Orf1B controls secretion of T3SS proteins and contributes to *Edwardsiella piscicida* adhesion to epithelial cells

Long Kun Wang¹,²†, Shan Shan Sun²,³†, Shu Ya Zhang²,³, Pin Nie² and Hai Xia Xie²*

**Abstract**

*Edwardsiella piscicida* is a Gram-negative enteric pathogen that causes hemorrhagic septicemia in fish. The type III secretion system (T3SS) is one of its two most important virulence islands. T3SS protein EseJ inhibits *E. piscicida* adhesion to epithelioma papillosum cyprini (EPC) cells by negatively regulating type 1 fimbria. Type 1 fimbria helps *E. piscicida* to adhere to fish epithelial cells. In this study, we characterized a functional unknown protein (Orf1B) encoded within the T3SS gene cluster of *E. piscicida*. This protein consists of 122 amino acids, sharing structural similarity with YscO in *Vibrio parahaemolyticus*. Orf1B controls secretion of T3SS translocon and effectors in *E. piscicida*. By immunoprecipitation, Orf1B was shown to interact with T3SS ATPase EsaN. This interaction may contribute to the assembly of the ATPase complex, which energizes the secretion of T3SS proteins. Moreover, disruption of Orf1B dramatically decreased *E. piscicida* adhesion to EPC cells due to the increased steady-state protein level of EseJ within *E. piscicida*. Taken together, this study partially unraveled the mechanisms through which Orf1B promotes secretion of T3SS proteins and contributes to *E. piscicida* adhesion. This study helps to improve our understanding on molecular mechanism of *E. piscicida* pathogenesis.

**Keywords:** T3SS, Orf1B, adhesion, T3SS protein secretion, *Edwardsiella piscicida*

**Introduction**

*Edwardsiella piscicida* PPD130/91, previously known as *Edwardsiella tarda* PPD130/91 [1], is an intracellular bacterium, belonging to the family of Enterobacteriaceae. Based on phenotypic characterization, DNA-DNA hybridization and phylogenetic analysis, *E. tarda* strains were further classified into three species *E. tarda*, *E. piscicida* and *E. anguillarum* [1–3]. *E. tarda* infects humans and possesses neither the T3SS nor type VI secretion system (T6SS) gene cluster [1]; *E. piscicida* infects a wide range of fish species of both freshwater and marine, causing hemorrhagic septicemia mainly in Japanese flounder (*Paralichthys olivaceus*) and turbot (*Scophthalmus maximus*), and only one set of T3SS and T6SS gene cluster is distributed in its genome [1–4]; *E. anguillarum* infects eel, it has acquired the locus for enterocyte effacement (LEE) genes and contains 2 sets of T3SS and 3 sets of T6SS gene clusters [1].

*E. piscicida* is able to invade and replicate in epithelial and phagocytic cells, such as Epithelioma papillomatosum cyprini (EPC) cells, HEp-2 cells, and murine macrophage J774A.1 cells [5–7]. The Type III secretion system (T3SS) is one of the two most important virulence factors in *E. piscicida* [6, 8]. T3SS is a membrane-spanning macromolecular machine that comprises more than 20 different proteins, through which effectors are delivered into host cells to cause infection [9]. Three translocon proteins (EseB, EseC and EseD) and two effector
proteins (EseJ and EseG) are encoded within the *E. piscicida* T3SS gene cluster [6, 10, 11]. In the presence of host cells, EseB, EseC and EseD can form pores on the host membrane, through which effectors are translocated. In the absence of host cells, EseB forms filamentous appendages and mediates autoaggregation and biofilm formation in *E. piscicida* [12]. EsaN is an ATPase that energizes the transportation of T3SS substrates [10]. Deleting a translocon gene, such as *eseB*, *eseC*, *eseD* or an ATPase gene *esaN*, increases the 50% lethal dose (LD50) by approximately 1 log in blue gourami fish [6, 10]. EseJ inside *E. piscicida* inhibits its adhesion to EPC cells through negative regulation on type 1 fimbriae [11, 13]. *E. piscicida* type 1 fimbrial major subunit FimA is involved in its adhesion to fish epithelial cells [14–16].

The function of orf1B within T3SS gene cluster of *E. piscicida* remains obscure. Orf1B shares similarity with EscO of enteropathogenic *Escherichia coli* (EPEC), Spa13 of *Shigella flexneri*, SpaM/InvI of *Salmonella SPI-1* (*Salmonella* Pathogenicity Island 1). Moreover, its structure resembles YscO of *Vibrio para-haemolyticus* and flagellar FliJ. EscO, encoded by a gene in the locus of enterocyte effacement (LEE) pathogenicity island, is essential for secretion of all categories of T3SS substrates in EPEC, moreover, EscO interacts with the ATPase EscN and stimulates EscN enzymatic activity [17]. Spa13 interacts with the ATPase Spa47, the C-ring protein Spa33, and the inner-membrane protein Spa40. Moreover, Spa13 can stabilize the needle protein MixH, thus influencing the secretion of type III proteins [18]. SpaM is required for efficient secretion of translocon proteins SipB and SipC and effector proteins SipA, SipD and SptP by *Salmonella SPI-1* [19]. *Yersinia YscO* interacts with needle length control protein YcsP to control Yop secretion [20, 21], while YscO is not required for the assembly of the ATPase YscN [22]. FliJ of the *Salmonella enterica* serovar Typhimurium is remarkably similar to the γ subunit of FoF1-ATP synthase, FliJ promotes the formation of FliJ (ATPase) hexamer rings by binding to the center of the ring [23, 24].

Orf1B was functionally characterized in this study, and it was revealed that Orf1B promotes the secretion of T3SS proteins probably by interacting with T3SS ATPase EsaN, contributing to its adhesion to epithelial cells by controlling the steady-state protein level of EseJ inside *E. piscicida*.

### Materials and methods

#### Bacterial strains and culture

The bacterial strains and plasmids used in this study are listed in Table 1. *E. piscicida* PPD130/91 [25] were grown in tryptic soy broth (TSB, BD Biosciences) at 28 °C and *E. coli* strains in Luria–Bertani broth (LB, BD Biosciences) at 37 °C. To activate T3SS, *E. piscicida* strains were cultured in Dulbecco modified Eagle medium (DMEM, Invitrogen) at 25 °C under a 5% (vol/vol) CO2 atmosphere.

### Table 1  Bacterial strains and plasmids used in this study.

| Strains or plasmids | Description and/or genotype | References or source |
|---------------------|-----------------------------|---------------------|
| **Strains**         |                             |                     |
| *E. piscicida*       |                             |                     |
| WT                  | PPD130/91, Km, Col, Amp    | [25]                |
| ΔesaN               | In-frame deletion of aa 21–421, PPD130/91 | [10]                |
| Δorf1B              | In-frame deletion of aa 7–117, PPD130/91 | This study         |
| WT/pACYC184         | WT strain transformed with pACYC184 | This study         |
| Δorf1B/pACYC184     | Δorf1B strain transformed with pACYC184 | This study         |
| Δorf1B/orf1B        | Δorf1B with pACYC184-HA-orf1B | This study         |
| WT esaN:3 × FLAG    | PPD130/91, with chromosomal expression of EsaN-3 × FLAG, Amp', Km' | This study         |
| WT esaN:3 × FLAG/orf1B | WT esaN:3 × FLAG with pACYC184-HA-orf1B | This study         |
| WT eseE:3 × FLAG    | PPD130/91, with chromosomal expression of EseE-3 × FLAG, Amp', Km' | This study         |
| WT eseE:3 × FLAG/orf1B | WT eseE:3 × FLAG with pACYC184-HA-orf1B | This study         |
| **Plasmids**        |                             |                     |
| pACYC184            | Tet', Cm'                   | Amersham           |
| pACYC184-HA-orf1B   | pACYC184 with HA-orf1B      | This study         |
| pRE112              | pGP704 suicide plasmid, pir dependent; Cm, onT, onV, sacB | [27]                |
| pKD46               | Red helper plasmid, Amp'    | [30]                |
| pKD4                | Template plasmid with FLP recognition target site, Km' | [30]                |

Superscripts—r, resistance; s, sensitivity.
Col, colistin; Amp, ampicillin; Tet, tetracycline; Km, kanamycin; Cm, chloramphenicol.
Antibiotics were supplemented at the following concentrations when required: 12.5 μg/mL colistin (Col; Sigma), 34 μg/mL chloramphenicol (Cm; Sigma), 50 μg/mL gentamicin (Gem; Sigma), and 50 μg/mL kanamycin (Km; Sigma).

**Cell and culture condition**

EPC cells [26] were grown at 28 °C in M199 medium (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) under a 5% (vol/vol) CO₂ atmosphere.

**Construction of deletion mutants, complementation, and plasmids**

Primers used in this study are listed in Table 2. Non-polar orf1B deletion mutants were generated by sacB-based allelic exchange [27]. Briefly, primer pairs orf1B-for/orf1B-int-rev, orf1B-int-for/orf1B-rev were used to generated an 845-bp fragment containing the upstream region of orf1B, and a 754-bp fragment containing the downstream region of orf1B from PPD130/91 genomic DNA. A 16 bp overlapping sequence introduced into the flanking DNA fragments permitted the fusion of the upstream and downstream regions together by a second PCR with primers orf1B-for and orf1B-rev. The resulting PCR product was ligated into the suicide vector pRE112 [27] before being transformed into E. coli S17-1λpir. Through conjugation, a Δorf1B strain was screened on 10% sucrose–tryptic soy agar plates and was verified by PCR and sequencing. The mutant strains show no defect in growth when cultured in TSB or DMEM.

To obtain the DNA sequence covering ribosome binding site, hemagglutinin tag (HA tag, sequence: YPYDVPDYA) and orf1B gene, two PCR fragments were first obtained from PPD130/91 genomic DNA by primer pairs orf1B-com-for plus orf1B-com-int-rev, orf1B-com-int-for plus orf1B-com-rev, which were overlapped with the primer pair of orf1B-com-for plus orf1B-com-rev before being inserted into pACYC184 (Amersham). The pACYC184-HA-orf1B obtained was introduced into the Δorf1B strain to obtain the Δorf1B/orf1B strain; expression of HA-Orf1B was confirmed by immunoblotting with mouse anti-HA antibody.

**Adhesion assay by confocal microscopy**

Adhesion assay was performed as described by Xie et al. [11]. Briefly, EPC monolayers were washed once with pre-warmed M199 medium (28 °C) and infected at a multiplicity of infection (MOI) of 10 with E. piscicida PPD130/91 strains wt/pACYC184, Δorf1B/pACYC184, and Δorf1B/orf1B (Table 1). After 30 min of infection, the EPC monolayers were washed three times and fixed in 4% paraformaldehyde (PFA) and stained with mouse anti-LPS antibody and Alex 488 Goat anti-mouse IgG (Invitrogen). The adherence rates of each infection condition were examined from two wells in triplicate experiments. Fourteen images were photographed randomly from two replicates under a confocal laser scanning microscope.

---

**Table 2 Primers used in this study.**

| Primers       | Sequences (5′−3′)     |
|---------------|-----------------------|
| orf1B-for     | ATGTTACCCCGGCTGGCGATCCCTTCCT |
| orf1B-int-rev | GCGTAGCTGCCGTCAGCATGG    |
| orf1B-int-for | GCTGACGGAGCTAGGAGGAGAGGAATGA |
| orf1B-rev     | ATGTTACCCGAGCCAGCAGCATGCCAC |
| orf1B-com-for | CACTGTGCCGACCGGTTTGGCCGCGGCTGGGCAATACGGAGC |
| orf1B-com-int-rev | ATATCGGTACGTCCGACCGGCTGGTCAGCGATAT |
| orf1B-com-int-for | AGGCCAGCTACATACGATGTCCGATAGCTGCCAGCAGATAT |
| orf1B-com-rev | GCCGAAAAGCGGCGAGAGGCGCTCGCGGA |
| pACYC184-for  | GCCCTCCAACGCTACTGCGGGCTGCTTC |
| pACYC184-rev  | GCGAAAGGGCGACGCGACGGCGCTCCGAGA |
| RT-16S-for    | ACTGAGACACGGCTCGGACGCTTCAGCTTCAG |
| RT-16S-rev    | TTAACGTTTACACCTTCTCCCTACG |
| RT-esN-for    | CTGCTGGGCGACCTTACCTCCAC |
| RT-esN-rev    | AGATCTCCGCGATGCTGCTT |
| esaN:3 × FLAG-for | GCCACCCCTCCGCGGACATGACGAGGCGACCATCGACCTACAAGACGAGCATAGACGCTGG |
| esaN:3 × FLAG-rev | CTTGGAGCGCTCGGCGACATGACGAGCATAGACGCTGG |
| eseE:3 × FLAG-for | ATCGTCGGGAGATGCAGGCCGTCGGGGCGTTCAGGACGACGAGCATAGACGCTGG |
| eseE:3 × FLAG-rev | GATATATATATATACAGGGCGTCGCGCCCCCGGGCCTGGGAACGACGAGCATAGACGCTGG |
(Leica SP8). Representative images and mean ± standard deviations (SD) from one representative experiment were shown. Student’s t test was used to analyse the difference between the two groups.

To prepare mouse anti-LPS antibody, LPS was isolated according to the manufacturer’s protocol (Bestbio) from 30 mL E. piscicida PPD130/91 cultured in TSB, and the LPS antibody was prepared as described by Gao et al. in eight 6-week-old naive C57BL/6 mice by the company of Daian, China [12].

Quantitative reverse transcription-PCR
Overnight cultures of E. piscicida WT strain, Δorf1B strain, and Δorf1B/orf1B strain were subcultured at 1:100 into 10 mL DMEM and grown at 25 °C under a 5% (vol/vol) CO2 atmosphere for 24 h. Bacteria from three replicates of each strain were harvested. Total RNA was isolated according to the manufacturer’s protocol with the RNeasy Mini Kit (Qiagen), followed by DNase I treatment for the elimination of genomic DNA contaminants. Upon the RNA reverse transcription, nine cDNA libraries (WT-1, WT-2, WT-3, Δorf1B-1, Δorf1B-2, Δorf1B-3, Δorf1B/orf1B-1, Δorf1B/orf1B-2, and Δorf1B/orf1B-3) were obtained. Real-time PCR was performed on a CFX96 real-time system (Bio-Rad) using the SYBR green reagent. The qPCR was run at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s, 72 °C for 30 s, and 75.5 °C for 5 s. The mRNA expression level was normalized against the level of 16S rRNA gene expression. The relative transcriptional level of eseJ in the Δorf1B and Δorf1B/orf1B strains compared to that in the WT was then calculated using the formula 2^\(-\Delta\Delta CT\), where CT is the threshold cycle and ΔCT is equal to the change in the CT (ΔC_T) for the Δorf1B or Δorf1B/orf1B strain minus ΔC_T for the wild type. The t test implemented in SPSS was used to calculate the P value with a hypothetical mean of 1.0. The data shown are the means ± SD from triplicate samples.

Expression and secretion assay
Overnight cultures of E. piscicida strains were subcultured at 1:100 into DMEM and grown static at 25 °C for 24 h. The total bacterial proteins (TBP) and the extra-cellular proteins (ECP) were prepared as described by Zheng and Leung [8] before being subjected to immunoblotting with mouse antibodies against DnaK at 1:2000 (Stressgen), and with rabbit antibodies against EseJ at 1:2000 [11], EseG at 1:2000 [10], EseB at 1:2000 [28], EseC at 1:2000 [29], EseD at 1:2000 [29] and EvpC at 1:2000 [8]. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or Goat anti-rabbit IgG (Millipore) were used at a dilution of 1:5000.

Co-immunoprecipitation (Co-IP) assay
The chromosomal copy of esaN or eseE in E. piscicida was tagged with the 3× FLAG epitope by the Red recombination system [30]. pACYC184-HA-orf1B was introduced into each constructed strain. The WT esaN::3×FLAG/orf1B and WT eseE::3×FLAG/orf1B strains were verified by immunoblotting with anti-FLAG antibody. Bacterial pellet from 20 mL culture of WT esaN::3×FLAG/orf1B or WT eseE::3×FLAG/orf1B strain was resuspended with phosphate buffered saline (PBS) supplemented with 1.0 mM phenylmethanesulfonyl fluoride (PMSF). After sonification, the cell lysates were centrifuged at 16 000g for 10 min and the supernatants were mixed with NP-40 at a final concentration of 1% for 30 min before being pre-cleared with protein G-immobilized beads (Thermo) for 1 h at 4 °C. The pre-cleared cell lysates were precipitated with anti-HA antibody for 4 h before being incubated with protein G immobilized beads overnight. The beads were washed four times before being resuspended in 1× SDS sample buffer, and analyzed by immunoblotting against HA tag and FLAG tag.

Single-strain infection in blue gourami fish
Single-strain infection in naïve blue gourami fish [Trichogaster trichopterus (Pallas)] (11.92 ± 1.62 g) was performed to investigate the contribution of Orf1B in pathogenesis of E. piscicida. Healthy blue gourami fish were maintained in 25 ± 0.5 °C. The E. piscicida WT and Δorf1B strains were subcultured at 1:20 into TSB overnight and grown at 28 °C for 3 h. The bacteria were washed three times with PBS, and the OD540 values were adjusted to 0.5. Equal amounts of bacteria were injected intramuscularly (i.m.) at 4.5 × 107 CFU into each fish, using 20 fish per group. The experiment was performed independently three times, and one representative data are shown. Differences in fish survival were assessed using the Long-rank (Mantel–Cox) test. The experiments with fish were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Sciences. The protocol was approved by the Ethics Committee on Animal Experiments of the Institute of Hydrobiology (Permit Number E0110305).

Results
Sequence analysis of Orf1B and EsaN
The orf1B gene is located upstream of esaP and esaQ and downstream of esaN, eseV, and esaM in the E. piscicida T3SS gene cluster (Figure 1). It encodes a 14.20 kDa protein with pl of 8.04. Using the SWISS-MODEL analysis [31], Orf1B was shown to structurally share 13.45%
identity with a putative type III protein YscO from *V. parahaemolyticus*, and 9.09% identity with FliJ protein, a flagellar type III export apparatus from *S. enterica* sero-var Typhimurium. Similarity analysis on Orf1B and EsaN revealed that Orf1B protein shares similarity with EscO of enteropathogenic *Escherichia coli* (EPEC), Spa13 of *Shigella flexneri*, SpaM/InvI of *Salmonella* SPI-1, and YscO of *Yersinia pseudotuberculosis* by 26.4%, 24.1%, 20.8%, and 32.5%, respectively, while T3SS ATPase EsaN shares similarity with InvC of *Salmonella*, Spa47 of *Shigella*, YscN of *Yersinia*, and EscN of EPEC by 41.6%, 43.4%, 53.5%, and 49.3%, respectively (Table 3).

Table 3 Similarity matrix for Orf1B and EsaN.

| Protein     | Homologous protein (% similarity) | E. piscicida | Salmonella | Shigella | Yersinia | EPEC |
|-------------|-----------------------------------|--------------|------------|----------|----------|------|
| Orf1B       | SpaM (20.8%) Spa13 (24.1%) YscO (32.5%) EscO (26.4%) | E. piscicida | Salmonella | Shigella | Yersinia | EPEC |
| EsaN        | InvC (41.6%) Spa47 (43.4%) YscN (53.5%) EscN (49.3%) | E. piscicida | Salmonella | Shigella | Yersinia | EPEC |

The percentage of similar amino acid residues was calculated using MegAlign program within the DNASTAR package (DNASTAR, Madison, USA). The corresponding protein sequences are retrieved through the following Genbank accession numbers: Orf1B (WP_034171802.1); SpaM (WP_001638823.1); Spa13 (WP_024260642.1); YscO (WP_00212952.1); Esco (WP_02929174.1); EsaN (AAX52538.1); InvC (AAA74038.1); Spa47 (AAP79012.1); YscN (AAN37557.1), and EsaN (AAL57542.1).

Table 3 reveals that Orf1B has high identity with YscO from *V. parahaemolyticus*, and 9.09% identity with FliJ protein, a flagellar type III export apparatus from *S. enterica* sero-var Typhimurium. Similarity analysis on Orf1B and EsaN revealed that Orf1B protein shares similarity with EscO of enteropathogenic *Escherichia coli* (EPEC), Spa13 of *Shigella flexneri*, SpaM/InvI of *Salmonella* SPI-1, and YscO of *Yersinia pseudotuberculosis* by 26.4%, 24.1%, 20.8%, and 32.5%, respectively, while T3SS ATPase EsaN shares similarity with InvC of *Salmonella*, Spa47 of *Shigella*, YscN of *Yersinia*, and EscN of EPEC by 41.6%, 43.4%, 53.5%, and 49.3%, respectively (Table 3).

Orf1B plays a pivotal role in T3SS protein secretion in *E. piscicida*

To explore the phenotype of Orf1B protein, *E. piscicida* WT strain, Δorf1B strain, Δorf1B/orf1B strain, and ΔesaN strain were subcultured into DMEM and the autoaggregation of each strain was compared at 24 h post-subculture. EsaN is an ATPase that energizes the transportation of T3SS substrates [10]. As shown in Figure 2A, the culture of WT and Δorf1B/orf1B strain settled to the bottom of the glass tubes, and their supernatants became clear, however, the culture of the Δorf1B and ΔesaN strains remained cloudy. This indicates that disruption of Orf1B abolishes its autoaggregation. Autoaggregation of *E. piscicida* in DMEM is mediated by its T3SS translocon protein EseB [12]. To investigate whether deletion of orf1B influences the secretion of EseB, the culture supernatants of the four *E. piscicida* strains aforementioned were collected and their extracellular protein profiles were compared on an SDS-PAGE gel stained with Coomassie blue. As shown in Figure 2B, neither T3SS translocon proteins EseB, EseC, EseD nor T3SS effector EseJ was secreted from Δorf1B or ΔesaN strains; complementation of Δorf1B strain with pACYC184-HA-orf1B restored their secretion to the level of the wild-type strain.

Does deletion of orf1B influence the steady-state protein levels of T3SS translocon and effectors? To address this question, similar amounts of bacterial pellets (TBP) and extracellular proteins (ECP) from those four strains were immunoblotted. EvpC, a major protein secreted via T6SS but not T3SS was used as a loading control [8]. Increased protein levels of T3SS translocon EseB/EseC/EseD and effector EseJ were observed in TBP of Δorf1B strain or ΔesaN strain compared to that in the WT strain. Complementation of Δorf1B strain with pACYC184-HA-orf1B restored T3SS translocon and effectors to the levels of the wild-type strain (Figure 2C, left panel). Neither T3SS translocon EseB/EseC/EseD nor T3SS effectors EseG and EseJ were secreted from Δorf1B strain or ΔesaN strain (Figure 2C, right panel). DnaK is a bacterial cytosolic marker. DnaK was not detected in any ECP, showing that detection of T3SS translocon and effectors is not due to leakage from bacterial pellets. Taken together, these data indicate that, like EsaN, Orf1B is necessary for the activity of *E. piscicida* T3SS, and disruption of Orf1B blocks the secretion of T3SS translocon and effectors, resulting in their accumulation inside *E. piscicida*. 

---

**Figure 1** Schematic representation of the escA-esaR region of *E. piscicida* T3SS. Arrows represent each of the open reading frames, the bent arrow represents the putative promoter region.
Figure 2  Orf1B is required for efficient secretion of EseJ, EseG, EseB, EseC, and EseD. A Autoaggregation of E. piscicida strains at 24 h post-subculture into DMEM. B Secretion profiles of E. piscicida strains. Samples of ECP from similar amounts of E. piscicida strains grown in DMEM were separated using SDS-PAGE gel and stained with Coomassie blue. EseJ, EseC, EseB, and EseD are T3SS proteins, EvpI, EvpP, and EvpC are T6SS proteins. C Orf1B is required for efficient secretion of T3SS translocon proteins and effectors. Total bacterial proteins (TBP; left panel) and extracellular proteins (ECP; right panel) from similar amounts of the WT strain, Δorf1B strain, Δorf1B/Δorf1B strain and ΔesaN strain were probed with EseB, EseC, EseD, EseG, EseJ, DnaK, and EvpC antibodies. EvpC, a protein secreted by T6SS but not by T3SS, was used as a loading control. The immunoblotting data shown are representative of three independent experiments.
**Orf1B interacts with EsaN as revealed by co-immunoprecipitation**

Considering that several homologues of Orf1B interact with ATPase [17–19, 24], the interaction between Orf1B and the T3SS ATPase EsaN was investigated through immunoprecipitation. WT \(esaN::3 \times \text{FLAG/orf1B}\) and WT \(eseE::3 \times \text{FLAG/orf1B}\) strains cultured in DMEM were harvested and sonicated to produce cell lysates. These cell lysates were immunoprecipitated (IP) with an anti-HA antibody (rabbit, left panel). In parallel, we performed co-IP with the pre-immune serum from rabbits (as a control, right panel) (Figure 3). HA-Orf1B protein was detected by the anti-HA antibody, EsaN-3 \times \text{FLAG} or EseE-3 \times \text{FLAG} were detected with an anti-FLAG antibody. It was observed that EsaN-3 \times \text{FLAG} was precipitated by HA-Orf1B, while EseE-3 \times \text{FLAG} was also precipitated by HA-Orf1B. Meanwhile, the interaction of HA-Orf1B with EseE-3 \times \text{FLAG} is non-specific, as co-IP with the pre-immune serum of rabbit also detected this interaction. This is probably due to the overwhelming protein level of EseE inside \(E.\ piscicida\). Taken together, Orf1B specifically interacts with EsaN in \(E.\ piscicida\).

**Orf1B contributes to \(E.\ piscicida\) adhesion to EPC cells**

\(Edwardsiella\ piscicida\) is able to adhere to and invade \(E.\ piscicida\) cells [6]. To investigate whether or not Orf1B plays a role in this process, EPC cells were infected with the WT strain, \(\Delta orf1B\) strain and \(\Delta orf1B/orf1B\) strain and cell-associated \(E.\ piscicida\) strains were quantified. It was found that the cell-associated \(\Delta orf1B\) strain was 11 ± 0.74 folds less than that of the wild-type strain. Complementation of the \(\Delta orf1B\) strain partially increased its adhesion to the level of the wild-type strain (Figure 4A and B). This demonstrates that Orf1B contributes to \(E.\ piscicida\) adhesion to EPC cells.
**Figure 4** Orf1B contributes to *E. piscicida* adhesion to fish EPC cells by facilitating secretion of EseJ. 

**A** Representative images of infected EPC cells. Fish EPC cells were infected at an MOI of 10. After a 30-min incubation, cells were fixed and stained with anti-LPS (*E. piscicida*) antibody and Alex 488 Goat anti-mouse antibody before being imaged under confocal microscope. Bars, 50 μm. 

**B** Quantification of cell-associated bacteria per view. Fourteen views from each infection were quantified, and the mean ± SD from one representative experiment are shown. ***P < 0.001.

**C** Increased steady-state protein level of EseJ upon disruption of Orf1B. Total bacterial proteins (TBP) and extracellular proteins (ECP) from similar amounts of WT strain, Δorf1B strain, and Δorf1B/orf1B strain cultured in TSB were probed with anti-EseJ and anti-EvpC antibodies. EvpC, a protein secreted by T6SS but not by T3SS, was used as a loading control. The immunoblotting shown are representative of three independent experiments.

**D** The transcription level of eseJ was not interfered by the disruption of Orf1B. The mRNA levels of eseJ in wild-type strain, Δorf1B strain, and Δorf1B/orf1B strain were examined by qRT-PCR. Gene expression levels in the Δorf1B strain, and Δorf1B/orf1B strain relative to the level in the wild-type strain are presented as relative fold changes in gene expression. 16S rRNA was used as the reference gene. Data are presented as means ± SD. The t test implemented in SPSS was used to calculate the P value with a hypothetical mean of 1.0. NS, not significantly different.
T3SS protein EseJ negatively regulates *E. piscicida* adherence to EPC cells from within bacteria [11]. By immunoblotting on TBP and ECP of the three strains, it was observed that the steady-state protein level of EseJ in the Δorf1B strain was much higher than that in the WT strain or Δorf1B/orf1B strain when cultured in TSB, and no EseJ was secreted by Δorf1B strain (Figure 4C). EvpC, a protein secreted via T6SS, was used as a protein loading control. The cytoplasmic protein DnaK was not detected in any ECP, suggesting that the EseJ that was detected from the ECP of WT strain or Δorf1B/orf1B strain was not due to leakage from the bacterial pellets. To investigate whether or not Orf1B regulates eseJ, the transcription levels of eseJ in the WT, Δorf1B strain, and Δorf1B/orf1B strains were compared. As shown in Figure 4D, no significant difference between each strain in eseJ transcription was detected. Taken together, these data indicate that the EseJ protein accumulated inside the Δorf1B strain because its secretion blockage led to the decreased adhesion of *E. piscicida* to EPC cells.

**Orf1B contributes to *E. piscicida* pathogenesis in vivo**

To determine the contribution of Orf1B in the pathogenesis of *E. piscicida*, the survival rates of blue gourami fish were assessed following bacterial infections. Each blue gourami fish was injected with 4.5 × 10^5 CFU of *E. piscicida*. Ten days post-infection, 10% of the fish infected with the wild-type strain (WT) survived, whereas 85% of the fish infected with *E. piscicida* Δorf1B strain survived. Of note, the blue gourami infected with WT strain began to die at 2 days post infection, and the death rate increased sharply between day 2 and day 4, while a low death rate occurred when infected with the Δorf1B strain. A significant difference (*P* < 0.001) was detected between the survival curve of blue gourami fish infected with the WT and Δorf1B strains (Figure 5). This indicates that deletion of orf1B significantly attenuates the virulence of *E. piscicida* PPD130/91 in vivo.

**Discussion**

Bacterial type III secretion machines are used to translocate effector proteins from the bacterial cytosol directly into eukaryotic cells [9], and secretion of T3SS translocon and effectors are tightly controlled [32–34]. In this study, we demonstrate that Orf1B controls secretion of T3SS translocon and effectors by interacting with ATPase protein EsaN, and Orf1B contributes to *E. piscicida* adhesion through facilitating the secretion of EseJ.

The autoaggregation of *E. piscicida* cultured in DMEM was abolished in either Δorf1B strain or ΔesaN strain because of their failure in EseB secretion. Secreted EseB forms filaments on the surface of *E. piscicida*, and the connection between EseB filaments among different bacteria mediates autoaggregation and biofilm formation in *E. piscicida* [12]. Besides EseB, neither the T3SS translocon protein EseC/EseD nor T3SS effector EseJ could be secreted by the Δorf1B strain; conversely, increased steady-state protein levels of EseB/EseC/EseD and EseJ were detected inside the Δorf1B strain when compared with that in the WT strain. Similar protein profiles were observed in the ΔesaN strain as in the Δorf1B strain. This leads us to speculate that Orf1B either functions as an apparatus or accompanies T3SS ATPase EsaN.

Disruption of Orf1B leads to the failure of T3SS protein secretion in *E. piscicida*, this is similar to the phenotypes of its homolog EscO, Spa13, YscO, SpaM, and FliI [17–19, 24]. EscN of EPEC forms an asymmetric ring, and the T3SS inner stalk protein EscO colid coli interacts with EscN at its C-terminal domain, which stimulates its ATP hydrolytic activity, energizing T3SS protein secretion [35, 36]. Consistently, Spa13 interacts with the ATPase Sp47 in *Shigella flexneri* [18]. However, *Yersinia* YscO is not required for the assembly of ATPase YscN [22], YscO interacts with chaperone SycD, and its interaction with the needle length control protein YscP is essential to type III protein secretion [35]. In this study, we provide evidence that Orf1B interacts with T3SS ATPase EsaN, it is speculated that by stimulating ATP hydrolytic activity of EsaN, Orf1B contributes to the secretion of T3SS proteins in *E. piscicida*.

Adhesion of bacteria to interfaces is the first step in pathogenic infection, and bacteria use a variety of surface structures to promote interfacial adhesion. Yfco fimbriae
enhances adherence and colonization of avian pathogenic Escherichia coli (APEC) in vivo and in vitro [37]. Fimbriation level alters the adhesion of Escherichia coli to interfaces [38]. Type 1 fimbria plays a pivotal role in E. piscicida adhesion to epithelial cells [13–16]. Through suppression of type 1 fimbriae, T3SS protein EseJ suppresses E. piscicida’s adhesion to host epithelial cells [13]. In this study, decreased adhesion was observed for Δorf1B strain when compared with WT strain. Based on the facts that the transcription level of eseJ did not change when Orf1B was disrupted, and that a higher steady-state protein level of EseJ was detected in the Δorf1B strain than in the WT strain, it was concluded that Orf1B promotes E. piscicida adhesion by facilitating EseJ secretion.

In summary, we have demonstrated that Orf1B interacts with T3SS ATPase protein EsaN. This interaction may provide energy for the secretion of T3SS translocan and effectors, thus contributing to E. piscicida adhesion and pathogenesis.

Acknowledgements

We are very grateful to Dr. Lu Yi Liu for technical assistance.

Author contributions

LKW and SSS performed the experiments, analyzed the data, and wrote the manuscript. SYZ constructed strains of WT esaN:3 × FLAG and WT eseJ:3 × FLAG. HXX and PN designed the experiment. SSS, LKW, and HXX modified the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by the National Natural Science Foundation of China (Grant No. 32073018), and Agriculture Research Systems of China (No. CARS-46).

Availability of data and materials

All data generated or analyzed in this study were included in this article.

Declarations

Competing interests

The authors declare that they have no competing interests.

Author details

1. Dalian Ocean University, Dalian 116023, Liaoning, China. 2. State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, Hubei, China. 3. College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing 101408, China.

Received: 2 February 2022 Accepted: 12 April 2022

Published online: 13 June 2022

References

1. Shao S, Lai Q, Liu Q, Wu H, Xiao J, Shao Z, Wang Q, Zhang Y (2015) Phylogenomics characterization of a highly virulent Edwardsiella strain ET080813(7) encoding two distinct T3SS and three T6SS gene clusters: propose a novel species as Edwardsiella anguillarum sp. nov. Syst Appl Microbiol 38:36–47

2. Abayneh T, Colquhoun DJ, Sorum H (2013) Edwardsiella piscicida sp. nov., a novel species pathogenic to fish. J Appl Microbiol 114:444–654

3. Yang M, Lv Y, Xiao J, Wu H, Zheng H, Liu Q, Zhang Y, Wang Q (2012) Edwardsiella comparative phylogenomics reveal the new intra/inter-species taxonomic relationships, virulence evolution and niche adaptation mechanisms. PLoS One 7:e36987

4. Bujañ N, Toranzo AE, Maganinhas B (2018) Edwardsiella piscicida: a significant bacterial pathogen of cultured fish. Dis Aquat Organ 131:59–71

5. Janda JM, Abbott SL, Oshiro LS (1991) Penetration and replication of Edwardsiella spp. in Hep-2 cells. Infect Immun 59:154–161

6. Tan YR, Zheng J, Tung SL, Rosenshine I, Leung KY (2005) Role of type III secretion in Edwardsiella tarda virulence. Microbiology 151:2301–2313

7. Okuda J, Arakawa Y, Takeuchi Y, Mahmoud MM, Suzuki E, Kataoka K, Suzuki T, Okinaka Y, Nakai T (2006) Intracellular replication of Edwardsiella tarda in murine macrophage is dependent on the type III secretion system and induces an up-regulation of anti-apoptotic NF-κappaB target genes protecting the macrophage from staurosporine-induced apoptosis. Microb Pathog 41:226–240

8. Zheng J, Leung KY (2007) Dissection of a type VI secretion system in Edwardsiella tarda. Mol Microbiol 66:1192–1206

9. Galán JE, Wolf-Watz H (2006) Protein delivery into eukaryotic cells by type III secretion machines. Nature 444:567–573

10. Xie HX, Yu HB, Zheng J, Nie P, Foster LJ, Mok YK, Finlay BB, Leung KY (2010) EseJ, an effector of the type III secretion system of Edwardsiella tarda, triggers microtubule destabilization. Infect Immun 78:5011–5021

11. Xie HX, Lu JF, Zhou Y, Ji J, Xu JX, Leung KY, Nie P (2015) Identification and functional characterization of the novel Edwardsiella tarda effector EseJ. Infect Immun 83:1650–1660

12. Gao ZP, Nie P, Lu JF, Liu YL, Xiao TY, Lu W, Liu JS, Xie HX (2015) Type III secretion system translocan component EseJ forms filaments on and mediates autoggregation of and biofilm formation by Edwardsiella tarda. Appl Environ Microbiol 81:6078–6087

13. Zhang Q, He TT, Li DY, Liu Y, Nie P, Xie HX (2019) The Edwardsiella piscicida type III effector EseJ suppresses expression of type 1 fimbriae, leading to decreased bacterial adherence to host cells. Infect Immun 87:e00187-e2019

14. Sakai T, Kanai K, Osatomi K, Yoshikoshi K (2003) Identification of a 19.3-kDa protein in MRHA-positive Edwardsiella tarda: putative fimbrial major subunit. FEBS Microbiol Lett 226:127–133

15. Srinivasa Rao PS, Lim TM, Leung KY (2003) Functional genomics approach to the identification of virulence genes involved in Edwardsiella tarda pathogenesis. Infect Immun 71:1343–1351

16. Sakai T, Kanai K, Osatomi K, Yoshikoshi K (2004) Identification and characterization of a fimbrial gene cluster of Edwardsiella tarda expressing mannosase-resistant hemagglutinin. Fish Pathol 39:857–93

17. Romo-Castillo M, Andrade A, Espinosa N, Feria JM, Soto E, Diaz-Guerrero M, Gonzalez-Pedrajo B (2014) EsCo, a functional and structural analog of the flagellar FlaU protein, is a positive regulator of Esf ATPase activity of the enteropathogenic Edenschia coli injectisome. J Bacteriol 196:2227–2241

18. Cherradi Y, Hanchani A, Allauoi A (2014) Spa13 of Shigella flexneri has a dual role: chaperone escort and export gate-activator switch of the type III secretion machine. Mol Microbiol 91:1225–1238

19. Evans LDB, Hughes C (2009) Selective binding of virulence type III effector importors by Flt effector orthologues InvL and YscO. FEBS Microbiol Lett 293:292–297

20. Payne PL, Straley SC (1998) YscO of Yersinia pestis is a mobile core component of the Yop secretion system. J Bacteriol 180:3882–3890

21. Ronnard KE, Schneewind O (2008) YscU cleavage and the assembly of Yersinia type III secretion machine complexes. Mol Microbiol 63:130–141

22. Diepold A, Wiesand U, Amstutz M, Cornelis GR (2012) Assembly of the flagellar type III protein export apparatus and F- and V-type ATPases. Nat Struct Mol Biol 18:277–282

23. Ling SHM, Wang XH, Xie L, Lim TM, Leung KY (2000) Use of green fluorescent protein (GFP) to track the invasive pathways of Edwardsiella tarda in the in vivo and in vitro fish models. Microbiology 146:7–19

24. Fijan N, Sullmanovic D, Bearzott M, Muzinic D, Zwillenberg LO, Chil- monczyk S, Vautherot JF, de Kinkel P (1983) Some properties of the
epithelioma papulosum cyprinid (EPC) cell line from carp *Cyprinus carpio*. Ann Inst Pasteur 134:207–220

27. Edwards RA, Keller LH, Schifferli DM (1998) Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene 207:149–157

28. Zhou Y, Liu LY, He TT, Laghari ZA, Nie P, Gao Q, Xie HX (2016) *Edwardsiella tarda* EsaE (Orf19 protein) is required for the secretion of type III substrates, and pathogenesis in fish. Vet Microbiol 190:12–18

29. Lu JF, Wang WN, Wang GL, Zhang H, Zhou Y, Gao ZF, Nie P, Xie HX (2016) *Edwardsiella tarda* (Orf13 protein) is a type III secretion system-secreted protein that is required for the injection of effectors, secretion of translocators, and pathogenesis in fish. Infect Immun 84:2–10

30. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645

31. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195–201

32. Wang D, Roe AJ, McAteer S, Shipston MJ, Gally DL (2008) Hierarchal type III secretion of translocators and effectors from *Escherichia coli* O15:7:H7 requires the carboxy terminus of SepL that binds to Tir. Mol Microbiol 69:1499–1512

33. Yu XJ, McGourty K, Liu M, Unsworth KE, Holden DW (2010) pH sensing by intracellular Salmonella induces effector translocation. Science 328:1040–1043

34. Takaya A, Takeda H, Tashiro S, Kawashima H, Yamamoto T (2019) Chaperone-mediated secretion switching from early to middle substrates in the type III secretion system encoded by *Salmonella* pathogenicity island 2. J Biol Chem 294:3783–3793

35. Mukerjea R, Ghosh P (2013) Functionally essential interaction between *Yersinia* YscO and the T3SS domain of YscP. J Bacteriol 195:4631–4638

36. Majewski DD, Worrall LJ, Hong C, Atkinson CE, Vuckovic M, Watanabe N, Yu Z, Strynadka NCJ (2019) Cryo-EM structure of the homohexameric T3SS ATPase-central stalk complex reveals rotary ATPase-like asymmetry. Nat Commun 10:626

37. Lu Y, Wang H, Ren J, Chen L, Zhu Ge X, Hu L, Li D, Tang F, Dai J (2016) The Yfco fimbriae gene enhances adherence and colonization abilities of avian pathogenic *Escherichia coli* in vivo and in vitro. Microb Pathog 100:56–61

38. McLay RB, Nguyen HN, Jaimes-Lizcano YA, Dewangan NK, Alexandrova S, Rodrigues DF, Cinno PC, Conrad JC (2017) Level of fimbriation alters the adhesion of *Escherichia coli* bacteria to interfaces. Langmuir 34:1133–1142

**Publisher’s Note**

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