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Edwardsiella tarda-Induced Cytotoxicity Depends on Its Type III Secretion System and Flagellin

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Many Gram-negative bacteria utilize a type III secretion system (T3SS) to translocate virulence proteins into host cells to cause diseases. In responding to infection, macrophages detect some of the translocated proteins to activate caspase-1-mediated cell death, called pyroptosis, and secretion of proinflammatory cytokines to control the infection. *Edwardsiella tarda* is a Gram-negative enteric pathogen that causes hemorrhagic septicemia in fish and both gastrointestinal and extraintestinal infections in humans. In this study, we report that the T3SS of *E. tarda* facilitates its survival and replication in murine bone marrow-derived macrophages, and *E. tarda* infection triggers pyroptosis of infected macrophages from mice and fish and increased secretion of the cytokine interleukin 1β in a T3SS-dependent manner. Deletion of the flagellin gene *fliC* of *E. tarda* results in decreased cytotoxicity for infected macrophages and does not attenuate its virulence in a fish model of infection, whereas upregulated expression of FliC in the *fliC* mutant strain reduces its virulence. We propose that the host controls *E. tarda* infection partially by detecting FliC translocated by the T3SS, whereas the bacteria downregulate the expression of FliC to evade innate immunity.

Edwardsiella tarda is a Gram-negative food- and waterborne pathogen that is recognized worldwide as a causative agent of hemorrhagic septicemia in fish. Humans with underlying immune disorders can be infected or colonized with *E. tarda* through consumption of undercooked seafood or by contact with infected marine life (1, 2). As in many other Gram-negative bacteria, the type III secretion system (T3SS) of *E. tarda* is vital for its pathogenesis (3, 4). Deletion of its T3SS ATPase gene *esaN* increased the 50% lethal dose (LD_{50}) by approximately 1 log in the blue gourami infection model (5). The T3SS facilitates the survival and replication of different *E. tarda* isolates in different eukaryotic cells, such as Hep-2 epithelial cells (6), the epithelioma papillosum of carp cells (3), J774A.1 macrophages (4), and fish primary macrophages (3, 7).

In response to intracellular bacterial infection, at least two major types of cell death might occur: pyroptosis and apoptosis (8, 9). Through detection of bacterial components such as flagellin and the conserved inner rod protein of T3SS, infected macrophages are induced to assemble inflammasome complexes in which caspase-1 is activated. Active caspase-1 triggers pyroptosis and proteolytic maturation and secretion of the proinflammatory cytokines interleukin 1β (IL-1β) and IL-18 (10). Unlike pyroptosis, apoptosis is triggered typically through activation of caspase-3 and -7 rather than caspase-1 (11). Bacteria released from either pyroptotic or apoptotic cells can be phagocytosed and killed. This provides a mechanism to inhibit replication and dissemination of intracellular bacteria. However, some bacterial pathogens are able to modulate and/or avoid constantly triggering host cell death to establish bacterial infection. For instance, *Salmonella enterica* serovar Typhimurium translocates the effector AvrA through its pathogenicity island 1 (SPI-1)-encoded T3SS into host cells to dampen the proapoptotic innate immune response (12). To avoid activating caspase-1, intracellular *S.* Typhimurium downregulates expression of flagellin and SPI-1 genes (13, 14). *E. tarda* is a flagellated bacterium. The T3SS of *E. tarda* induces an upregulation of anti-apoptotic NF-κB target genes to protect J774A.1 murine macrophages from staurosporine-induced apoptosis, and this ability is required for intracellular replication in J774A.1 macrophages (4). However, nothing is known about pyroptosis and *E. tarda* infection.

In this study, we explored the possibility of using murine bone marrow-derived macrophages (BMDMs) as a model to study infection of *E. tarda* PPD130/91, a strain which has been used to investigate virulence factors, gene regulation of *E. tarda*, and interactions between *E. tarda* and hosts (15). We found that *E. tarda* infection induces pyroptosis of murine and fish macrophages in a T3SS-dependent manner.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. *E. tarda* PPD130/91 (16) and its derived strains were grown in tryptic soy broth (TSB; BD Biosciences), or Dulbecco's modified Eagle medium (DMEM) to induce T3SS at 30°C (for the *in vitro* study) and 25°C (for the *in vivo* study), while *E. coli* strains were cultured in Luria-Bertani broth (LB; BD Biosciences) at 37°C. Cultivation of bacteria in DMEM was carried out in a 5% (vol/vol) CO_2 atmosphere. When required, appropriate antibiotics were supplemented at the following concentrations: carbenicillin or ampicillin (50 μg/ml or 100 μg/ml), colistin (12.5 μg/ml), gentamicin (100 μg/ml), tetracycline (15 μg/ml), and chloramphenicol (34 μg/ml).

Cells and culture conditions. BMDMs were isolated from the femurs of 6- to 8-week-old C57BL/6 mice (Charles River) and grown in complete medium as described by Helaine et al. (17): RPMI (Gibco) supplemented

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TABLE 1 Strains and plasmids used in this study[a]

Strain or plasmid	Description and/or genotype	Reference or source
E. tarda strains		
PPD130/91	Wild type; Km^s Col^r Amp^s; $LD_{50} = 10^{5.0}$	16
Δ*esaN* strain	PPD130/91, in-frame deletion of *esaN*	5
Δ*esaN*/*esaN* strain	Δ*esaN* strain with pACYC-*esaN*	5
wt/GFP	PPD130/91 transformed with pFPV25.1	This study
GFP-tagged Δ*esaN* strain	Δ*esaN* strain transformed with pFPV25.1	This study
GFP-tagged Δ*esaN*/*esaN* strain	Δ*esaN*/*esaN* strain transformed with pFPV25.1	This study
Δ*fliC* strain	PPD130/91, in-frame deletion of *fliC*	This study
Δ*fliC*/pACYC-*fliC* strain	Δ*fliC* strain with pACYC-*fliC*	This study
wt/pACYC184	PPD130/91 with pACYC184	This study
wt/pWSK29	PPD130/91 with pWSK29	This study
Δ*fliC*/pWSK29 strain	Δ*fliC* strain with pWSK29	This study
Δ*fliC*/pWSK-*fliC* strain	Δ*fliC* strain with pWSK-*fliC*	This study
Δ*flhB* strain	PPD130/91, in-frame deletion of *flhB*	This study
Δ*flhB* Δ*esaN* strain	PPD130/91, in-frame deletion of *flhB* and *esaN*	This study
wt/pACYC-*fliC*2HA	PPD130/91 with pACYC-*fliC*2HA	This study
Δ*flhB*/pACYC-*fliC*2HA strain	Δ*flhB* strain with pACYC-*fliC*2HA	This study
Δ*esaN*/pACYC-*fliC*2HA strain	Δ*esaN* strain with pACYC-*fliC*2HA	This study
Δ*flhB* Δ*esaN*/pACYC-*fliC*2HA strain	Δ*flhB* Δ*esaN* strain with pACYC-*fliC*2HA	This study
E. coli strains		
DH5α	α complementation	Stratagene
MC1061 λ*pir*	(λ*pir*) *thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara14 xyl5 supE44* λ*pir*	21
S17-1 λ*pir*	RK2 *tra* regulon, λ*pir*, host for *pir*-dependent plasmid	22
Plasmids		
pFPV25.1	Derivative of pBR322 with *gfpmut3A* under the control of the constitutive promoter	24
pMD-18T	Amp^r	TaKaRa
pACYC184	Tet^r Cm^r	Amersham
pACYC-*fliC*	pACYC184 with wide-type *fliC*	This study
pACYC-*fliC*2HA	pACYC184 with C-terminal 2HA-tagged wild-type *fliC*	This study
pRE112	pGP704 suicide plasmid, *pir* dependent; Cm^r *oriT oriV sacB*	19
pRE112-$fliC_{\Delta 1-416aa}$	pRE112 with *fliC*-flanking fragment, 1 to 416 amino acids deleted	This study
pRE112-$flhB_{\Delta 4-430aa}$	pRE112 with *flhB*-flanking fragment, 4 to 430 amino acids deleted	This study
pWSK29	Amp^r	23
pWSK-*fliC*	pWSK29 with *orf29/30* promoter and wild-type *fliC*	This study

[a] Km, kanamycin; Col, colistin; Amp, ampicillin; Cm, chloramphenicol; Tet, tetracycline; r, resistance; s, sensitivity.

with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 mM HEPES, 50 μM β-mercaptoethanol, 20% (vol/vol) L929 cell-conditioned medium (National Institute for Medical Research), and 100 U/ml penicillin-streptomycin if necessary. J774A.1 macrophages were cultured in DMEM (Invitrogen) with 10% FBS and 10 mM L-glutamine.

Healthy mandarin fish (*Siniperca chuatsi*) were obtained from a commercial fish farm and maintained in well-aerated dechlorinated water at 25 ± 2°C. Leukocytes were isolated from the head kidneys of mandarin fish and purified following the procedure of Secombes (18) with slight modifications. Briefly, head kidneys were removed aseptically, pressed through a 100-μm nylon mesh (BD), and resuspended in DMEM supplemented with 2% FBS and 25 U/ml heparin. The cell suspensions were then layered onto a 51/34% discontinuous Percoll (Pharmacia) density gradients and centrifuged at 400 × *g* for 30 min. The band of leukocytes lying at the interface was collected, and the cells were washed three times with DMEM supplemented with 2% FBS and 10 U/ml heparin. Leukocytes were diluted in DMEM supplemented with 0.1% FBS and were seeded at 1.5×10^6 cells per well of a 96-well plate for 2 to 3 h to allow to adhere. Old medium and unattached cells were aspirated and DMEM with 1% FBS applied before infection.

Construction of mutants and plasmids. Nonpolar deletion mutants of *fliC* and *flhB* were generated by *sacB*-based allelic exchange (19) as described previously (20). For example, two PCR fragments were generated from PPD130/91 genomic DNA for the construction of the Δ*fliC* strain. The primer pairs *fliC*-for plus *fliC*-int-rev and *fliC*-int-for plus *fliC*-rev were used. The resulting products were a 689-bp fragment containing the upstream region of *fliC* and a 682-bp fragment containing the downstream region of *fliC*. A 17-bp overlapping sequence introduced into the flanking DNA fragments made it possible to fuse them together by a second PCR with the primers *fliC*-for and *fliC*-rev. The resulting 1,354-bp product, deleting the whole coding sequence of *fliC*, was digested and ligated into the KpnI site of the suicide vector pRE112 (19) to create pRE112-$fliC_{\Delta 1-416aa}$ in *E. coli* MC1061 λ*pir* (21). pRE112-$fliC_{\Delta 1-416aa}$ was then transferred into S17-1 λ*pir* (22) to conjugate with *E. tarda* PPD130/91. Deletion mutants were screened on 10% sucrose–tryptic soy agar (TSA) plates. Mutants were verified by PCR (primer pair fliC-com-for and fliC-check-rev). The double-deletion Δ*flhB* Δ*esaN* mutant was screened based on Δ*esaN* (5). No mutants showed growth defects when cultured in TSB or DMEM. All primers used for the construction of mutants are listed in Table 2.

To construct the complementing plasmid pACYC-*fliC*, the *fliC* gene and its upstream sequence were amplified with the primers *fliC*-com-for and *fliC*-com-rev and ligated into the BamHI and SphI sites of pACYC184 (Amersham). To study the secretion of *fliC*, we constructed another complementing plasmid, pACYC-*fliC*-2HA, with *fliC*-com-rev-2HA as the reverse primer. For creating pWSK-*fliC*, overlapping PCR was used to

TABLE 2 Oligonucleotides used in this study

Designation	Sequence (5′–3′)
fliC-for	AAGCATGCTCACCGACGGTAATGGTCA
fliC-int-rev	AGTGTGCTTTCCTTCGAATGTTG
fliC-int-for	TCGAAGGAAAGCACACTTCGCGCATTACCGCGTGC
fliC-rev	AAGCATGCACCTTCACGCCGTTGAAGTC
pRE112-For	CAACAGTACTGCGATGAGT
pRE112-Rev	GGTGTAAGTGAACTGCATGA
fliC-check-rev	GCGGCAACCTTAGTCGTGTC
fliC-com-for	CGGGATCCCGTGCCAAGGGGAGCGCCGATA
fliC-com-rev	ACATGCATGCATGTTTAACGCAGCAGAGACAGGAC
fliC-2HA-rev	CGGAATTCTTACTAGAGGCTAGCATAATCAGGAAC ATCATACGGATAACGCAGCAGAGACAGGACG
$P_{orf2930}$-for	CCAAGCTTAATGCCCTTTGGAGTATGGA
$P_{orf2930}$-int-rev	GACACAAGGCACCGGTCGTTCGCCGGAACATGG TGCGA
$P_{orf2930}$-fliC-int-for	CGACCGGTGCCTTGTGTCATGGCACAAGTAATTA ATACCAAC
flhB-for	GGTACCCACGAACACCAGCGTCGGCTTCTCG
flhB-int-rev	AGCCGCCACGCTTAAGGCTATTTTAAGTTTCTC
flhB-int-for	CCTTAAGCGTGGCGGCTGACTCCGAAGAAGGAAC ACCG
flhB-rev	GGTACCGGCACCAGCCCTAACAGTCC
flhB-check-for	ATGTTCTCCAGCCAGGTGTTG
flhB-check-rev	GCGTAGTGGGTCGGGTTAGT

fuse the promoter of effector gene *orf29/30* with *fliC* by using the primers $P_{orf2930}$-for, $P_{orf2930}$-int-rev, $P_{orf2930}$-*fliC*-int-for, and *fliC*-com-rev. The resulting PCR product was ligated into the HindIII and EcoRI sites of pWSK29 (23). All the plasmids constructed were verified by DNA sequencing.

Replication assay by CFU. BMDMs from C57BL/6 mice were seeded at a density of 5×10^5 cells per well in 24-well tissue culture plates 24 h before use. Overnight wild-type cultures, Δ*esaN* mutant cultures, and Δ*esaN*/*esaN* cultures expressing green fluorescent protein (GFP) from pFPV25.1 (24) in DMEM at 30°C were opsonized in DMEM with 10% normal mouse serum for 20 min at room temperature (RT). Bacteria were added to the monolayers at a multiplicity of infection (MOI) of ~2.5, centrifuged at 170 × *g* for 5 min at RT, and incubated in a 5% CO_2 incubator at 30°C for 30 min. The macrophages were washed once with prewarmed phosphate-buffered saline (PBS) and incubated in complete RPMI medium with 100 μg/ml gentamicin for 1 h. The macrophages were then incubated in complete RPMI medium supplemented with 16 μg/ml gentamicin for the rest of the experiment. For enumeration of intracellular bacteria at 1 h, 2 h, and 6.5 h after uptake, macrophages were washed four times with PBS and lysed with 0.1% Triton X-100 for 10 min, and a dilution series was spread onto TSB agar plates. Three replicates for each infection condition were analyzed, and the results were averaged. Replication of the wild-type strain expressing GFP was also assayed at 37°C following the procedure described for 30°C.

Replication assay by counting GFP-labeled bacteria. BMDMs were seeded onto coverslips and infected as described for the CFU assay. At 0 h, 2 h, and 6.5 h after uptake, the cell monolayer was washed and fixed in 4% paraformaldehyde in PBS for 15 min at RT. Monolayers were then washed three times with PBS, permeabilized and blocked with 10% horse serum (Sigma) in 0.1% saponin–PBS (Sigma), and stained with rhodamine phalloidin (Invitrogen) at a 1:400 dilution for 1 h. After 3 washes, coverslips were mounted on glass microscope slides using ProLong Gold anti-fade reagent (Invitrogen), and bacterial replication was analyzed by enumerating the GFP-labeled bacteria against the cytoskeleton background under a fluorescence microscope equipped with a 100× objective lens (BX50; Olympus).

Cytotoxicity assay by propidium iodide uptake. BMDMs were seeded at a density of 1×10^6 cells per glass bottom cell culture dish (20-mm diameter) 24 h before use. Infection was performed as described for replication assays. At 2 h after uptake, cells were stained live with 2 μg/ml propidium iodide (PI) in Opti-MEM (Invitrogen) for 20 min at RT in the dark. Cytotoxic cells were identified and scored for PI uptake into the nucleus. PI uptake was counted from more than 10 fields of view for each infection. Images were taken using a confocal laser scanning microscope (LSM510; Zeiss). The data presented are the averages from three independent experiments.

Cytotoxicity assays by lactate dehydrogenase (LDH) release. BMDMs from C57BL/6 mice were seeded onto 24-well plates at a density of 2×10^5 cells per well and incubated overnight at 30°C. Infection was performed as described for the replication assay. At time zero h and 1 h and 2 h after uptake, supernatants from triplicate wells per infection condition were collected and analyzed for lactate dehydrogenase release using the cytotoxicity detection kit plus (LDH) (Roche), following the manufacturer's instructions. Spontaneous LDH release was measured from supernatants of uninfected cells, and total LDH release was measured from lysed uninfected cells. The optical density at 600 nm (OD_{600}) was subtracted from the OD_{490} for each infection condition, and the percentage of LDH per infection condition was calculated as follows: percent LDH release = [(infected cell LDH release − spontaneous LDH release)/(total LDH release − spontaneous LDH release)] × 100.

For fish leukocytes, cells were infected with *E. tarda* at an MOI of 5.

To test the effect of a caspase-1 inhibitor on infection-induced cytotoxicity, cells were pretreated with 317 μM Ac-YVAD-AOM (acetyl–Tyr-Val-Ala-Asp–2,6-dimethylbenzoyloxymethyl ketone; Calbiochem) in 1% FBS-DMEM. The LDH release was measured at 2 h after uptake.

Detection of activated caspase-1. BMDMs were infected as described for the replication assay. After 30 min of infection, the cell monolayers were washed twice and incubated in 200 μl of serum-free DMEM supplemented with gentamicin (50 μg/ml). After 2 h, the cell supernatants were collected, and the cells were lysed with 200 μl 4× lysis buffer. The supernatants and cell lysates were subjected to NuPAGE 12% bis-Tris gel (Novex) electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (pore size, 0.2 μm). The membrane was probed with mouse anti-caspase-1 (p20) monoclonal antibody (Adipogen) at a 1:2,000 dilution and rabbit anti-β-actin polyclonal antibody (Proteintech) at a 1:5,000 dilution.

Cytokine measurements. Levels of IL-1β and tumor necrosis factor alpha (TNF-α) secreted into tissue culture media during infection assays were measured with the Quantikine mouse IL-1β and TNF-α immunoassay kit following the manufacturer's instructions (R&D Systems). Supernatants from three replicate wells per infection condition were collected at 6.5 h after uptake, centrifuged to remove cellular debris, transferred to new tubes, and stored at −80°C until analyzed. A seven-point standard curve of 2-fold dilutions from 1,000 pg/ml to 15.625 pg/ml of recombinant mouse IL-1β and mouse TNF-α was used. A volume of 100 μl of the standards and samples were added to a 96-well microtiter plate. Standard curves were used to estimate levels of cytokines in each sample. The replicate values for each infection condition were averaged.

Secretion of FliC. To examine the secretion of FliC, overnight cultures of *E. tarda* PPD130/91 (wild type), the Δ*flhB* and Δ*esaN* mutants, and the Δ*flhB* Δ*esaN* strain transformed with pACYC-*fliC*2HA were diluted 1:200 in TSB with 34 μg/ml chloramphenicol. Bacteria were then grown without shaking for 12 h at 25°C. Each bacterial culture was centrifuged. The supernatant was filtered (0.22-μm pore size; Millipore) and concentrated with an Amicon Ultra-15 centrifugal filtration device with a 10-kDa-molecular-mass-cutoff filter (Millipore). The bacterial pellet was resuspended in PBS and labeled as the total bacterial protein (P). Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were probed with rabbit anti-hemagglutinin (HA) antibody (Proteintech) diluted at 1:2,000 and goat anti-rabbit IgG–horseradish peroxidase (HRP) at 1:3,000 (Millipore), with mouse anti-DnaK monoclonal antibody (Abcam) at 1:2,000 and goat anti-mouse IgG-HRP at

1:5,000 (Millipore), or with rabbit anti-EvpC antibody (25) diluted at 1:5,000 and goat anti-rabbit IgG-HRP.

Competitive index (CI) in blue gourami fish. Mixed competitive infections in naive blue gourami (9.20 ± 1.55 g) were performed to determine the contribution of FliC to pathogenesis. Bacteria inoculated from a fresh plate were grown overnight at 25°C in TSB and subcultured at 1:40 for 4 h. The bacteria were then washed three times in PBS, and the OD_{540} was adjusted to 0.5. Equal amounts of bacteria were mixed together and injected intramuscularly (i.m.) at 5×10^4 CFU per fish. At 72 h postinoculation, livers were harvested and homogenized, and a series dilution was spread onto TSA plates supplemented with colistin. The colonies were then patched onto TSA colistin-chloramphenicol plates or TSA colistin-ampicillin plates to determine the ratio of different strains to the wild type. The CI is defined as the ratio of the test and wild-type strains within the output divided by their ratio within the input (26).

Statistical analysis. Statistical tests were applied to data from at least three independent experiments. Probability (*P*) values were calculated by two-way analysis of variance and least-significant-difference (LSD) or Student's *t* test, as stated in the legend to Fig. 6, and were considered significant if they were less than 0.05.

RESULTS

Replication and survival of *E. tarda* PPD130/91 in BMDMs depends on temperature and its T3SS. Replication of *E. tarda* PPD130/91 in fish epithelial cells and primary macrophages depends on its T3SS (3), whose expression is regulated by the ambient cultivating temperature *in vitro* (5, 27, 28). To recapitulate *E. tarda* replication in BMDMs and investigate the effect of temperature on this process, the *E. tarda* wild-type strain PPD130/91 was grown at 30°C to activate the T3SS and used to infect BMDMs at 37°C or 30°C. At different time points (1 h, 2 h, and 6.5 h) after uptake, intracellular bacteria were plated for CFU enumeration (Fig. 1A). At 37°C, the number of intracellular bacteria was drastically reduced at 6.5 h after uptake compared with the number at 1 h or 2 h after uptake. In contrast, bacterial numbers slightly increased over the time course at 30°C. This result suggests that temperature affects the intracellular survival/replication of *E. tarda* PPD130/91 in BMDMs, possibly through the regulation of its T3SS.

To test the effect of T3SS on bacterial growth in BMDMs at 30°C, an isogenic *esaN* mutant was used for replication assays (Fig. 1A). Both the mutant and wild-type bacteria were internalized at similar level (1 h after infection; $P = 0.869$). However, the proportion of the *esaN* mutant dropped to approximately 56.7% at 2 h and 16.1% at 6.5 h after internalization of bacteria. The growth defect of the *esaN* mutant was rescued by expressing EsaN from a plasmid (Fig. 1A). As an independent test for T3SS-dependent intracellular growth, we used fluorescence microscopy to count bacteria. To do this, BMDMs were infected at 30°C with bacterial strains expressing GFP from pFPV25.1, fixed at different time points after uptake, and labeled with rhodamine phalloidin for analysis. According to the number of bacteria in each cell, the infected cells were categorized into three groups: (i) no more than 5 bacteria; (ii) more than 5 but no more than 15; (iii) more than 15 bacteria. The percentages in each group are shown in Fig. 1B. This shows that wild-type bacteria replicated over the course of the experiment, while the Δ*esaN* mutant did not. Taken together, our results indicate that T3SS is required for growth of *E. tarda* PPD130/91 in BMDMs at 30°C as it is in fish primary macrophages (3).

***E. tarda*-induced pyroptosis in murine macrophages depends on the T3SS but not temperature.** In the replication assays

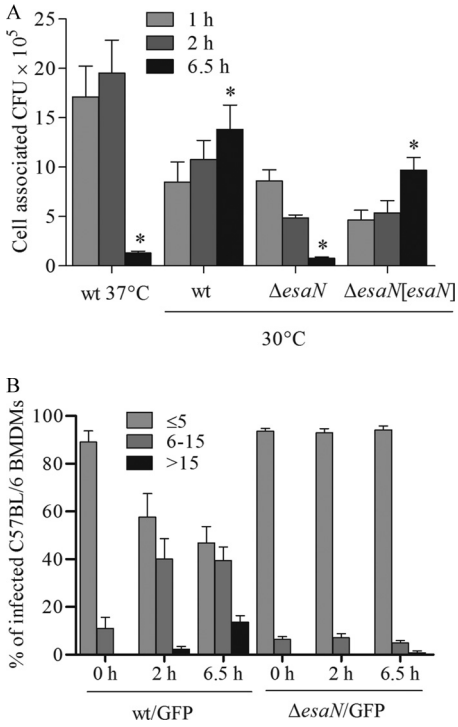

FIG 1 Survival and replication of *E. tarda* PPD130/91 in C57BL/6 BMDMs. (A) *E. tarda* PPD130/91 fails to replicate in BMDMs at 37°C, but T3SS-dependent replication is observed at 30°C. BMDMs were infected with opsonized bacteria at an MOI of ~2.5. Intracellular bacteria were determined by CFU counting at the indicated times after infection. Experiments were performed in quadruplicate wells for each infection, and the data are from one representative infection. *, $P < 0.05$ (relative to the 1-h value). (B) Microscopic analysis of *E. tarda* PPD130/91 replication in BMDMs at 30°C. BMDMs were infected with bacteria expressing GFP and fixed at different time points. Cells were labeled with rhodamine phalloidin and subjected to confocal microscopy. At least 300 infected cells per infection were counted for the number of intracellular bacteria. Data are means ± standard errors of the means (SEM) from three independent experiments.

at both 37°C and 30°C, we noticed that wild-type-infected BMDMs frequently became rounded and displayed membrane blebbing, a sign of apoptosis or pyroptosis (29, 30, 31). Therefore, propidium iodide (PI) staining was done to distinguish the type of cell death caused by *E. tarda* infection. BMDMs were infected at 30°C with different strains expressing GFP for 2 h, PI was then added, and cells were incubated for 20 min. Images were acquired with a confocal microscope (Fig. 2A), and PI-positive cells were scored. In infected cells, both wild-type and complemented Δ*esaN*/*esaN* strains exhibited a significantly higher ratio of PI-positive cells (57.89 ± 4.03% and 53.75 ± 10.58%, respectively) than the Δ*esaN* mutant (16.14 ± 6.0%) (Fig. 2B). This indicates that *E. tarda* induces pyroptosis of BMDMs in a T3SS-dependent manner.

One feature of pyroptosis is rapid lysis of the affected cells and release of the cytosolic contents into the extracellular space. Hence, release of cytosolic LDH by infected BMDMs was also examined. Consistent with the above observations, release of LDH by BMDMs infected with the Δ*esaN* mutant at 30°C was much lower than that by cells infected with the wild type and the complemented strains at 2 h after uptake (Fig. 2C). This was not due to the different number of intracellular bacteria, as a similar result

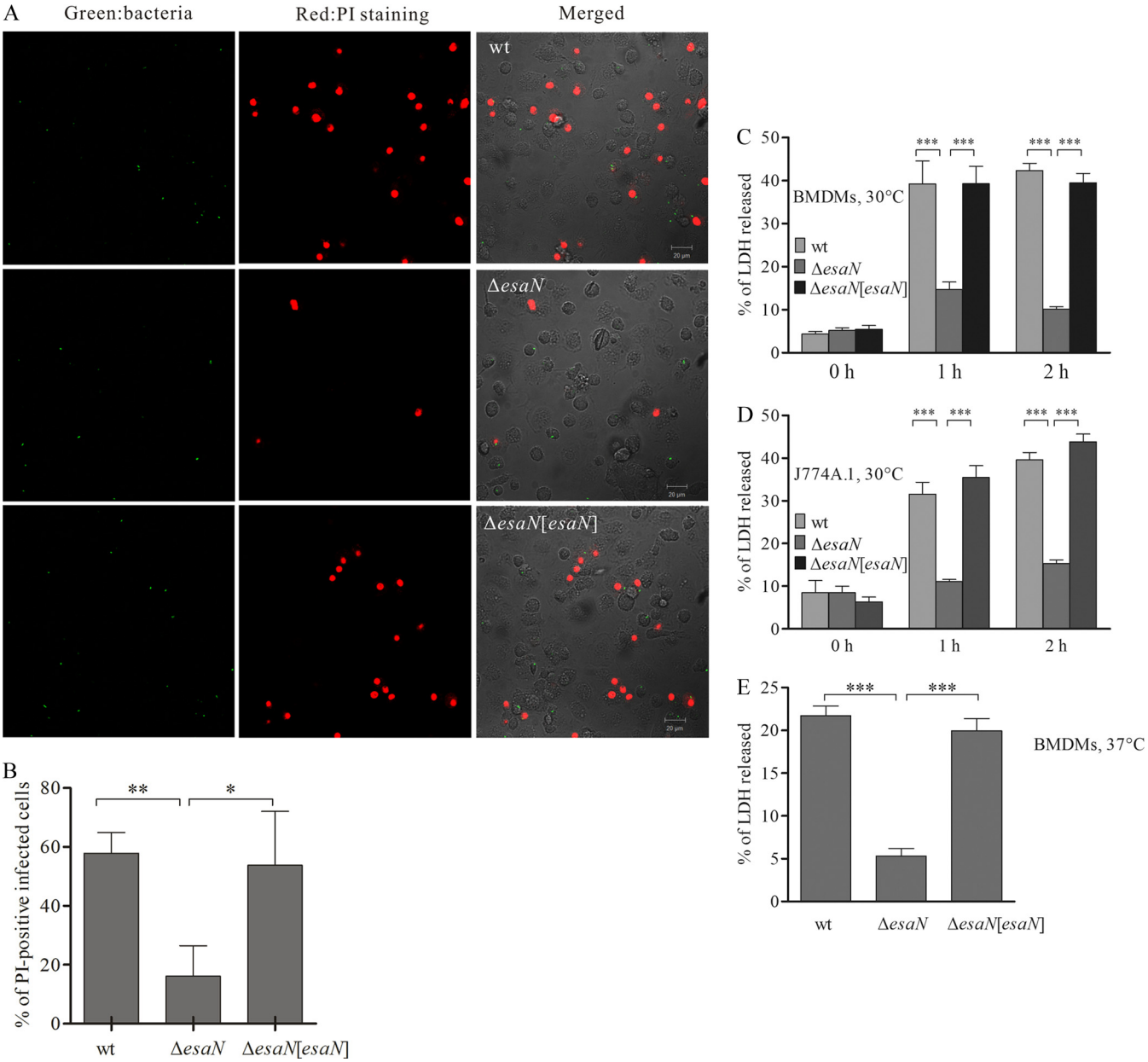

FIG 2 T3SS-dependent cytotoxicity in BMDMs and J774A.1 cells. (A) Confocal micrographs of PI uptake by BMDMs. BMDMs were infected with GFP-expressing strains for 2 h and stained with PI in Opti-MEM without fixation. Green, bacteria; red, PI-positive BMDMs. Bar, 50 μm. (B) Quantification of PI-positive infected cells. More than 300 infected cells for each infection were counted. (C) T3SS-dependent cytotoxicity of *E. tarda* PPD130/91 in BMDMs at 30°C determined by LDH assay. BMDMs were infected with different bacterial strains, and the culture supernatants were collected and processed for the LDH release assay. (D) T3SS dependent cytotoxicity of *E. tarda* PPD130/91 in J774A.1 at 30°C. (E) T3SS-dependent cytotoxicity of *E. tarda* PPD130/91 in BMDMs at 37°C. Samples were collected 1 h after uptake for analysis. Means ± SEM from three experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

was obtained at 1 h after uptake (Fig. 2C), when wild-type and mutant strains had similar amounts of intracellular bacteria (Fig. 1A). The T3SS-dependent cytotoxicity occurred not only in BMDMs but also in J774A.1 macrophages (Fig. 2D). Furthermore, when the infection of BMDMs was processed at 37°C, a similar phenotype was also observed (Fig. 2E). Collectively, these results indicate that *E. tarda*-induced pyroptosis in murine macrophages depends on its T3SS but not the infection temperature.

Pyroptosis-triggered release of cytosolic LDH can be blocked by the caspase-1 inhibitor Ac-YVAD-AOM. To test if a caspase-1 inhibitor could inhibit LDH release by *E. tarda*-infected BMDMs, cells were pretreated with Ac-YVAD-AOM and then infected with wild-type *E. tarda* for 2 h. The supernatants were then analyzed for release of cytosolic LDH. As shown in Fig. 3A, Ac-YVAD-AOM inhibited the LDH release by *E. tarda*-infected BMDMs. To verify that caspase-1 of BMDMs is activated upon *E. tarda* infection, culture supernatants were collected from uninfected or infected BMDMs for immunoblotting. A cleaved product of caspase-1 (p20) was detected in the supernatants of BMDMs infected with the wild type (wt) or the complemented *esaN* strain but not in the

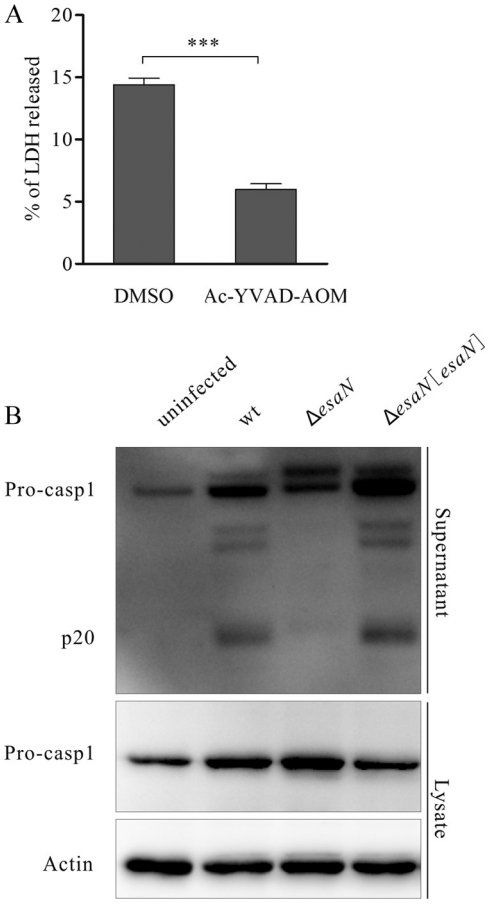

FIG 3 Caspase-1 of BMDMs is activated during *E. tarda* infection. (A) Effect of the caspase-1 inhibitor Ac-YVAD-AOM on the release of LDH. BMDMs were pretreated with 317 μM Ac-YVAD-AOM or dimethyl sulfoxide (DMSO) for 90 min and then infected with the wild type for 2 h. Culture supernatants were collected to determine LDH release. ***, $P < 0.001$. (B) *E. tarda*-induced activation of caspase-1. BMDMs were either mock infected or infected with indicated strains for 2 h. Cell culture supernatants were collected, and cell monolayers were lysed with 4× SDS sample buffer. Membrane was probed with anti-caspase-1 (p20) mouse monoclonal antibody. β-Actin was used to show the same loading of the cell lysates.

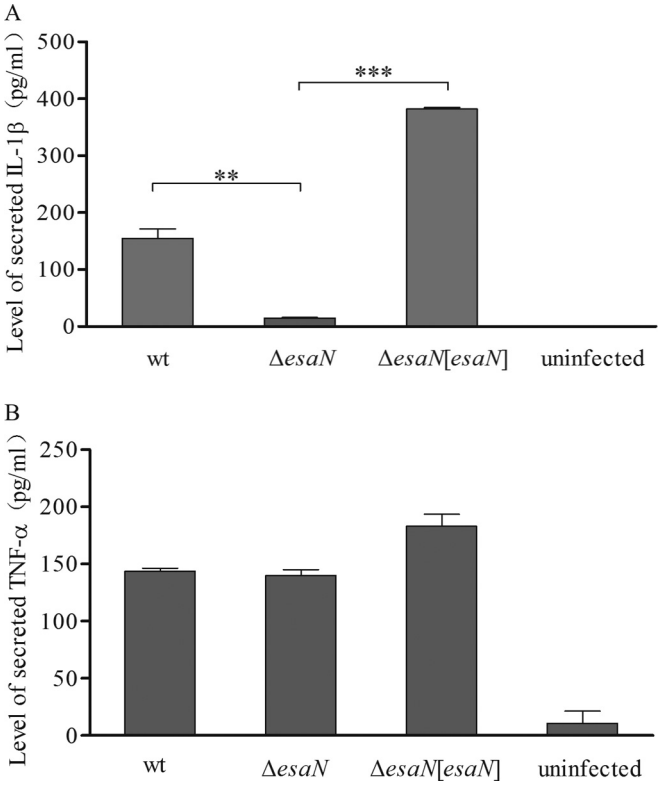

FIG 4 The *E. tarda* T3SS is required for secretion of IL-1β but not TNF-α by BMDMs. BMDMs were infected with different strains for 6.5 h, and supernatants were collected for ELISA. Supernatant from a noninfected cell culture was used as a control. Values are means ± standard deviations (SD) for triplicate cultures. **, $P < 0.01$; ***, $P < 0.001$. (A) Secretion of IL-1β. (B) Secretion of TNF-α.

supernatants of uninfected BMDMs or those infected with the Δ*esaN* strain (Fig. 3B). These results demonstrate that the T3SS of *E. tarda* is required to activate caspase-1 to induce pyroptosis of infected cells.

Secretion of IL-1β by infected BMDMs depends on the T3SS of *E. tarda*. Next, we tested secretion of IL-1β by infected BMDMs, as pyroptosis is induced by activation of caspase-1, which also results in secretion of the proinflammatory cytokines IL-1β and IL-18. For this, BMDMs were infected with different strains for 6.5 h, and supernatants were collected for enzyme-linked immunosorbent assay (ELISA). There was much more IL-1β secreted by BMDMs infected with both the wild type and the complemented strains than in uninfected BMDMs or cells infected with the Δ*esaN* mutant (Fig. 4A). As a control, BMDMs were infected with the wild-type, Δ*esaN* mutant, and complemented mutant strains, and the levels of secreted TNF-α were measured. There was no difference between the Δ*esaN* mutant- and wild-type-infected BMDMs in the levels of this cytokine (Fig. 4B). These data suggest that specific secretion of IL-1β by BMDMs infected with *E. tarda* depends on its T3SS, consistent with the dependence of pyroptosis on the T3SS described above (Fig. 2 and 3).

Requirement of T3SS and flagellin FliC for pyroptosis of fish macrophages. Having demonstrated that the T3SS of *E. tarda* is required to induce pyroptosis of murine macrophages, we asked if this was the case for fish macrophages and if flagellin protein FliC was required for pyroptosis, as has been shown for pyroptosis induced by other bacteria, such as *Salmonella*, *Pseudomonas aeruginosa*, and *Legionella* spp. (32, 33, 34, 35, 36). To this end, a nonpolar deletion mutant of *fliC* was created by allelic exchange and tested for its infection efficiency in fish primary macrophages. After 1 h infection, intracellular bacteria were enumerated by plating. There was no difference between the *fliC* mutant and the wild type (see Fig. S1 in the supplemental material), indicating that the infection efficiencies of the *fliC* mutant and the wild type are similar. Next, we checked the release of LDH by fish primary macrophages infected with different *E. tarda* strains. As shown in Fig. 5A, both Δ*esaN* and Δ*fliC* mutants induced significantly less release of LDH than did the wild-type strain or the *fliC*-complemented strain, in which *fliC* is constitutively expressed from pACYC-*fliC*. Interestingly, fish primary macrophages infected with the Δ*fliC* mutant released larger amounts of LDH than cells infected with the Δ*esaN* mutants did at 2 h after infection. Consistent with the role of caspase-1 in the release of cytosolic LDH by BMDMs, the

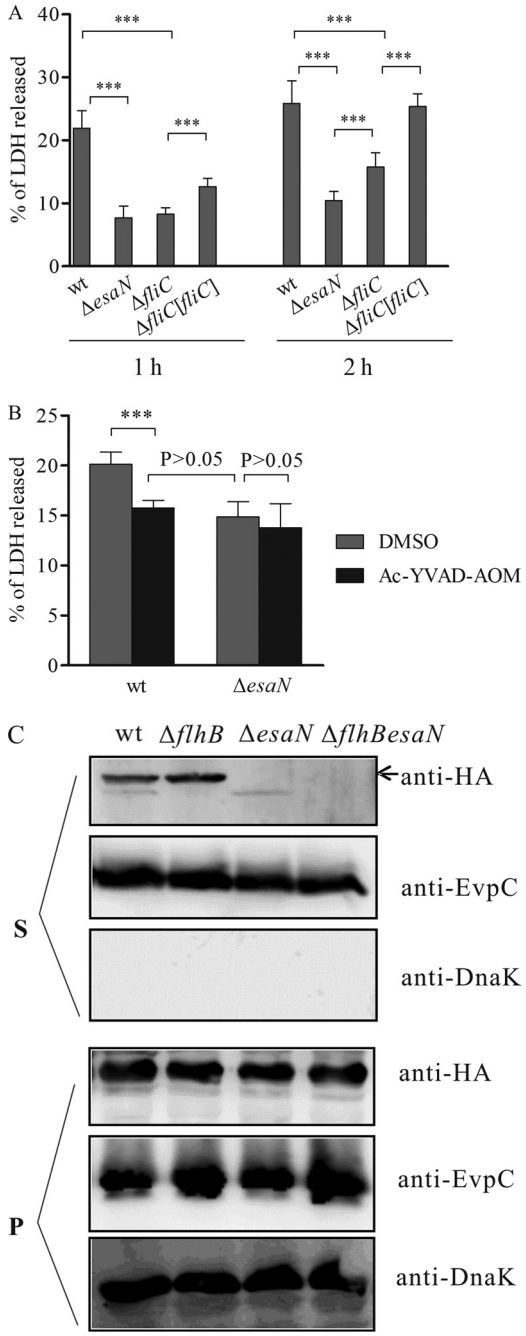

FIG 5 *E. tarda* T3SS and FliC are required for cytotoxicity for fish leukocytes. (A) Fish leukocytes isolated from head kidney were left noninfected or were infected at an MOI of 5 with different strains. After 1 h and 2 h, the levels of LDH released were analyzed. Values are means ± SD for 8 replicate cultures. ***, $P < 0.001$. (B) The release of LDH by fish leukocytes is inhibited by Ac-YVAD-AOM. Primary leukocytes were pretreated with 317 μM Ac-YVAD-AOM or DMSO for 90 min and then infected with the wild type or the *esaN* mutant for 2 h. Culture supernatants were collected to determine LDH release. ***, $P < 0.001$. (C) FliC2HA is secreted into the culture supernatant in a T3SS-dependent manner. Bacterial strains carrying pACYC-*fliC2HA* were grown in TSB for preparing protein samples from bacterial pellets (P) or culture supernatants (S). Membranes were probed with anti-HA for FliC2HA, anti-DnaK (a bacterial cytosolic marker), and anti-EvpC (a T6SS protein, which served as an internal loading control for different strains). DnaK was not detected in any culture supernatants, showing that detection of FliC2HA is not due to contamination from bacterial cells.

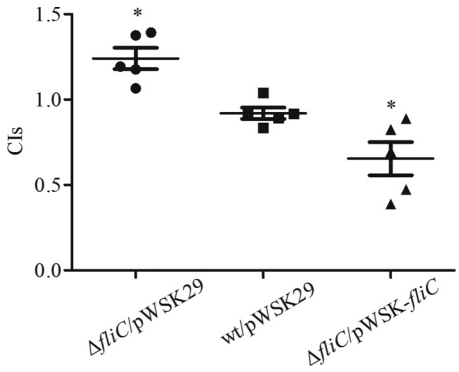

FIG 6 Competitive index analysis. Five naive blue gourami fish were used for each group. Fish were injected intramuscularly with mixtures of equal numbers of cells of the indicated strains and the wild-type strain and sacrificed 72 h after injection. CIs from livers for individual fish are presented, and the mean ± SD is also shown. Student's *t* test was used to calculate the *P* value with the hypothetical mean of 1.0 (*, $P < 0.05$).

caspase-1 inhibitor Ac-YVAD-AOM prevented the T3SS-induced release of LDH by wt-infected fish primary macrophages (Fig. 5B). These results suggest that FliC and other proteins translocated by the T3SS of *E. tarda* are required for inducing pyroptosis in infected fish macrophages.

To test if FliC can be secreted by the T3SS of *E. tarda*, we constructed plasmid pACYC-*fliC*2HA and transformed the plasmid into the wild type or its isogenic mutant strains. Expression and secretion of FliC2HA were tested *in vitro* by immunoblotting using an antibody against the HA epitope. As shown in Fig. 5C, secreted FliC2HA was detected in the culture supernatants of both the wt and a flagellar-secretion-defective (Δ*flhB*) mutant but not in the Δ*esaN* mutant or the double Δ*flhB* Δ*esaN* mutant. Unlike FliC2HA, a type VI secretion system substrate, EvpC, was secreted by all strains tested, and the intrabacterial protein DnaK was not secreted by any of the strains. These data suggest that a C-terminal 2HA tag of FliC blocks its secretion by the flagellar secretion system but that it can be secreted in a T3SS-dependent manner.

Role of FliC in *E. tarda* virulence. To test the effect of deleting FliC on *E. tarda* virulence, mixed infection in the naive blue gourami model was performed to determine the competitive index (CI) between the wt and the Δ*fliC* mutant. For this, pWSK29 was transformed into the Δ*fliC* mutant to distinguish the mutant from the wild-type strain. When equal numbers of wild-type and Δ*fliC* pWSK29 bacteria were mixed and used to infect naive blue gourami, the Δ*fliC* pWSK29 strain was slightly more competitive than the wild type (Fig. 6) (CI = 1.24 ± 0.14; $P = 0.018$). As a control, introducing pWSK29 into the wild-type strain did not affect its virulence. This shows that deletion of *fliC* does not attenuate its virulence *in vivo* but rather leads to slightly increased virulence.

Next, we investigated whether increased production of FliC could attenuate *E. tarda* virulence. As introducing pACYC184-based plasmid into *E. tarda* resulted in its attenuation in the naive blue gourami model (data not shown), we constructed plasmid pWSK-*fliC*, in which expression of FliC is under the control of the promoter controlling expression of the T3SS effector Orf29/30, and transformed the plasmid into the Δ*fliC* mutant. The Δ*fliC* pWSK-*fliC* strain was less competitive than the wild-type strain (Fig. 6) (CI = 0.65 ± 0.22). This result suggests that FliC-medi-

ated pyroptosis is a host defense mechanism in the naive blue gourami fish model.

DISCUSSION

In this work, we demonstrated that *E. tarda* replicates in BMDMs and triggers macrophage pyroptosis and increased secretion of IL-1β in a T3SS-dependent manner. We also found that secretion of FliC could occur through the T3SS and that host cells respond to this by undergoing pyroptosis. This might be an important means by which *E. tarda* infection is controlled in the *E. tarda*/naive blue gourami infection model.

T3SS is a multiproteinaceous machinery that mediates the injection of effector proteins from the bacterial cell into the host cell. Through the action of different effectors, the T3SS of different bacterial pathogens enables them to invade nonphagocytic cells or inhibit phagocytosis by phagocytes, to downregulate innate immunity or modulate intracellular trafficking, and to establish a survival/replication niche. The T3SS of *E. tarda* has been shown to be essential for invading fish epithelial cells (3) and for replication in fish primary macrophages and J774A.1 macrophages. In this work, we demonstrated that the T3SS of *E. tarda* PPD130/91 is required for bacterial survival and replication in more restrictive BMDMs at 30°C (Fig. 1). The dependence of *E. tarda* PPD130/91 survival/replication in BMDMs on temperature is consistent with the role of temperature in the regulation of its T3SS (5, 27, 28). At 37°C, the expression and secretion of T3SS were suppressed via the downregulated expression of the two-component system regulatory protein EsrB by the PhoP-PhoQ system (28).

S. Typhimurium T3SSs not only translocate effectors into host cells to benefit bacterial infection but also occasionally translocate flagellin into host cells (33, 37, 38). Secretion of FliC of *S.* Typhimurium depends on its chaperone, FliS (39), which binds to the C-terminal region of FliC (40, 41). Spontaneous mutations in the C-terminal region of FliC result in its secretion defect in *S.* Typhimurium (42). Here, we showed that FliC2HA is unable to be secreted by the *E. tarda* flagellar system (Fig. 5C), indicating that the 2HA tag has an effect on FliC that prevents it from interacting with its chaperone and hence impairs FliC2HA secretion by the flagellar system. However, FliC2HA is secreted by the T3SS of *E. tarda in vitro* (Fig. 5C), suggesting that FliC can be translocated by the T3SS of *E. tarda in vivo*.

Translocated flagellin interacts with NAIP5 to activate NLRC4 inflammasomes (43, 44). Caspase-1 is then activated by NLRC4 inflammasomes, induces pyroptosis, and processes IL-1β and IL-18 to their active forms. One feature of pyroptosis is that the membrane-impermeative dye PI is able to enter through the pores in the cell membrane and stain pyroptotic cells (31). We showed here that wild-type-infected BMDMs have significantly higher numbers of PI-positive cells than those infected with the T3SS-null-mutant Δ*esaN* strain (Fig. 2B), and the level of secreted IL-1β by infected cells is significantly different between wild-type and the mutant strains (Fig. 4). Furthermore, the wild type is more cytotoxic than the Δ*esaN* mutant in the different macrophages tested (Fig. 2 and 5), and FliC plays a major role in inducing cytotoxicity (Fig. 5). The Δ*fliC* mutant is more cytotoxic to fish primary macrophages than the Δ*esaN* mutant (Fig. 5), suggesting that in addition to FliC, other proteins translocated by T3SS are able to induce cytotoxicity. The conserved inner rod protein EsaI and needle protein EsaG of T3SS might be candidates for such a role, as their homologs in other bacteria can also trigger pyroptosis by interacting with different NLRC4 inflammasome receptors (38, 43, 44). We showed here that, consistent with the role of caspase-1 in pyroptosis, the caspase-1 inhibitor Ac-YVAD-AOM blocks the LDH release induced by *E. tarda* infection (Fig. 3A and 5B), and a cleaved product of caspase-1 is detected in wt-infected but not *esaN* mutant-infected BMDMs (Fig. 3B). Caspase-1 homologs have been detected in sea bream and zebrafish (45, 46). Intriguingly, the caspase-1 inhibitor impaired *S.* Typhimurium-induced cell death of sea bream macrophages but did not prevent processing of IL-1β (47), whereas the processing of IL-1β by *Francisella noatunensis*-infected zebrafish primary leukocytes was considerably abrogated (48). It will be interesting to determine if *E. tarda* infection and *E. tarda*-induced pyroptosis induce processing of IL-1β in our model. Based on the data obtained here, we propose that *E. tarda*-induced pyroptosis depends on its T3SS and flagellin FliC.

Pyroptosis and secretion of proinflammatory cytokines IL-1β and IL-18 induced by pathogens play a vital role in controlling bacterial infection. To avoid inducing this innate immunity, intracellular *S.* Typhimurium downregulates the expression of flagellin FliC (14, 49). *E. tarda* possibly uses the same mechanism to avoid activating caspase 1, as the expression of flagellar genes in infected macrophages was greatly decreased (50). When FliC was constantly expressed from the promoter of the effector gene *orf29/30*, it attenuated *E. tarda* in the blue gourami fish model, as indicated by mixed infection (Fig. 6). Therefore, detection of FliC by host cells is likely to provide a mechanism to control *E. tarda* infection. This is consistent with the finding that constant expression of FliC by the promoter of *sseJ*, an effector gene of SPI-2 T3SS, attenuates *S.* Typhimurium *in vivo* (33). It was reported recently that FliC was essential for growth of *E. tarda* H1 in culture medium and normal secretion of putative translocator proteins EseC and EseB (51). These observations probably explain why the *E. tarda* H1 Δ*fliC* mutant was attenuated in the zebrafish model, as measured by the LD_{50}. However, our *E. tarda* PPD130/91 Δ*fliC* mutant had a growth curve and amounts of secreted EseC and EseB similar to those of the wild-type *E. tarda* strain PPD130/91 (see Fig. S2 in the supplemental material) and was not attenuated in the blue gourami fish model (Fig. 6). Indeed, deletion of *fliC* tends to increase *E. tarda* virulence, as the CIs for livers were slightly higher than 1 ($P = 0.018$). This further supports the notion that *E. tarda* FliC-induced innate immunity controls *E. tarda* infection.

In conclusion, we have shown that *E. tarda* is able to survive and replicate in murine BMDMs in a T3SS- and temperature-dependent manner. This provides a useful model for future studies on the host factors that restrict intracellular *E. tarda* growth. FliC-triggered cytotoxicity of infected macrophages has an important role in controlling *E. tarda* infection.

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