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TcpA, a novel *Yersinia ruckeri* TIR-containing virulent protein mediates immune evasion by targeting MyD88 adaptors

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ABSTRACT

TIR domain-containing protein is an important member for some bacterial pathogens to subvert host defenses. Here we described a fish virulent *Yersinia ruckeri* SC09 strain that interfered directly with Toll-like receptor (TLR) function by a TIR-containing protein. Firstly, the novel TIR-containing protein was identified by bioinformatics analysis and named as TcpA. Secondly, the toxic effects of TcpA in fish was demonstrated *in vivo* challenge experiments through knockout mutant and complement mutant of *tcpA* gene. Thirdly, The study *in vitro* revealed that TcpA could down-regulate the expression and secretion of IL-6, IL-1β and TNF-α. Finally, we demonstrated that TcpA could inhibit the TLR signaling pathway through interaction with myeloid differentiation factor 88 (MyD88) in experiments such as NF-κB dependent luciferase reporter system, co-immunoprecipitation, GST pull-down and yeast two-hybrid. The study revealed that TcpA was essential for virulence and was able to interact with the TIR adaptor protein MyD88 and inhibit the pre-inflammatory signal of immune cells and promote the intracellular survival of pathogenic *Yersinia ruckeri* SC09 strain. In conclusion, our results showed that TcpA acted as a new virulence factor in *Y. ruckeri* could suppress innate immune response and increase virulence by inhibiting TLR and MyD88-mediated specific signaling, highlighting a novel strategy for innate immune evasion in bacteria.

1. Introduction

Yersinia ruckeri is the etiological agent of enteric redmouth disease (ERM) in rainbow trout and has been reported worldwide from many different species [1]. Over the past few decades, ERM disease has induced substantial economic losses in fish due to the associated mortality and veterinary costs [2]. Despite the general administration of an effective vaccine in fish, outbreaks continue to occur, which are mainly attributed to specific strains [3,4]. However, very little is known about the virulence mechanisms of *Y. ruckeri* even ERM is serious in fish. Many potential virulence factors have been reported, such as extracellular toxins [5,6], a high affinity iron uptake system named ruckerbactin [5], flagellar motility genes [7], two-component system [8], etc. To further systematically understand *Y. ruckeri* and its virulence, we had reported the complete genome of *Y.ruckeri* SC09, a highly virulent strain isolated from diseased fish with severe septicaemia in China [9]. In the genome of SC09, we found a great deal of horizontal gene transfer (HGT) events in *Y. ruckeri*, and these HGT carried many genes related to immune evasion, which prompted us to further study the correlation between immune evasion and the virulence of *Y. ruckeri*.

Among the gene horizontal transfer elements in the genome of SC09, the most representative one is a class called Integrative and conjugative elements (ICEs). ICEs are a class of newly recognized mobile DNA elements in prokaryotes [10]. Many ICEs harbor a tyrosine recombinase gene and are flanked by direct repeats corresponding to the 3′ end of a conserved gene (e.g., a tRNA gene), which suggest integration happen via site-specific recombination in the 3′ end of this gene [11]. Furthermore, ICEs typically have a core of conserved modular structures that mediate their integration, excision, conjugation, and regulation, interspersed with “accessory regions” that are variably present across members of a species [12]. A mobile element named ICE (r2) in SC09 (NJ56_RS12425-NJ56_RS12600) was integrated between an intact or partial tRNA-Asn copy. Interestingly, a class of bacterial proteins which were homology to the Toll/IL-1 receptor (TIR) domain

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had been identified, it was encoded by the ICE(r2) and acted as "accessory genes". One of these proteins had been named as TcpA, encoded by NJ56_RS12465, which was specific in SC09 and absent in other *Y. ruckeri* strains, such as RS41 (NCBI RefSeq: NZ_CQBN00000000.1), OMBL4 (NCBI RefSeq: NZ_CPUZ00000000.1) and CSF007-82 (NCBI RefSeq: NZ_CCYO00000000.1) strains. The TIR domain is essential for the interaction between the Toll-like receptor (TLR) and adaptor, and is onset of a signalling cascade resulting in nuclear translocation of the transcription factor, NF-κB, followed by the production of inflammatory cytokines and type I interferons [13]. TIR domain interactions play a key role in activating conserved cellular signal transduction pathways in response to pathogen signals [14]. Accordingly, SC09 may target TLR signaling to facilitate its escape from the host innate immune response and enhance virulence by TcpA. In fact, the intracellular survival of *Y. ruckeri* had been detected in previous studies [15].

In the present study, a novel *Y. ruckeri* TIR-containing virulent protein named as TcpA were identified through *in vivo* and *in vitro* experiments. The results showed that TcpA efficiently inhibited TLR signaling and contributed to toxicity *in vitro* and *in vivo*. Besides, we proposed TIR-containing protein TcpA represent a new class of virulence factors in *Y. ruckeri* and modulated host inflammatory responses during infection.

2. Materials and methods

2.1. Bacterial strains

Wild-type *Y. ruckeri* SC09 was isolated from diseased fish in a commercial farm in Jianyang, Sichuan province of China, and was routinely cultured in Luria-Bertani (LB) medium at 28 °C. The virulent *Y. ruckeri* SC10 strain was isolated from the aquatic environment.

2.2. Construction of Y. ruckeri ΔtcpA mutant and complemented strains

Gene knockout was previously described by Luo et al. [16]. The *tcpA* gene sequence (gene accession number: NJ56_RS12465) of the *Y. ruckeri* SC09 strain (genome accession number: NZ_CP025800) is available in GenBank. The left and right homology arm primer sequences of *tcpA* were GGAATCTAGACCTTGAGTCGAAGAACATTTGCG GGTATAGAGTG/AGATGACGCTAGTGTTGATGGAGTTTCAAGGAGGTA ATGTCGTATGCTAA (upstream, A) and TTAGCATACGACATTACCTCC TTGAAACTCCATCAACACTAGCGTCATCT/ACAGCTAGCGACGATATGT CTGTAAATGGTGTCAAGTCTTCGGTA (downstream, B). The left and right homology arms AB of the *tcpA* gene were constructed, and AB was cloned into pLP12 (Guangzhou KnoGen Biotech Co., Ltd.) to form the pLP12-*tcpA* construct. pLP12-*tcpA* was transformed into competent the *E. coli* strain β2163 (Guangzhou KnoGen Biotech Co., Ltd.) by electrotransformation. A positive strain resistant to chloramphenicol was isolated and designated pLP12-*tcpA*-β2163. The co-culture of β2163 cells containing pLP12-*tcpA* positive clones with *Y. ruckeri* SC09 resulted in conjugation and allowed screening for the first homologous recombination in the mutant SC09 strain in LB plates (20 μg/mL CM + 0.3% D-glucose). The SC09 strains with the insertion mutation occurred was screened on LB plates (0.4% L-arabinose) to obtain a Δ*tcpA* strain with a second homologous recombination. As previously described [16], the SC09 and SC10 strains were re-transformed with the *tcpA*-pBAD33cm-rp4 vector [17], and the expression of *tcpA* gene was induced with arabinose.

2.3. Fish infection model

In the logarithmic growth phase, the wild-type *Y. ruckeri* SC09 and recombinant SC09Δ*tcpA* were inoculated intraperitoneally (dose: 5×10^7 CFU) into 15 random rainbow trout (weight, 60–100 g), and fish death was determined. Rainbow trout survival curve analysis and mapping were performed using GraphPad Prism software version 8.0. The growth curves of *Y. ruckeri* SC09 and SC09Δ*tcpA* were determined to rule out the effect of the difference in growth ability of the knockout strain on the infection model. To investigate infection and histological differences in immune organs after fish infection, the liver, spleen, and kidney of dead rainbow trout were homogenized under aseptic conditions, and the homogenates were diluted 10 times. Bacterial counts were performed using conventional plate counting methods. Bacteria cells were counted on a plate containing nutrient agar supplemented with triphenyl tetrazolium chloride, and the bacterial load was determined in each organ. Furthermore, the liver, kidney, and spleen of rainbow trout were harvested in mid-infection (5 days after infection) for routine paraffin embedding and HE staining.

2.4. Bacterial adherence, invasion, and intracellular survival assays

Head kidney macrophages cells of rainbow trout were separated according to the method of Jiang et al [18] and used to determine the effects of the *Y. ruckeri tcpA* gene on bacterial adherence, invasion, and intracellular survival. The cells were seeded at about 2×10^5 cells per well in 24-well plates and grown in Medium 199 (M199) (Hyclone) with 10% fetal bovine serum (FBS) (Hyclone) at 20 °C for 24 h. For the adherence assay, the cell monolayer was washed twice with M199 and infected with *Y. ruckeri* SC09 or *Y. ruckeri* SC09Δ*tcpA* at a multiplicity of infection (MOI) of 1.5. Bacteria were centrifuged onto the cells at $400 \times g$ for 5 min, and cells were then incubated at 20 °C for 1 h. Non-adherent bacteria were removed by rinsing the wells twice with D-PBS (Hyclone). The cells were released from the plate by adding 100 μl of 0.2% Triton X-100 in sterile water, and subsequently 900 μl of M199 was added. This cell suspension was 10-fold serially diluted with D-PBS and spread onto LB plates to determine the number of viable bacteria. For the invasion assay, cell culture, bacterial infection, and bacterial counting were performed as described above for the bacterial adherence assay, except that the extracellular bacteria were killed by incubation of the monolayers with M199 containing gentamicin (100 μg/ml) for 1 h after incubation with bacteria and twice washes with D-PBS. For the bacterial intracellular survival assay, cell culture and bacterial counting were performed as described above for the bacterial adherence assay. Rainbow trout head kidney macrophages cells were infected with *Y. ruckeri* SC09 or *Y. ruckeri* SC09Δ*tcpA* at a MOI of 1.5 and incubated in M199 with 3% FBS and 50 g/ml gentamicin. The cells were then washed and lysed at 1, 4, 8, 12, 18, 24, 48 and 72 h post-infection (hpi) to determine the amount of bacterial recovery. All assays were performed in sextuplicate wells, and the results are averages for at least six independent experiments. Further, the intracellular survival state of the cells 48 h after infection of the wild type bacteria was observed by a transmission electron microscope. In order to verify that gene knockout would result in loss of TcpA protein capacity in the cells, we used rabbit anti-TcpA to test the ability of knockout, wild-type and replenished strains to produce proteins. At the same time, in order to prove the effectiveness of the replenishing strain, we further complemented the expression plasmid of *tcpA* in the SC10 strain without *tcpA* in the chromosome, and also used rabbit anti-TcpA to test the protein production.

2.5. Enzyme-linked immunosorbent assays and qPCR

To quantify IL-6, IL-1β and TNF-α in culture supernatants, we applied ELISA sets of fish (Jiangsu Meimian industrial Co., Ltd). For qPCR, the tissue homogenate and the supernatant from infected cells were prepared for extraction of total RNA with RNAiso Plus (Takara, Dalian, China) according to the manufacturer's standard protocol. cDNA was synthesized from the extracted RNA using the Prime Script RT reagent Kit (TaKaRa, Dalian, China). The gene expression levels of immune-related genes (IL-6, IL-1β, TNF-α) were analysed by real-time PCR. Thus, β-actin of rainbow trout was selected because of the less variability presented. Specific primers used for rainbow trout were: IL-1β-F:

TGAGAACAAGTGCTGGGTCC and IL-1β-R: GGCTACAGGTCTGGCTT CAG (Product 148 bp); IL-6-F: GAGTTTCAGAAGCCCGTGGA and IL-6-R: AGCTGGTACACTTGCAGACC (Product 149 bp); TNF-α-F: CACACT GGGCTCTTCTTCGT and TNF-α-R: CAAACTGACCTTACCCCGCT (Product 155 bp).

2.6. Construction of prokaryotic expression vectors

The full-length *Y. ruckeri* SC09 *tcpA* was cloned into pET28a (Invitrogen) generating the pET28-*tcpA*. The following primers were used: 5′-ATGGCTAGAGAAATTAA-3′ and 5′-GCATACGACATTAC CTC-3′. pET28-*tcpA* was used for production of TcpA recombinant protein. The full-length *Y. ruckeri* SC09 *tcpA* was also cloned into pGEX-6P-1 (Invitrogen) generating the pGEX-*tcpA*, which carries the tac promoter, N-terminal GST tag and ampicillin resistance gene. The following primers were used: 5′-CGGGATCCATGGCTAGAGAAATTAA-3′ and 5′- CGCTCGAGGCATACGACATTACCTC-3′. pGEX-*tcpA* was used for production of TcpA-GST recombinant protein.

2.7. Construction of eukaryotic expression vectors

pCMV-HA-MyD88 plasmid was purchased from Wuhan Miaoling Bioscience & Technology Co., Ltd. TcpA was cloned into the eukaryotic expression plasmids pCMV-Flag-2B (Stratagene) generating the plasmids pTcpA-Flag using the following PCR primers: 5′-ACCATGGATTA CAAGGATGAATGGCTAGAGAAATTAA-3′ and 5′- GGGCCCCCCCTCGA GGTCGAGCATACGACATTACCTC-3'.

2.8. Transfection and NF-κB-dependent luciferase reporter assay

Rainbow trout head kidney macrophages cells were transiently transfected using Lipofectamine™ 3000 Transfection Reagent (Life) for 12 h, according to manufacturer's instructions, for a total of 0.3 μg of DNA consisting of 50 ng TLR-4 and TLR-2 plasmids (Wuhan Miaoling Bioscience & Technology Co.,Ltd), 200 ng of pBIIXLuc reporter plasmid and 50 ng of FLAG-*tcpA* expression vector. The total amount of DNA was kept constant by adding empty vector. Where indicated, cells were treated with *E. coli* LPS (Invivogen) and Pam2CSK4 (Invivogen), for 8 h and then cells were lysed and luciferase activity measured using Dual-Glo® Luciferase Assay System (Promega). The *tcpA* constructs were obtained from SC09 genomic DNA by recombinase Exnase II (ClonExpress II, Vazyme) and then cloned in pCMV-Tag 2B.

2.9. Co-immunoprecipitations (CO-IP)

Rainbow trout head kidney macrophages were infected with the wild-type SC09 for 12 h. Cells were washed twice in ice-cold PBS, harvested and cell lysis buffer was added. Cell lysis and processing for co-immunoprecipitation were performed using the Pierce™ Co-Immunoprecipitation Kit (Thermo Scientific). The eluted IP samples were detected by Western blotting using rabbit anti-TcpA (prepared in this study) or anti-MyD88 (Abcam) antibody.

2.10. Pull-downs from cell extracts

Rainbow trout head kidney macrophages were transiently transfected with pCMV-HA-MyD88 plasmid using Lipofectamine™ 3000 Transfection Reagent (Life). Eighteen hours after infection, cells were washed in ice-cold PBS, harvested and resuspended in RIPA buffer (Sigma). Extracts were then centrifuged at 16,000 g at 4 °C for 20 min. The supernatant was incubated with TcpA-GST recombinant protein during 3 h at 4 °C, then incubated within gravity flow column containing Glutathione-Sepharose column (GE) during 1 h beforehand washed in water and pre-equilibrated in equilibrium buffer 20 mM Tris–HCl. The column was washed successively two times in equilibrium buffer and eluted in 1% Triton PBS. Proteins eluted were separated by SDS–PAGE, transferred to a PVDF membrane, incubated with anti-HA (abcam), anti-MyD88 (abcam) or anti-TcpA antibodies for 50 min and detected with horseradish peroxidase (HRP)-conjugated secondary antibodies.

2.11. Yeast two-hybrid assay

The plasmids used for the Y2H were constructed by the recombinase Exnase II (ClonExpress II, Vazyme). *myD88* and *Y. ruckeri* SC09 *tcpA* were amplified by PCR respectively from the pCMV-HA-MyD88 vector and from SC09 genomic DNA with Exnase II primers. PCR products were then separately cloned into the entry vector pGBKT7 downstream of the Gal4 DNA-binding domain (BD) (Clontech) with screening marker gene *trp* and pGADT7 downstream of the Gal4 activation Domain (AD) with screening marker gene *leu*. Y2HGold Yeast competent cells were transformed with BD and AD fusion protein vectors, respectively. Diploid yeasts carrying both plasmids were obtained by mating and selected on synthetic dextrose medium (SD) lacking leucine (*leu*) and tryptophan (*trp*). Protein interactions were assessed on medium lacking histidine (*his*). The β-galactosidase expression filter assay using the LacZ reporter gene. The primers used for two-hybrid constructs were: GWMyD88F- CATATGGCCATGGAGGCCGAGCTGCAG GAGGTCCCGGCGC; GWMyD88R- GCGGCCGCTGCAGGTCGACGTCAG GGCAGGGACAAGGCCT; GW*tcpA*F- CATATGGCCATGGAGGCCAGATG GCTAGAGAAATTAA; GW*tcpA*R- CTGCAGCTCGAGCTCGATGGGCATA CGACATTACCTC.

3. Results

3.1. Genomic location of tcpA in Yersinia ruckeri

Integrative and conjugative elements (ICEs) are widespread mobile DNA in prokaryotes [19]. Numerous ICEs harbor a tyrosine recombinase gene and are flanked by direct repeats corresponding to a conserved gene [20]. ICEs typically have some modular structures that mediate their integration, excision, conjugation, and regulation, interspersed with “accessory regions” that are variably present across different species [21]. The mobile element ICE(r2) in SC09 (NJ56_RS12425-NJ56_RS12600) was integrated between an intact or partial tRNA-Asn copy. ICE(r2) is flanked on one side by an integrase gene and carries a VirB/VirD4 type IV secretion system operon (T4SS, NJ56_RS12510-NJ56_RS12550) that may mediate conjugation from donor to recipient cells analogous to conjugative plasmid translocation. A bacterial protein with homology to the Toll/IL-1 receptor (TIR) domain encoded by SC09-ICE(r2) that act as “accessory genes” has additionally been identified. This protein have been designated TcpA, encoded by NJ56_RS12465 (genes are colored in red, Fig. 1). The TIR domain is essential for Toll-like receptor (TLR) and adaptor interactions. TIR domain interactions play a key role in activating conserved cellular signal transduction pathways in response to pathogen signals [22]. Therefore, it is reasonable to hypothesize that TIR domain-containing proteins (Tcps) from *Y. ruckeri* SC09 disrupt the TLR signal transduction system in host cells to facilitate escape from the host innate immune and enhance bacterial survival.

3.2. TcpA is required for Yersinia ruckeri SC09 virulence during infection in vivo and in vitro

To analyze the virulence of TcpA during infection, we constructed an in-frame *tcpA*-deletion mutant of SC09 and the Δ*tcpA* + pTcpA mutant, which is complemented with a plasmid containing the *tcpA* gene controlled by its promoter. We used an acute *in vivo* infection model of rainbow trout to evaluate the involvement of TcpA in *Y. ruckeri* induced fish disease. The SC09 wild-type strain caused 50% lethality 6 days after inoculation (Fig. 2A). In contrast, we found that the SC09Δ*tcpA* mutant showed a slight but significant ($P < 0.01$; **)

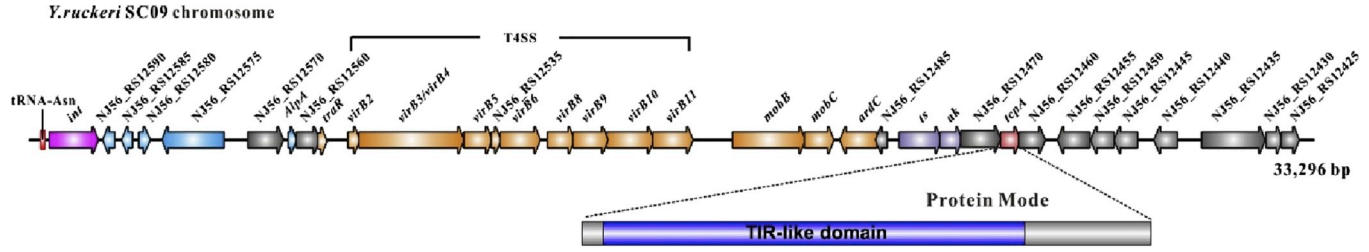

Fig. 1. ICE(r2) with putative ICE regions in bacterial genomes. Operon structure of Integrative and conjugative elements (ICEs). Int: integrase. T4SS: Type 4 secretory system.

attenuation in virulence in fish (Fig. 2A). Fish infected with *E. coli* DH5α with low-toxicity to fish act as a negative control (Fig. 2A). These differences were not due to an *in vitro* growth defect of the mutant (Fig. 2B). SC09Δ*tcpA* infected fish showed decreased cell recruitment and an enhanced bacterial clearance in liver, spleen and kidney compared to the wild-type strain (Fig. 2C–E, $P < 0.01$; **). Tissue damage was also detected in fish after infection with wild-type SC09, but not with the SC09Δ*tcpA* mutant. Histopathological examination of the infected fish showed marked changes in the liver, kidney, and spleen (Fig. 2E–G). Severe vacuolar degeneration and necrosis of the hepatic cells was observed in liver (Fig. 2F); glass-like substrate was observed in kidney tubules (Fig. 2G); the spleen medulla of fish became loose and exhibited edema (Fig. 2H). Together these results show that TcpA is required for *Y. ruckeri* SC09 infection *in vivo*.

Given the innate immune-evasion molecule TcpA may interfere with the host's innate immune response, which may further increase the intracellular survival capacities of bacteria. To test this hypothesis we investigated the ability of bacterial adherence, invasion and intracellular survival *in vitro*. Rainbow trout head kidney macrophages were infected with the wild-type SC09, SC09Δ*tcpA* mutant and Δ*tcpA* + pTcpA mutant, respectively. The results showed that no significant difference between the wild-type SC09 and SC09Δ*tcpA* mutant was found for bacterial adherence (Fig. 3A), invasion (Fig. 3B), or invasion ratio (number of invasive bacteria/number of adherent bacteria) (Fig. 3C). Thus, *tcpA* does not appear to have a role in *Y. ruckeri* adherence to and invasion into macrophages of rainbow trout. However, an intracellular survival assay demonstrated reduced survival of Δ*tcpA* mutant in rainbow trout head kidney macrophages compared to that of wild-type SC09 and Δ*tcpA* + pTcpA mutant after 18 hpi (Fig. 3D), indicating a role of *tcpA* in intracellular survival within rainbow trout macrophages. Moreover, as visualized using TEM, intact wild-type SC09 strains were detected inside the macrophages at 48 h post infection and several bacteria were contained in autophagocytic vacuoles (Fig. 3E). In addition, we have found that western blots couldn't be detected the TcpA protein in Δ*tcpA* mutant, while the protein in Δ*tcpA* + pTcpA mutant could be detected again (Fig. 3F). The SC10 (non-*tcpA Y. ruckeri* strain) couldn't be detected the TcpA protein, but we can express TcpA protein in SC10 and detected TcpA again (Fig. 3F). Together these results show that TcpA is required for *Y. ruckeri* SC09 infection *in vitro*.

3.3. TcpA affect the secretion and transcription of proinflammatory cytokines

The intracellular survival assay in macrophages stimulated us to test whether this was due to immune evasion or inactivation of innate

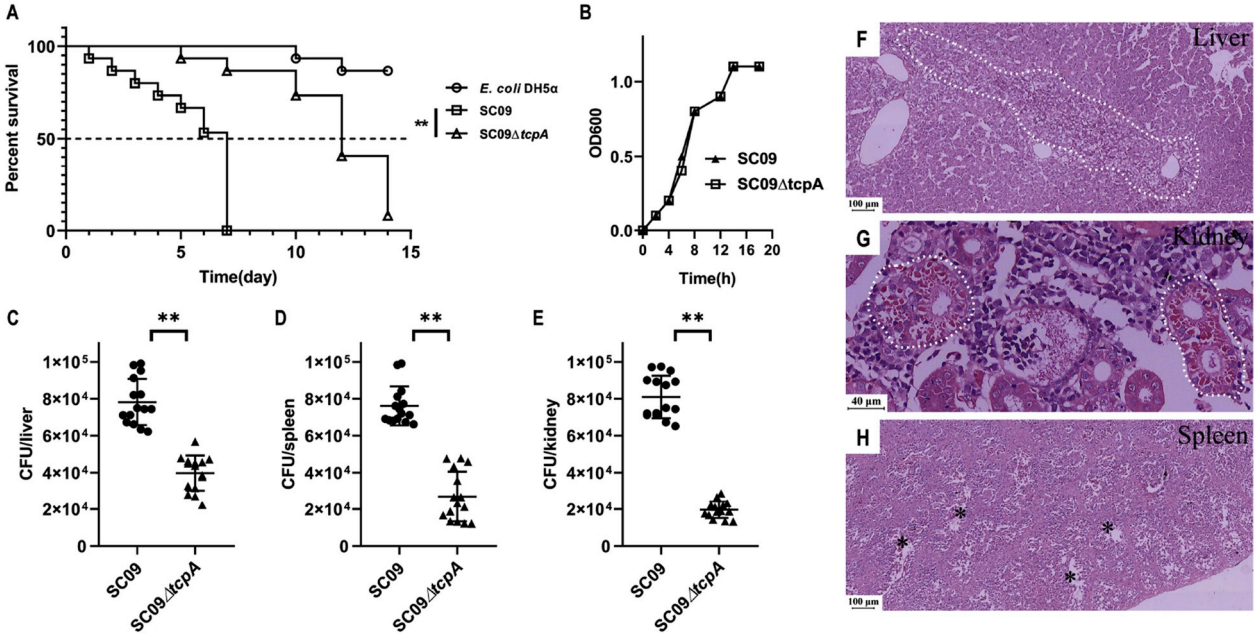

Fig. 2. TcpA is required for *Yersinia ruckeri* SC09 virulence *in vivo*. **A**. Rainbow trout survival curve. Rainbow trout were infected with *Y. ruckeri* SC09Δ*tcpA* and with wild-type *Y. ruckeri* SC09. Test of Mantel–Cox was used with **$P < 0.0001$. Fish infected with *E. coli* DH5α with low-toxicity to fish act as a negative control. **B**. Growth curve of wild-type *Y. ruckeri* SC09 and SC09Δ*tcpA*. **C-E**. Rainbow trout were infected with *Y. ruckeri* SC09 or SC09Δ*tcpA* strains (n = 15/group). Bacterial load in the liver (C), spleen (D) and kidney (E) were assessed through cultured the tissue homogenate. Non-parametric two-tailed T test was carried out with (C) **$P < 0.01$, (D) **$P < 0.01$ and (E) **$P < 0.01$. **F-H**. Pathological lesions of rainbow trout infected with wild-type *Y. ruckeri* SC09. Necrosis areas in liver (F, White dotted line); glass-like substrate in kidney tubules (G, White dotted line); edema in spleen medulla (H, asterisk).

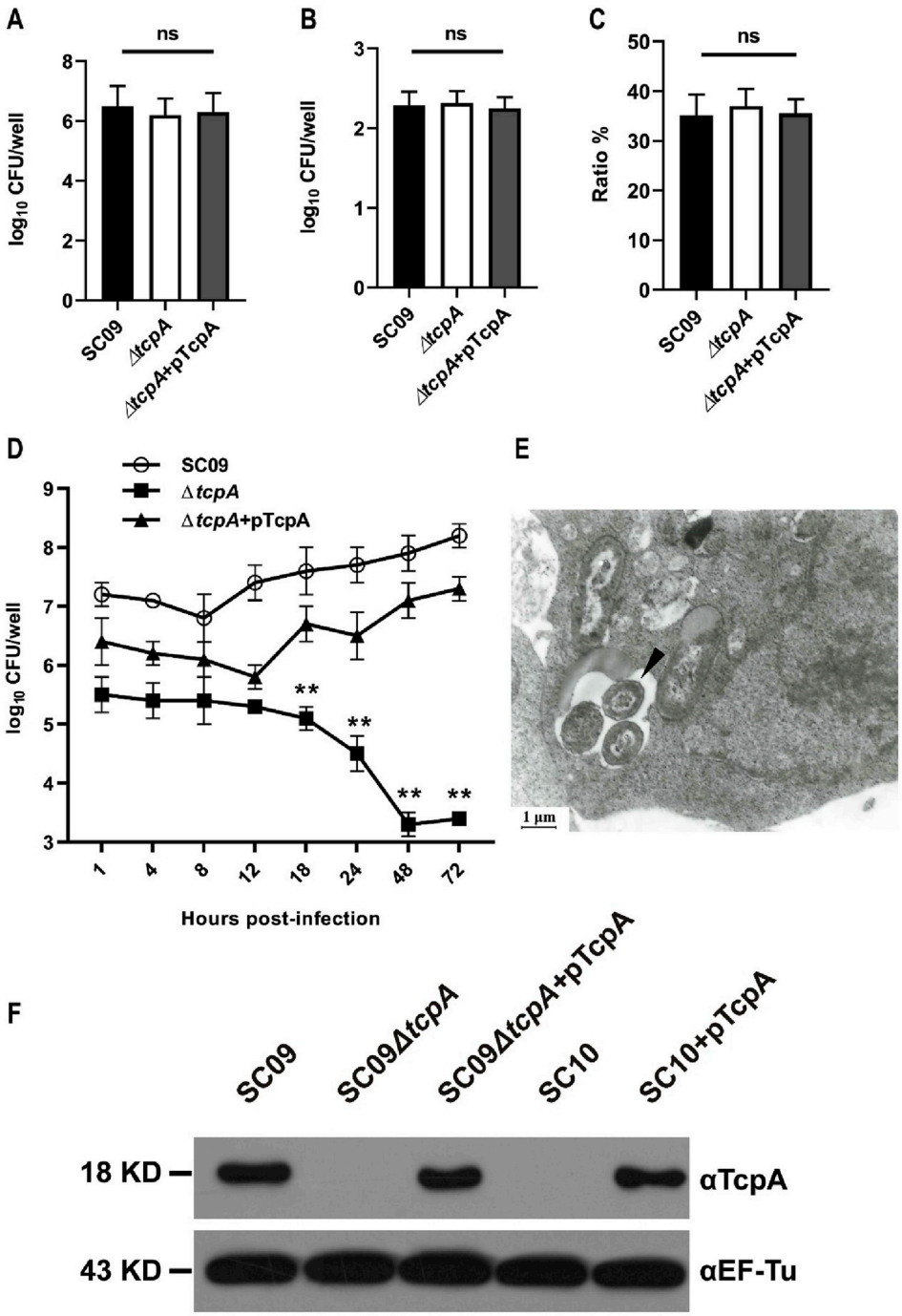

Fig. 3. The *tcpA* deletion mutant from the *Yersinia ruckeri* SC09 affects intracellular survival, but not adherence and invasion capacities in primary rainbow trout head kidney macrophages. Adherence (A), invasion (B), and invasion ratios (C) for the wild-type SC09, SC09Δ*tcpA* mutant and Δ*tcpA* + pTcpA mutants in primary rainbow trout head kidney macrophages, with no significant changes seen for infections at an MOI of 1.5. The invasion ratio was evaluated as the number of internalized bacteria/number of adherent bacteria. ns, no significant difference. (D) Bacterial intracellular survival of the SC09Δ*tcpA* mutant was significantly decreased compared to that of the wild-type SC09 and Δ*tcpA* + pTcpA mutants at 18, 24, 48 and 72 hpi, for infections at an MOI of 1.5. **P < 0.01. (E) Transmission electron micrographs of primary rainbow trout head kidney macrophages fixed at 48 h after infection *in vitro* with wild-type *Y. ruckeri* SC09. (F) Western blots from representative inocula used for the infection experiments, showing expression of TcpA (18 kDa) in the different *Y. ruckeri* strains visualized using a polyclonal rabbit anti-TcpA with control blot against the standard cytoplasmic protein EF-Tu (43 kDa) below.

immunity. To test this hypothesis, the innate response to wild-type SC09, SC09Δ*tcpA* mutant and Δ*tcpA* + pTcpA mutant were further studied in the rainbow trout head kidney macrophages. We first quantified the IL-6, IL-1β and TNF-α in culture supernatants by ELISA Sets. The results showed that infection with the Δ*tcpA* mutant stimulated a much higher IL-6 (Fig. 4A), IL-1β (Fig. 4B) and TNF-α response (Fig. 4C) in macrophages cells at 18, 24 and 48 hpi, respectively, than the wild-type SC09 strain or the Δ*tcpA* + pTcpA complemented mutant strain. To further confirm the effect of *tcpA* on macrophages was due to decreasing of the cytokine transcription levels. We also tested the expression of the IL-6, IL-1β and TNF-α in the rainbow trout head kidney macrophages. These data clearly indicated that infection with the Δ*tcpA* mutant caused a much higher expression of IL-6 (Fig. 4D), IL-1β (Fig. 4E) and TNF-α response (Fig. 4F) in macrophages cells at 18, 24 and 48 hpi, respectively, than the wild-type SC09 strain or the Δ*tcpA* + pTcpA complemented mutant strain. Together these results show that TcpA could affect the secretion and transcription of proinflammatory cytokines.

3.4. *Yersinia ruckeri* TcpA affect TLR signaling

Given the results above and the results of bioinformatics analysis (TcpA belongs to the family of bacterial TLRs), we then investigated the ability of TcpA to specifically interfere with TLR signaling using an *in vitro* NF-κB-dependent luciferase reporter system. Ectopic expression of *tcpA* in rainbow trout head kidney macrophages transfected with the NF-κB luciferase reporter vector and the plasmid encoding TLR4 or TLR2 was stimulated by LPS or PAM. TcpA inhibited the TLR4-

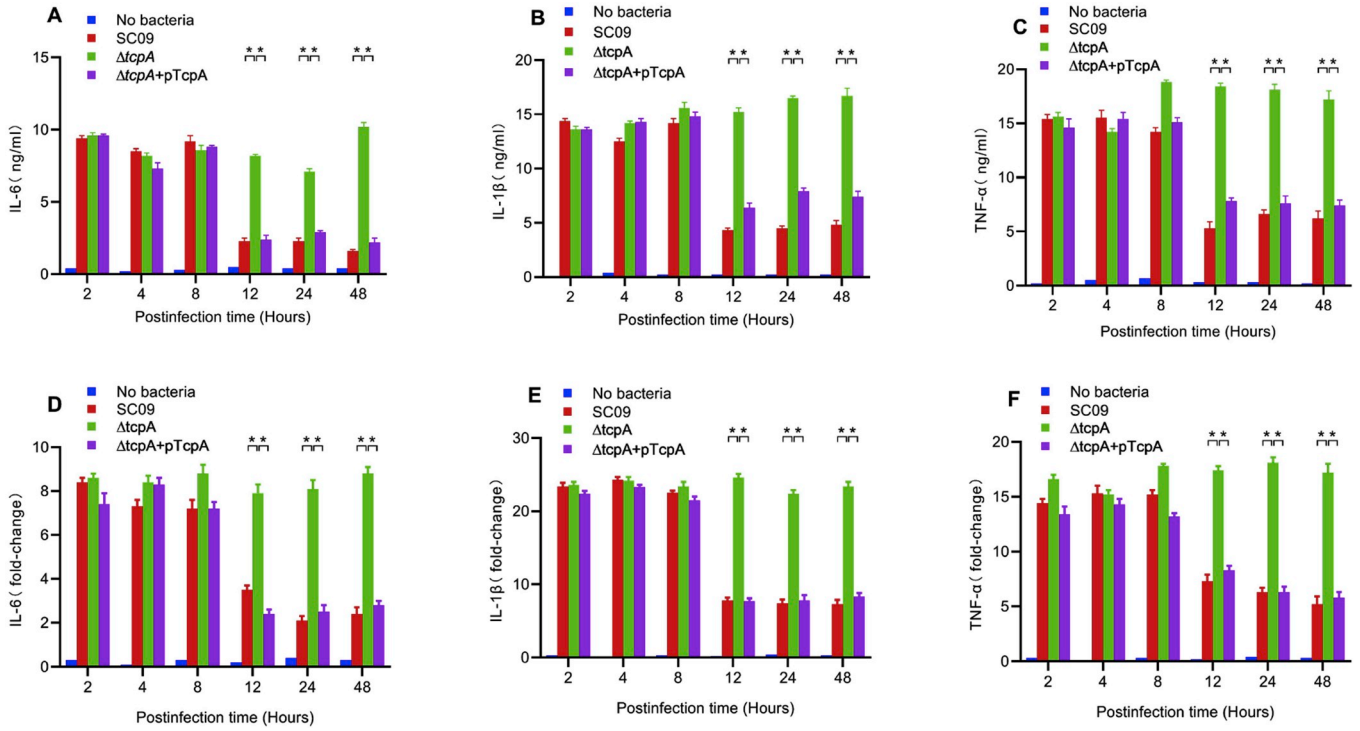

Fig. 4. TcpA reduce cytokine secretion and expression of macrophages cells. (A–C): IL-6, IL-1β and TNF-α secretion by rainbow trout head kidney macrophages cells that were either not infected or infected with *Y. ruckeri* wild-type SC09, *Y. ruckeri* SC09Δ*tcpA*, the Δ*tcpA* + pTcpA mutant were determined by ELISA. Cytokine levels were determined at 2 h, 4 h, 8 h, 12 h, 24h and 48h of infection. **(D–F)**: The expression of IL-6, IL-1β and TNF-α in macrophages cells that were either not infected or infected with *Y. ruckeri* wild-type SC09, *Y. ruckeri* SC09Δ*tcpA*, the Δ*tcpA* + pTcpA mutant were determined by qPCR. Cytokine expression levels were determined at 2 h, 4 h, 8 h, 12 h, 24h and 48h of infection. Error bars indicate s.d. of six individual cultures. **P < 0.01, ANOVA on ranks.

mediated NF-κB response to LPS (Fig. 5A). TcpA also impaired the NF-κB response to the potent TLR2 agonist PAM in cells transfected with TLR2 (Fig. 5B). These results suggest that TcpA may interfere with a common molecule of these TLR pathways, such as MyD88. We then tested whether TcpA could interact with MyD88. Rainbow trout head kidney macrophages were infected with the wild-type SC09 and co-immunoprecipitated (CO-IP) suggesting TcpA and MyD88 could be part of the same complex (Fig. 5C). This association was confirmed using purified GST-tagged TcpA immobilized on Ni-NTA resin, which retained HA-MyD88 (Fig. 5D). In addition, we observed by directed yeast two-hybrid that TcpA was able to interact with MyD88 (Fig. 5E).

4. Discussion

In the current study, we found that fish pathogen *Yersinia ruckeri* SC09 produced TLR homologs to inhibit TLR signaling and promoted immune evasion. Previously we used whole-genome sequencing to obtain the genome-wide sequence of *Y. ruckeri* SC09 [9], and by comparative genomics we found specific ICE elements in the bacterial genome. ICE elements are currently thought to mediate the horizontal transfer of genes in bacteria [23]. This element can provide the host with some specific “cargo” genes to help the host better adapt to a variety of complex environments, or to provide certain toxic capabilities to the host bacteria [24]. We identified the ICE(r2) element of Y. ruckeri SC09 genome carries a *tcpA* gene (NJ56_RS12465) associated with immune evasion, and we guess that the gene provides the host with the ability to toxic and immune evasion. We first demonstrated the correlation between *tcpA* gene and fish disease through *in vivo* experiments. TcpA was also shown to increase bacterial burden and tissue damage in fish. Meanwhile, *tcpA* from *Y. ruckeri* SC09 promoted the intracellular accumulation of bacteria *in vitro*. Further, *tcpA* can affect the transcription and secretion of some important proinflammatory cytokines during infection of fish primary immune cells. Finally, we found that TcpA could interact with MyD88, resulting in the inhibition of MyD88-induced activation. Therefore, we believe that this gene acts as a virulence factor for bacteria that impairs the signal transduction of the innate immune system, thereby helping the bacteria to escape from immunity and replicate in large amounts in host cells. This is the first virulence gene found in *Y. ruckeri* with immune evasion. This finding helps us to better understand aquatic pathogens and propose more effective means of prevention and control.

Toll/interleukin-1 receptor (TIR) domain plays an important role in the innate immune response of animals, including fish [13]. In recent years, the TIR domains of many microorganisms have been gradually identified and found to destroy the animal's natural immune system [22,25]. In fact, the TIR-homologous genes is not only found in *Y. ruckeri*, but current research has found that the genes are ubiquitous in *Escherichia coli* [26], *Brucella* [27], *Yersinia pestis* [28], *Pseudomonas* [29] and *Staphylococcus aureus* [30]. Studies of the TIR-homologous genes in other bacteria have also demonstrated its toxicity and ability to provide immune evasion to host bacteria [26–30]. It is worth noting that in these bacteria, only the highly virulent strains carry the TIR-homologous genes [26–30], while the gene knockout of the virulent strain is significantly less toxic. It was also found in our study that the highly virulent *Y. ruckeri* SC09 strain carries the TIR-homologous gene (*tcpA* gene), while the non-toxic environmental bacteria *Y. ruckeri* SC10 do not have this gene or TIR-homologous genes.

Some products of bacteria, including lipopolysaccharide, peptidoglycan, etc., can be used as a dangerous signal in the infection area [31]. When these signals interact with the TLR, the cells are activated to exercise the corresponding immune function [13,14]. The danger signal is present to alert the host to the natural immune system. The TIR of bacteria can play a competitive role in blocking signal transmission because it is similar to the intracellular portion of TLR [22,25,27,29]. Bacterial TIR may cause immune signals to be blocked by competitively binding to the MyD88 linker protein in the NF-κB signaling pathway

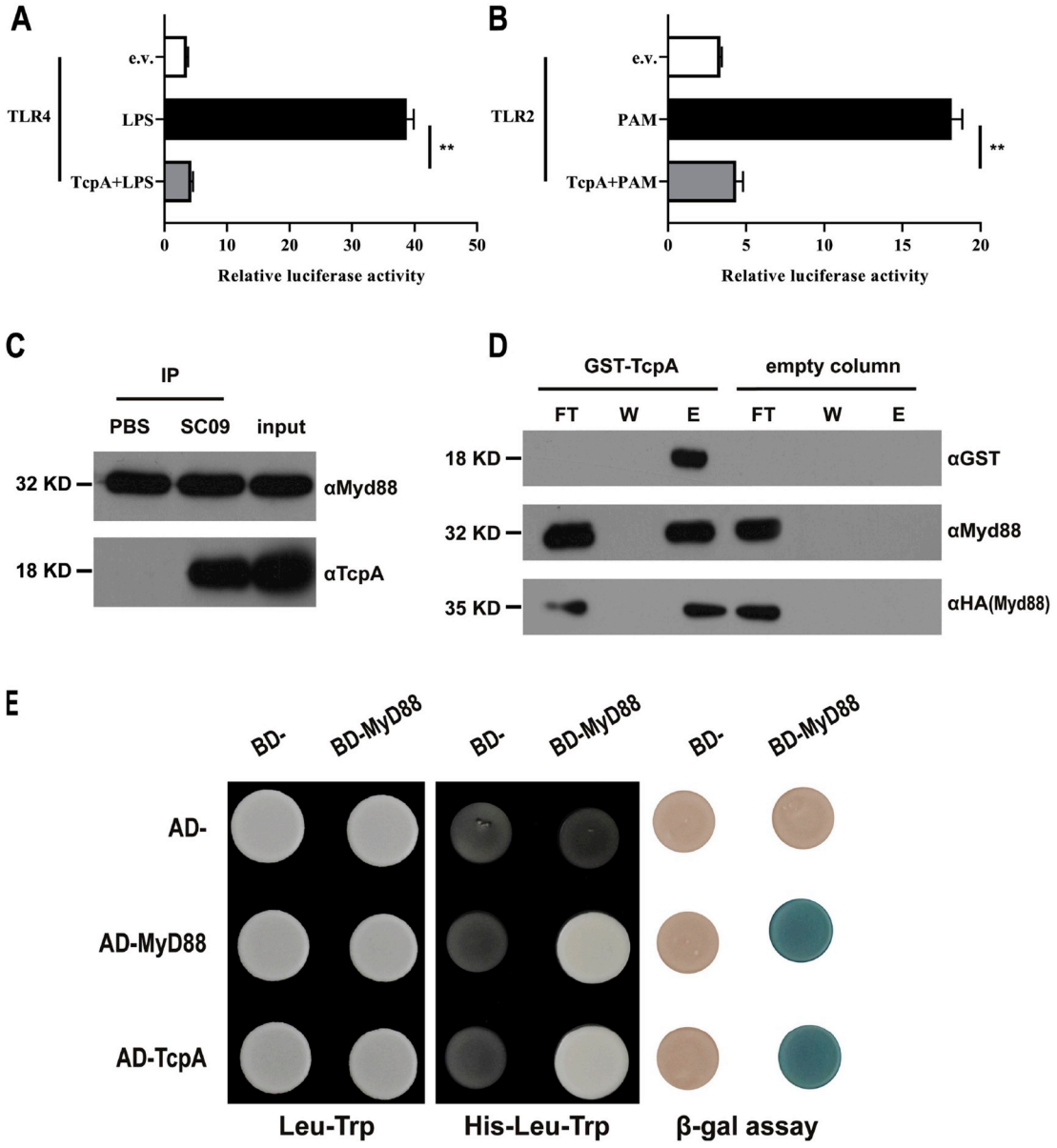

Fig. 5. TcpA impairs TLR signaling by binding to MyD88. (A) The luciferase reporter plasmid and the TLR4 plasmid were transiently transfected into rainbow trout head kidney macrophages with or without the TcpA plasmid. After 24 h, the cells were stimulated with LPS for 6 h, and luciferase activity was determined. The white band represents the negative control (empty vector), the black band represents LPS-stimulated cells, and the gray band represents cells transfected with the TcpA plasmid and stimulated by LPS. Data correspond to median ± standard errors of the relative luciferase activity from five independent experiments. **(B)** This part of the experiment was similar to (A), except that the plasmid was TLR2, and the cells were stimulated with PAM. **(C)** Co-immunoprecipitation (co-IP) experiments were performed on cells infected with wild-type SC09 or PBS (control). After co-IP, the anti-TcpA antibody was used to detect protein interactions, and the anti-MyD88 antibody was used to detect proteins bound to beads, and the inputs were detected with anti-MyD88 antibody and anti-TcpA antibody, respectively. **(D)** Pull-down experiments were performed by the *in vitro* expression of HA-MyD88 protein and the prokaryotic expression of GST-TcpA protein immobilized on a Ni-NTA resin. An empty vector was used as a control. The anti-HA and anti-MyD88 antibodies were used to detect protein interactions (lower blot) by Western blotting, and the anti-GST antibody was used to detect the binding of the GST-TcpA protein to the resin. The flowthrough (FT), two washes (W), and elution (E) are shown in each lane. **(E)** Recombinant plasmids containing Gal4 BD and Gal4 AD were co-transformed into yeast cells and screened on plates lacking leucine (Leu) and tryptophan (Trp) (left panel). Gal4 BD- and Gal4 AD-recombinant plasmids were co-transformed into yeast cells and screened on plates lacking histidine (His) in the presence of 20 mM 3AT (middle panel). Transformants grown on this medium present an interaction between TcpA and MyD88. The expression of the reporter β-galactosidase gene in yeast produces blue yeast, and indicated the occurrence of protein interactions (right panel). Empty vectors for AD- and BD-plasmids served as negative controls, and MyD88 homodimerization served as a positive control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

[27,29]. Our study showed that the TcpA protein in the *Y. ruckeri* SC09 strain does interact with the MyD88 protein. In addition, *in vitro* experiments also demonstrated the ability of the TcpA protein to block the TLR2-and TLR4-mediated activation of NF-κB. NF-κB is an important transcription factor in the fish immune system that initiates the expression of many cytokines that mediate inflammation [32]. If the NF-κB signaling pathway is blocked, the production of downstream inflammatory cytokines may also be affected. Our data suggest that the *tcpA* gene does significantly affect NF-κB-mediated transcription and production of some cytokines. These are beneficial for the bacteria to further infect the host and survive and replicate within the host cells. Our observations also corroborate the intracellular viability of the wild

type *Y. ruckeri* SC09 and the colonization ability of the host organs.

TcpA as a TIR-homologue is a very interesting protein. Our study showed that TcpA was adjacent to the type IV secretion system (T4SS) in the ICE element, suggesting that the secretion of TcpA may be related to the T4SS. In the TIR-homologous genes study of *E. coli* [26] and *Brucella* [27], it was found that the secretion of these genes was mediated by the T4SS. Therefore, it was a meaningful research to uncover the underlying mechanism of the interaction between TcpA and T4SS in *Y. ruckeri* in the future study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.069.

References

[1] M. Ohtani, K.R. Villumsen, H.K. Strøm, A.H. Lauritsen, B. Aalbæk, I. Dalsgaard, B. Nowak, M.K. Raida, A.M. Bojesen, Effects of fish size and route of infection on virulence of a Danish Yersinia ruckeri O1 biotype 2 strain in rainbow trout (Oncorhynchus mykiss), Aquaculture 503 (2019) 519–526.
[2] G. Kumar, S. Menanteau-Ledouble, M. Saleh, M. El-Matbouli, Yersinia ruckeri, the causative agent of enteric redmouth disease in fish, Vet. Res. 46 (1) (2015).
[3] I. Altinok, E. Capkin, H. Boran, Comparison of molecular and biochemical heterogeneity of Yersinia ruckeri strains isolated from Turkey and the USA, Aquaculture 450 (2016) 80–88.
[4] D. Cascales, J.A. Guijarro, A.I. García-Torrico, J. Méndez, Comparative genome analysis reveals important genetic differences among serotype O1 and serotype O2 strains of Y. ruckeri and provides insights into host adaptation and virulence, MicrobiologyOpen 6 (4) (2017).
[5] L. Fernandez, I. Marquez, J.A. Guijarro, Identification of specific in vivo-induced (ivi) genes in Yersinia ruckeri and analysis of ruckerbactin, a catecholate siderophore iron acquisition system, Appl. Environ. Microbiol. 70 (9) (2004) 5199–5207.
[6] L. Fernandez, M. Prieto, J.A. Guijarro, The iron- and temperature-regulated haemolysin YhlA is a virulence factor of Yersinia ruckeri, Microbiology 153 (Pt 2) (2007) 483–489.
[7] J.P. Evenhuis, S.E. Lapatra, D.W. Verner-Jeffreys, I. Dalsgaard, T.J. Welch, Identification of flagellar motility genes in Yersinia ruckeri by transposon mutagenesis, Appl. Environ. Microbiol. 75 (20) (2009) 6630–6633.
[8] I. Dahiya, R.M. Stevenson, The UvrY response regulator of the BarA-UvrY two-component system contributes to Yersinia ruckeri infection of rainbow trout (Oncorhynchus mykiss), Arch. Microbiol. 192 (7) (2010) 541–547.
[9] T. Liu, K.Y. Wang, J. Wang, D.F. Chen, X.L. Huang, P. Ouyang, Y. Geng, Y. He, Y. Zhou, J. Min, Genome sequence of the fish pathogen Yersinia ruckeri SC09 provides insights into niche adaptation and pathogenic mechanism, Int. J. Mol. Sci. 17 (4) (2016) 557.
[10] F. Delavat, R. Miyazaki, N. Carraro, N. Pradervand, J.R. van der Meer, The hidden life of integrative and conjugative elements, FEMS Microbiol. Rev. 41 (4) (2017) 512–537.
[11] X. Bellanger, S. Payot, N. Leblond-Bourget, G. Guedon, Conjugative and mobilizable genomic islands in bacteria: evolution and diversity, FEMS Microbiol. Rev. 38 (4) (2014) 720–760.
[12] H.M. Seth-Smith, M.C. Fookes, C.K. Okoro, S. Baker, S.R. Harris, P. Scott, D. Pickard, M.A. Quail, C. Churcher, M. Sanders, J. Harmse, G. Dougan, J. Parkhill, N.R. Thomson, Structure, diversity, and mobility of the Salmonella pathogenicity island 7 family of integrative and conjugative elements within enterobacteriaceae, J. Bacteriol. 194 (6) (2012) 1494–1504.
[13] Y. Palti, Toll-like receptors in bony fish: from genomics to function, Dev. Comp. Immunol. 35 (12) (2011) 1263–1272.
[14] S.M. Quiniou, P. Boudinot, E. Bengten, Comprehensive survey and genomic characterization of Toll-like receptors (TLRs) in channel catfish, Ictalurus punctatus: identification of novel fish TLRs, Immunogenetics 65 (7) (2013) 511–530.
[15] J. Ryckaert, P. Bossier, K. D'Herde, A. Diez-Fraile, P. Sorgeloos, F. Haesebrouck, F. Pasmans, Persistence of Yersinia ruckeri in trout macrophages, Fish Shellfish Immunol. 29 (4) (2010) 648–655.
[16] P. Luo, X. He, Q. Liu, C. Hu, Developing universal genetic tools for rapid and efficient deletion mutation in Vibrio species based on suicide T-vectors carrying a novel counterselectable marker, vmi480, PLoS One 10 (12) (2015) e0144465.
[17] N. Carraro, D. Matteau, P. Luo, S. Rodrigue, V. Burrus, The master activator of IncA/C conjugative plasmids stimulates genomic islands and multidrug resistance dissemination, PLoS Genet. 10 (10) (2014) e1004714.
[18] J. Jiang, W. Zhao, Q. Xiong, K. Wang, Y. He, J. Wang, D. Chen, Y. Geng, X. Huang, P. Ouyang, W. Lai, Immune responses of channel catfish following the stimulation of three recombinant flagellins of Yersinia ruckeri in vitro and in vivo, Dev. Comp. Immunol. 73 (2017) 61–71.
[19] E. Skippington, M.A. Ragan, Lateral genetic transfer and the construction of genetic exchange communities, FEMS Microbiol. Rev. 35 (5) (2011) 707–735.
[20] M. Del Grosso, R. Camilli, E. Rizzi, A. Pietrelli, G. De Bellis, A. Pantosti, ICESpy009, a conjugative genetic element carrying mef(E) in Streptococcus pyogenes, Antimicrob. Agents Chemother. 60 (7) (2016) 3906–3912.
[21] R. Miyazaki, M. Minoia, N. Pradervand, S. Sulser, F. Reinhard, J.R. van der Meer, Cellular variability of RpoS expression underlies subpopulation activation of an integrative and conjugative element, PLoS Genet. 8 (7) (2012) e1002818.
[22] R.R. Rana, M. Zhang, A.M. Spear, H.S. Atkins, B. Byrne, Bacterial TIR-containing proteins and host innate immune system evasion, Med. Microbiol. Immunol. 202 (1) (2013) 1–10.
[23] D. Bi, Z. Xu, E.M. Harrison, C. Tai, Y. Wei, X. He, S. Jia, Z. Deng, K. Rajakumar, H.Y. Ou, ICEberg: a web-based resource for integrative and conjugative elements found in bacteria, Nucleic Acids Res. 40 (Database issue) (2012) D621–D626.
[24] A.P. Roberts, P. Mullany, A modular master on the move: the Tn916 family of mobile genetic elements, Trends Microbiol. 17 (6) (2009) 251–258.
[25] N.J. Patterson, D. Werling, To con protection: TIR-domain containing proteins (Tcp) and innate immune evasion, Vet. Immunol. Immunopathol. 155 (3) (2013) 147–154.
[26] C. Cirl, A. Wieser, M. Yadav, S. Duerr, S. Schubert, H. Fischer, D. Stappert, N. Wantia, N. Rodriguez, H. Wagner, C. Svanborg, T. Miethke, Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins, Nat. Med. 14 (4) (2008) 399–406.
[27] S.P. Salcedo, M.I. Marchesini, C. Degos, M. Terwagne, K. Von Bargen, H. Lepidi, C.K. Herrmann, T.L. Santos Lacerda, P.R.C. Imbert, P. Pierre, L. Alexopoulou, J.-J. Letesson, D.J. Comerci, J.-P. Gorvel, BtpB, a novel Brucella TIR-containing effector protein with immune modulatory functions, Front. Cell. Infect. Microbiol. 3 (2013).
[28] A.M. Spear, R.R. Rana, D.C. Jenner, H.C. Flick-Smith, P.C. Oyston, P. Simpson, S.J. Matthews, B. Byrne, H.S. Atkins, A Toll/interleukin (IL)-1 receptor domain protein from Yersinia pestis interacts with mammalian IL-1/Toll-like receptor pathways but does not play a central role in the virulence of Y. pestis in a mouse model of bubonic plague, Microbiology 158 (Pt 6) (2012) 1593–1606.
[29] P.R. Imbert, A. Louche, J.B. Luizet, T. Grandjean, S. Bigot, T.E. Wood, S. Gagne, A. Blanco, L. Wunderley, L. Terradot, P. Woodman, S. Garvis, A. Filloux, B. Guery, S.P. Salcedo, A Pseudomonas aeruginosa TIR effector mediates immune evasion by targeting UBAP1 and TLR adaptors, EMBO J. 36 (13) (2017) 1869–1887.
[30] S. Patot, P.R. Imbert, J. Baude, P. Martins Simoes, J.B. Campergue, A. Louche, R. Nijland, M. Bes, A. Tristan, F. Laurent, A. Fischer, J. Schrenzel, F. Vandenesch, S.P. Salcedo, P. Francois, G. Lina, The TIR homologue lies near resistance genes in Staphylococcus aureus, coupling modulation of virulence and antimicrobial susceptibility, PLoS Pathog. 13 (1) (2017) e1006092.
[31] A.M. Spear, N.J. Loman, H.S. Atkins, M.J. Pallen, Microbial TIR domains: not necessarily agents of subversion? Trends Microbiol. 17 (9) (2009) 393–398.
[32] Y. Wu, M. Guo, X. Hua, K. Duan, G. Lian, L. Sun, L. Tang, Y. Xu, M. Liu, Y. Li, The role of infectious hematopoietic necrosis virus (IHNV) proteins in the modulation of NF-kappaB pathway during IHNV infection, Fish Shellfish Immunol. 63 (2017) 500–506.