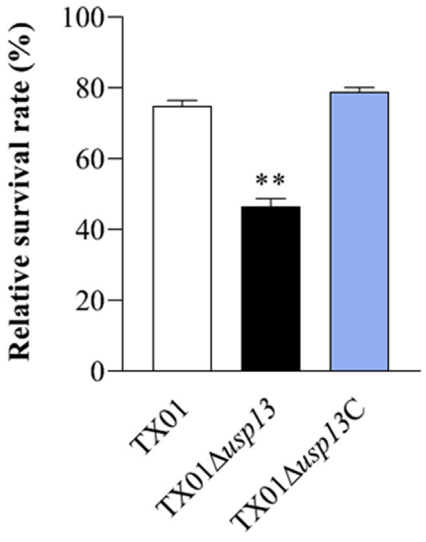

Fig. 7. Effects of *usp13* mutation on resistance to host serum. A, survival of *E. piscicida* in fish serum. TX01, TX01Δ*usp13*, or TX01Δ*usp13*C were incubated with non-immune tilapia serum or PBS (control). After incubation, the survival of the bacteria was determined by plate counting. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. *, $P < 0.05$.

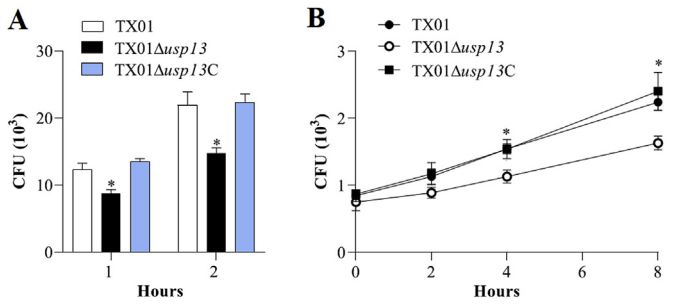

Fig. 8. Effects of *usp13* mutation on cellular infection and replication. A, the association and invasion of *E. piscicida* to flounder gill cells (FG cells). FG cells were infected with the same dose of TX01, TX01Δ*usp13*, or TX01Δ*usp13*C for various hour and washed with PBS. Then FG cells were lysed and the bacterial associated with and invaded into the host cells were determined. B, survival and replication of *E. piscicida* in mouse macrophage cell RAW264.7. RAW264.7 cells were hatched with TX01, TX01Δ*usp13* and TX01Δ*usp13*C for 2 h, then extracellular bacteria were killed. The cells were then incubated further for different hours, and the number of intracellular bacteria was determined by plate counting. Data are the means of three independent experiments and presented as means ± SEM (N = 3). N, the number of times the experiment was performed. *, $P < 0.05$.

carried out using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described above. The immune genes analyzed were genes encoding chemokine CC2, chemokine CXCL10, heat shock protein (Hsp70), interferon (IFN-γ), interleukin 6 (IL-6), IL-10, IL-8, tumor necrosis factor alpha (TNF-α). The primers used for the PCR are listed in Table 1. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of the immune genes was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with β-actin as a control. All data are given in terms of mRNA levels relative to that of β-actin and expressed as means plus or minus standard errors of the means (SE). The experiment was performed three times.

2.10. 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 $P < 0.05$.

3. Results

3.1. Classification of Usps and their phylogenetic relationships

There are 13 Usps classified as members of the Usp family by analyzing *E. piscicida* genomic information [21], and the 13 Usps were named to Usp1 to Usp13. Genomic organization of these Usp genes was shown in Fig. 1. To classify the Usps based on domain organization and their characteristics, we compared them to those of *E. coli*, which also belongs to Enterobacteriaceae family and its Usps were characterized well [25–27]. According to the phylogenetic tree, the 13 Usps grouped into four clusters (Fig. 2). The cluster I consists of Usp1, Usp2, Usp3, Usp9, Usp10, and Usp11. UspE, UspF, and UspG from *E. coli* also belong to cluster I. There are Usp4, Usp5, Usp6, and Usp8 in the cluster II, in which contains UspA, UspC, and UspD of *E. coli*. Only Usp7 is classified to cluster III, and UspB of *E. coli* also appears in this category. Usp12 and Usp13 are part of the cluster IV, which includes the Cpx envelope stress system protein CxpP of *E. coli*.

3.2. Expression analysis of usp genes under different environmental stress

To identify 13 *usp* genes expression profiles of *E. piscicida* in

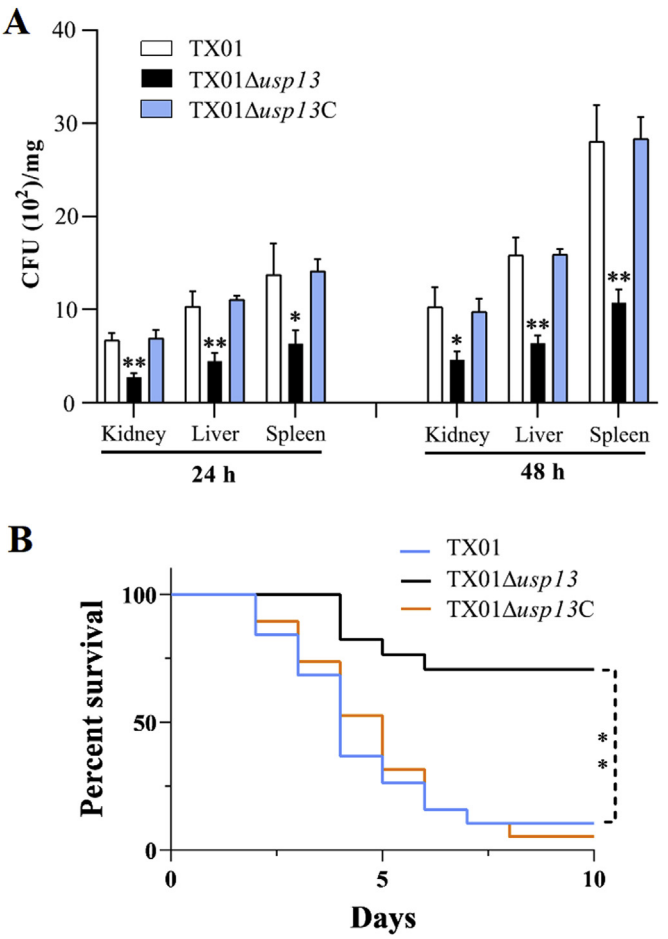

Fig. 9. *In vivo* infection of *Edwardsiella piscicida* in tilapia. A, bacterial dissemination in the fish tissues. Tilapia were infected with the same dose of TX01, TX01Δ*usp13*, or TX01Δ*usp13*C, bacterial recoveries from kidney, liver, and spleen were determined by plate count at 24 h and 48 h post-infection. B, host mortality induced by *E. piscicida*. Tilapia were infected with equivalent doses of TX01, TX01Δ*usp13* and TX01Δ*usp13*C, and accumulated mortality were monitored for a period of 20 days (only 10 days are shown since no more deaths occurred after 10 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. *$P < 0.05$, **$P < 0.01$.

different conditions, total RNA was separately isolated from bacteria grown in normal media (i.e., cultured in LB medium, Con) and in adverse conditions (i.e., cultured in high temperature, in low pH, in LB medium with H_2O_2, or incubated with fish serum) and used for RT-qPCR. The result showed that when bacteria cultured at high temperature (45 °C), three *usp* genes were significantly up-regulated. Among these up-regulated genes, *usp13* exhibited the highest expression level with 31.9-fold, *usp1* and *usp4* exhibited 7.4-fold and 4.5-fold increase, respectively. Other genes expressions were basically unchanged (Fig. 3A).

In the presence of hydrogen peroxide, the expressions of four genes (*usp1*, *usp4*, *usp10*, and *usp13*) were dramatically enhanced and *usp13* displayed the highest induction value (30.8-fold). However, the expression of another four genes (*usp2*, *usp3*, *usp6*, and *usp8*) were downregulated under oxidative stress circumstance (Fig. 3B).

When bacteria facing acid pressure, six *usp* genes (*usp2, usp4, usp5, usp7, usp12, usp13*) expressions were significantly upregulated. Amongst these upregulated genes, *usp5* and *usp13* expression were dramatically enhanced (18.5- and 16.2-fold, respectively). On the contrary, the expression of *usp3* was significantly downregulated in acid

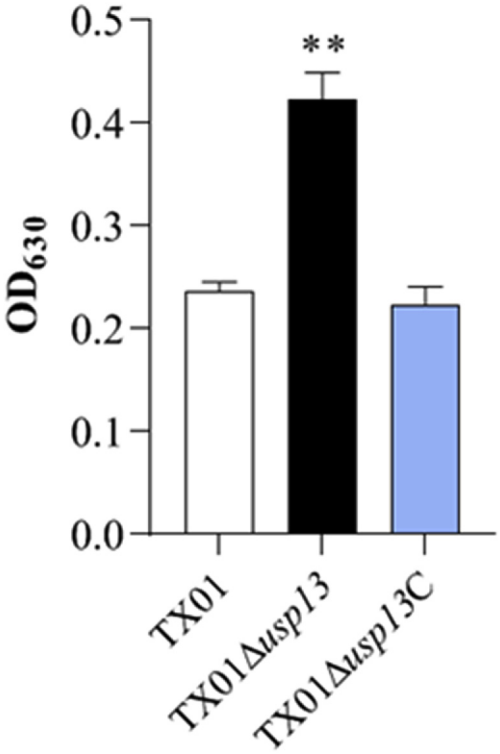

Fig. 10. Effect of TX01, TX01Δ*usp13*, and TX01Δ*usp13*C on the immune response of macrophages. Tilapia head kidney macrophages were infected with TX01, TX01Δ*usp13*, or TX01Δ*usp13*C, and reactive oxygen species productions in the cells were determined at 2 h post-infection. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. $^{**}P < 0.01$.

condition (Fig. 3C).

Since *E. piscicida* exhibits a remarkable ability to survive in host serum [48], we examined *usp* genes expression when bacteria incubated with fish serum. RT-qPCR result showed that when bacteria were under serum environment, nine *usp* genes expressions were significantly increased, four *usp* genes expressions remained unchanged (Fig. 3D).

3.3. Construction of an E. pisicida usp13 mutant

As mentioned above, *usp13* showed positively induced expression under various stress conditions except for serum stress, which indicated Usp13 may play an important role in response to adversity. To further examine its functional importance, the *usp13* of *E. piscicida* TX01 was knocked out by markerless in-frame deletion of a region encoding amino acid residues 33 to 142. The resulting mutant was named TX01Δ*usp13*.

3.4. Multiple effects of usp13 mutation

3.4.1. Effect on growth and survival under stress conditions

Firstly, we want to know whether the deletion of *usp13* has any effect on bacterial growth. Growth analysis showed that when cultured in normal LB medium, TX01Δ*usp13* exhibited a comparative generation time with TX01 at the logarithmic phase but reached higher cell densities than TX01 at the stationary phase (Fig. 4A). When cultured in high temperature, the growth of TX01 was retarded and maximum cell density was about 0.3 of OD_{600}. However, TX01Δ*usp13* was hardly able to grow at 42 °C condition. In LB medium with low pH, the growth of TX01Δ*usp13* was significantly slower than that of TX01 (Fig. 4B).

3.4.2. Effect on bacterial resistance against oxidation stress

Since the expression of *usp13* was markedly induced by H_2O_2-induced oxidation stress, the ability of TX01 and TX01Δ*usp13* to resist hydrogen peroxide was investigated with inhibition zone assay. As shown in Fig. 5A, the inhibition zone of H_2O_2 formed around TX01Δ*usp13* (average diameter 25.2 ± 1.4 mm) was significantly larger than that formed around TX01 (average diameter 17.1 ± 1.5 mm) or TX01Δ*usp13*C (average diameter 17.6 ± 1.2 mm). Similarly, when incubated with 6 mM H_2O_2, the survival rate of TX01Δ*usp13* was 29%, which was significantly lower than that of TX01 (50%), and more noticeable differences between TX01Δ*usp13* and TX01 was found when they were incubated with 8 mM H_2O_2 (Fig. 5B).

3.4.3. Effect on bacterial biofilm formation and motility

It is well known that bacteria biofilm communities increased tolerance to extracellular stresses [49], so we investigated the effect of *usp13* mutation on bacterial biofilm formation. For this purpose, TX01 and TX01Δ*usp13* were incubated in a 96-well polystyrene plate for 24 h. The biofilm growth of *E. piscicida* was analyzed and the result showed that the biofilm formation capacity of TX01Δ*usp13* was significantly lower than that of TX01 (Fig. 6A).

To examine the motility of TX01 and TX01Δ*usp13*, bacteria were dripped on soft LB agar plate. After culturing 24 h, the mobility was examined and the results showed that the motility zone diameter of

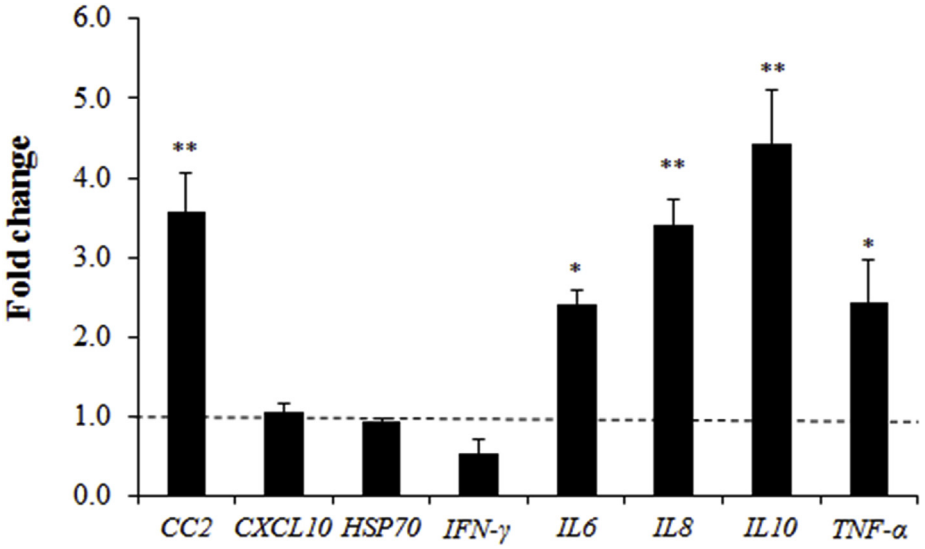

Fig. 11. Immune response induced by TX01 and TX01Δ*usp13*. Tilapia were administered via intramuscular injection with TX01, TX01Δ*usp13*, or PBS (control), and the expression of immune relevant genes in spleen was determined by RT-qPCR. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. $^{*}P < 0.05$, $^{**}P < 0.01$.

TX01Δ*usp13* was smaller (average diameter 18.0 ± 1.0 mm) than that of TX01 (average diameter 25.1 ± 2.1 mm) (Fig. 6B).

3.4.4. Effect on bacterial resistance to non-immune fish serum

Although *usp13* expression level remained unchanged when bacteria facing serum stress, we yet wanted to know whether *usp13* mutation affects the ability of serum tolerance. For this purpose, TX01 and TX01Δ*usp13* were incubated with non-immune tilapia serum, and the survival of bacteria was determined by plate count at different time point. The result showed that TX01 exhibited apparent serum resistance, as 74.6% of the cells survived after 1 h incubation with tilapia serum. However, only 46.3% of TX01Δ*usp13* cells survived after the same treatment, which is significantly lower than that of TX01; similar result was observed after 2 h of incubation with tilapia serum (Fig. 7).

3.4.5. Effect on cell invasion in vitro

To examine whether *usp13* mutation has any role in the infectivity of TX01, cultured FG cells were incubated with the same dose of TX01 or TX01Δ*usp13* and the bacterial cells associated with and invaded into the host cells were enumerated. The results showed that the amount of TX01Δ*usp13* recovered from FG cells was significantly lower than that of TX01 at 1 h and 2 h post-infection (Fig. 8A).

It is known that *E. piscicida* is able to survive and replicate in mouse macrophage cell line RAW264.7 [45]. To examine whether *usp13* mutation plays any role in the intracellular survival of TX01, cultured RAW264.7 cells were incubated with the same dose of TX01 or TX01Δ*usp13*, and extracellular bacteria were killed. The cells were then incubated further for different hours, and the number of intracellular bacteria was determined by plate count. The results showed that the number of TX01Δ*usp13* recovered from the intracellular RAW264.7 cells was significantly lower than those of TX01 at 4 h and 8 h post-infection (Fig. 8B).

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

To examine the effect of *usp13* mutation on tissue infectivity, tilapia were infected with the same dose of TX01 and TX01Δ*usp13*, and bacterial recoveries from spleen, liver, and kidney were determined at 24 and 48 h post-infection (hpi). The results showed that bacterial recoveries from TX01Δ*usp13*-infected fish were significantly lower than those from TX01-infected fish at 24 hpi and 48 hpi (Figur 9A).

To examine the effect of *usp13* mutation on the overall bacterial virulence, tilapia infected with TX01 or TX01Δ*usp13* were used to monitor for mortality. The results showed at the end of the monitored period (20 days), the survival rate of TX01Δ*usp13*-infected fish was 75.0%, which was significantly higher than that of TX01-infected fish (15.0%) (Fig. 9B).

3.5. Genetic complementation of usp13 deletion and its effect

To examine whether the stress resistance and virulence defect observed with TX01Δ*usp13* were indeed due to *usp13* deletion, the strain TX01Δ*usp13*C was created, which is a genetic variant of TX01Δ*usp13* that expresses *usp13* in trans from a plasmid. In contrast to TX01Δ*usp13*, TX01Δ*usp13*C exhibited a comparable ability of resistance against oxidative stress and non-immune fish serum to those of TX01 (Figs. 5 and 7). The biofilm growth of TX01Δ*usp13*C was similar to that of TX01 (Fig. 6). Following infection of FG cells, the amount of TX01Δ*usp13*C recovered from FG cells and RAW264.7 cells was comparative to that of TX01 (Fig. 8). Likewise, the bacterial dissemination capacity of TX01Δ*usp13*C in the fish tissues was comparable to that of TX01, and the survival rate of TX01Δ*usp13*C-infected fish was similar to that of TX01-infected fish (Fig. 9).

3.6. Difference of host immune response induced by TX01 and TX01Δusp13

To explore the mechanism of attenuated virulence caused by *usp13* deletion, we examined the effect of *usp13* mutation on the ability to block activation of host phagocytes. For this purpose, tilapia HK macrophages were infected with TX01 or TX01Δ*usp13*, and cellular productions of ROS were determined. The results showed that the level of ROS in TX01Δ*usp13*-infected cells was significantly higher than that in TX01-infected cells (Fig. 10).

To investigate whether *usp13* deletion had any effect on the expression of immune-related genes during host responding to *E. piscicida* infection, RT-qPCR was carried out to analyze the expression of the genes encoding chemokine CC2, chemokine CXCL10, Hsp70, IFN-γ, IL-6, IL-10, IL-8, and TNF-α. The results showed that the expression level of genes encoding IL6, IL-8, IL-10, TNF-α, and CC2 in TX01Δ*usp13*-infected fish was significantly higher than that in TX01-infected fish (Fig. 11).

4. Discussions

Bacteria are constantly exposed to stressful and challenging environment. To cope with adverse environment and survive, they have evolved intricate mechanisms to sense the surrounding milieu and to adequately respond. One of these mechanisms is universal stress proteins. It is reported that universal stress proteins play a role in different bacteria to respond to different stress conditions, such as heat, substrate starvation, acidic stress, and oxidative stress [29]. *E. coli* has six USP groups (UspA, UspB, UspC, UspD, UspE, and UspF), which are involved in various adversity adaptation [25]. *M. tuberculosis* has 10 Usps encoded in its genome [50] and *E. ictaluri* has 13 Usps encoded in its genome [51], and most of the Usps are related to cope with stress. There are also 13 Usps in *E. piscicida* genome [24], but their functions are totally unknown. In this study, we characterized these Usps in *E. piscicida*. Thirteen Usps were divided into four different classes based on their amino acid sequence, which is similar to the Usps in *E. coli* [52].

In their natural habitats, bacteria are inevitably exposed to high temperature environment. To explore the function of *usp* genes in *E. piscicida*, we examined the expression of 13 *usp* genes under high temperature environment, the results showed that *usp1*, *usp4*, and *usp13* were significantly up-regulated and *usp13* exhibited the highest expression level, indicating the three *usp* genes, especially *usp13*, play an important role in dealing with high temperature stress. For pathogenic bacteria, oxidative stress and acid stress are inevitable adversity in host cell, so we investigate *usps* expression under the two stress conditions. Compared to high temperature, another *usp* gene, *usp4,* was also significantly up-regulated under oxidative stress condition. Facing acid stress, six *usp* genes, *usp2*, *usp4*, *usp5*, *usp7*, *usp12*, and *usp13* displayed up-regulated expression. For pathogens, host serum is also an inescapable adversity in host. Incubating with host serum, bacteria up-regulated more *usps* genes, compared to other three stress conditions. Most *usps* genes exhibited up-regulated expression under these stress condition, indicating Usps played important roles in response to adverse environment, which is consistent with Usps in other pathogens [34,51]. Under the first three stresses conditions, *usp13* exhibited high expression level. However, *usp13* expression remained unchanged under serum stress. On the contrary, in *E. ictaluri*, serum stress enhanced dramatically the expression of *usp13*, which also encoded CpxP like protein [51]. In order to further explore *usp13* function, the mutant strain of *usp13*, TX01Δ*usp13*, was constructed.

Since the expression of *usp13* is induced by adversity condition, it probably participates in stress resistance. Our results showed that when *usp13* was inactive, the strain was hardly able to grow at high temperature, and its viability in acid stress was significantly weaken. Moreover, the mutant TX01Δ*usp13* exhibited a higher sensitivity to H_2O_2 than that of wild type TX01. These results indicated *usp13* played an important role in tolerance to high temperature, acid stress, and

oxidative stress. Similar results were reported in other pathogens. For example, in *A. baumannii*, *uspA* mutants demonstrated a significant increase in sensitivity to oxidative stress and low pH condition, but not high temperature [38]. In *Porphyromonas gingivalis*, a *usp* mutant was sensitive to oxidative stress and high temperature [53]. In *Listeria monocytogenes*, tolerance to acidic stress was clearly reduced in two *usp* gene mutant strains [29]. Our results, with other reports, suggested that bacterial Usps were closely related to adversity adaptation.

The capability of adaptation to adversity is usually one of the manifestations of bacterial pathogenicity. Except as an important factor of adaptation to adversity, Usps also play a key role in bacterial pathogenesis. It is reported that the UspA of *P. gingivalis* is required for optimal biofilm formation [53]. UspA in *Salmonella* contribute to bacterial virulence [39]. In *L. monocytogenes*, the survival within macrophages and the invasion into tissues were impaired in the absence of two *usp* genes [29]. In *E. ictaluri*, of thirteen Usps, six Usps were involved in virulence, including Usp13, a homologue of CpxP [40]. However, in *Citrobacter rodentium*, CpxP has nothing to do with virulence [54]. Our results showed that *usp13* mutation promoted slightly the growth of *E. piscicida* in normal condition, but harmed bacterial biofilm growth and attenuated bacterial motility. Similarly, although the expression of *usp13* was not up-regulated by the host serum stress, the capability of resistance against serum bactericidal activity was significantly declined when *usp13* was inactivated, which indicated that it has a role in serum resistance. Consistently, when infection with host cells, compared to TX01, TX01Δ*usp13* showed an impaired ability to infect cells, indicating that *usp13* is required for bacterial invasion of host cells. Moreover, *in vivo* experiment showed that TX01Δ*usp13* was severely reduced in the ability of infecting host tissues and inducing host mortality. All these evidences indicated that *usp13* is vital to the pathogenicity of *E. piscicida*.

In addition to the tolerance to host hostile environment, whether the participation of Usp13 in virulence embodies other aspects, such as overcoming the immune response of the host, there is no relevant report at present. For this purpose, host immune response induced by TX01 and TX01Δ*usp13* was examined, and we found that ROS in TX01Δ*usp13*-infected cells were significantly higher than those in TX01-infected cells. In contrast to TX01-infected fish, the expression of IL-6, IL-10, IL-8, CC2, TNF-α was significantly enhanced in TX01Δ*usp13*-infected fish. It is reported that *E. piscicida* is an important intracellular pathogen that can survive and replicate in host as an escape mechanism from the host defense [20,55–57]. Our finding suggested the deletion of *usp13* attenuated the ability of bacteria to inhibit the expression of immune-related factors. Of these up-regulated genes in TX01Δ*usp13*-infected fish, IL-6, IL-8, and TNF-a are pro-inflammatory cytokines that play an important role in fish response of microbial infection [58]. It was reported that IL-6 was a pleiotropic cytokine and involved in tilapia defense against bacterial infection [59]. IL-10 participated in the immune response of golden pompano to bacterial infection [60]. Chemokine CC2 were crucially involved in the early immune responses to pathogens [61]. The induction of these genes in TX01Δ*usp13*-administered fish may contribute to the clearance of *E. piscicida*.

In conclusion, we characterized 13 *usp* genes expression and identified the *usp13* function in *E. piscicida*. The results showed most of *usp* genes expression was up-regulated under stress conditions, indicating Usps generally participated in response to adversity. Of these *usp* genes, *usp13* exhibited high expression level in several stress conditions. Our finding suggested that Usp13 not only played an important role in coping with stress circumstances, but functioned as a factor that is essential to *E. piscicida* infection both at cellular level and in a live fish model. Moreover, the deletion of *usp13* attenuated the ability of bacteria to inhibit the host immune response to pathogen infection. These findings will help us understand the important roles of bacterial stress proteins in protecting the cells from various damages originated from environment and host.

Acknowledgments

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