Cellular Microbiology (2017) **19**(1), e12638

doi:10.1111/cmi.12638
First published online 26 July 2016

Identification and functional characterization of EseH, a new effector of the type III secretion system of *Edwardsiella piscicida*

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Summary

***Edwardsiella piscicida*, a bacterial pathogen in fish and humans, expresses a type III secretion system (T3SS) that is critical for pathogen virulence and disease development. However, little is known about the associated effectors and their functional importance. In this study, we identified the ETAE_1757 encoded protein, termed here *E. piscicida* secretion effector H (EseH) as a novel T3SS effector. We found that upon infection with *E. piscicida*, EseH is translocated into nucleus of host cells which required the T3SS. Homology modelling analysis suggests that EseH is an enzyme that belongs to the family of phosphothreothine lyases. Consistently, EseH inhibited phosphorylation of ERK1/2, p38α and JNK MAPK pathways in host cells, but had no effect on the NF-kB pathway. Furthermore, mutation of the critical amino acid residues predicted to confer phosphothreonine lyase activity abolished the ability of EseH to inhibit phosphorylation of ERK1/2, p38α and JNK MAPK pathways in host cells. In addition, we found an increase in transcript levels of TNF-α, IL-12, IL-10 and IFN-γ in zebrafish infected with the *eseH* mutant when compared with the wild type bacterium. Importantly, the virulence of *E. piscicida* deficient in *EseH* was highly attenuated in the zebrafish infection model which correlated with decreased loads of the mutant bacterium in both liver and kidney. Complementation of the *E. piscicida* mutant strain with EseH restored virulence in zebrafish. These results identified EseH as a critical T3SS effector that contributes to virulence by targeting MAPK signalling during *E. piscicida* infection.**

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Introduction

Gram-negative bacterial pathogens often use type III secretion systems (T3SS) to inject effector proteins into host cells, to target host signalling pathways and to promote pathogenicity (Abramovitch *et al.,* 2006; Alfano and Collmer, 2004; Galan and Wolf-Watz, 2006). Although the structural components of T3SS are relatively conserved, the amino acid sequences and functions of individual effectors secreted from T3SS can be highly divergent in different species of pathogenic bacteria (Galán and Collmer, 1999). In addition, pathogenic bacteria deliver a wide repertoire of effector proteins that can suppress host immunity through overlapping or distinct molecular mechanisms (Escoll *et al.,* 2016; Ham *et al.,* 2011; Saijo and Schulza-Lefert, 2008). Recent findings have uncovered novel strategies by which T3SS effector proteins target evolutionarily conserved host MAP kinase (MAPK) signalling cascades and suppress host immune responses (Shan *et al.,* 2007; Bhavsar *et al.,* 2007; Wei *et al.,* 2012).

Early immune responses are activated by sensing of different microbe-associated molecular patterns (MAMPs), such as bacterial flagellin (Akira *et al.,* 2006), lipopolysaccharide (Ausubel, 2005) and peptidoglycan (Dong *et al.,* 2002; He *et al.,* 2006; Nürnberger *et al.,* 2004) during pathogen infection. In plants and animals, stimulation of immune receptors by MAMPs leads to the activation of multiple signalling pathways and their associated transcriptional networks of which the activation of MAPK signalling is a frequent target of T3SS effectors (Akira *et al.,* 2006; Ausubel, 2005; Dong *et al.,* 2002; He *et al.,* 2006; Nürnberger *et al.,* 2004). Several studies have provided evidence that the *Shigella* T3SS effector OspF directly targets and inhibits the activation of host MAPK signalling cascades resulting in increased pathogen survival and

proliferation at the initial infection site (Arbibe *et al.,* 2007; Kramer *et al.,* 2007; Li *et al.,* 2007). OspF exhibits an unexpected phosphothreonine lyase activity on extracellular signal-regulated protein kinases (ERK) 1/2, p38 kinases and c-Jun NH2-terminal kinases (JNK), dampening the host immune response to the pathogen (Li *et al.,* 2007). Furthermore, *Salmonella enterica* serovar Typhimurium SPI-2 T3SS dependent effector, SpvC, was shown to have phosphothreonine lyase activity on full-length ERK phosphorylated at Thr202 and Tyr204, which mediate systemic virulence in mice (Mazurkiewicz *et al.,* 2008; Haneda *et al.,* 2012). In addition to SpvC, HopAI1 from *Pseudomonas syringae* (Li *et al.,* 2007; Jie *et al.,* 2007) possess comparable phosphothreonine lyase activity indicating that pathogens use T3SS effectors to inhibit host MAPK signalling pathways.

Edwardsiella piscicida (*Edwardsiella tarda*), a facultative intracellular bacterium, belonging to the family *Enterobacteriaceae*, is one of the most well-characterized species in the family (Mohanty and Sahoo, 2007; Shao *et al.,* 2015), which causes gastrointestinal and systemic infections such as myonecrosis, septic arthritis and wound infections in humans (Schlenker and Surawicz, 2009; Leung *et al.,* 2012). *E. piscicida* is able to invade and replicate in various cells, including Hep-2 (Stauss *et al*., 1997, Okuda *et al.,* 2008), HeLa (Osuna *et al.,* 1984), epithelioma papillosum of carp (EPC) (Tan *et al.,* 2005; Wang *et al.,* 2013), macrophage-like cells J774A.1 (Okuda *et al.,* 2006) and fish primary phagocyte (Tan *et al.,* 2005). Recently, *E. piscicida* was reported to induce caspase-1-dependent cell death in macrophages during infection (Xie *et al*., 2014). And we also found that *E. piscicida* replicates and induces a caspase-1-dependent cell pyroptosis in a murine macrophage model, revealed that the macrophage-released population gains enhance infectivity for host epithelial cells and increase resistance to multiple host defences and hence display significantly promoted virulence *in vivo* (Zhang *et al.,* 2016). Furthermore, this macrophage-released population displays a reprogrammed transcriptional profile with significantly up-regulated type III secretion system (T3SS)/T6SS-related genes (Zhang *et al.,* 2016). However, the roles of these potential virulence effectors during the bacterial infection remain unknown.

In this study, we first confirmed the up-regulation transcriptome profile of ETAE_1757 in macrophage-released *E. piscicida*, then identified this gene encoded protein as a new *E. piscicida* T3SS effector which was named as EseH (*E. piscicida* secretion effector H). EseH shares the phosphothreonine lyase activity of the bacterial T3SS effector family. We demonstrate that EseH inhibits the activation of ERK1/2, p38α and JNK MAPK pathways, but has no effect on the NF-kB pathway. In addition, we found that *E. piscicida* lacking *eseH* is highly attenuated in the zebrafish infection model. These findings suggest that the newly identified *E. piscicida* effector EseH promotes pathogen colonization and virulence by targeting host MAPK signalling pathways.

Results

Identification of the new E. piscicida *T3SS effector*

We first assessed the transcription of ETAE_1757 in *E. piscicida* released from infected murine macrophages cell lines (J774A.1 cells) and DMEM-cultured *E. piscicida*, and confirmed that ETAE_1757 expression was significantly up-regulated during infection (Fig. 1A, Zhang *et al.,* 2016). To investigate whether the ETAE_1757 encoded protein (EseH) is a secreted T3SS effector, we first constructed an *E. piscicida* strain in which a C-terminus HA-tagged EseH was inserted by homologous recombination on the plasmid puTt, which can replicate within *E. piscicida*. Then we assessed the production of EseH-HA by wild-type and T3SS mutant *E. piscicida* strains grown in DMEM. Robust induction of EseH-HA was detected in the supernatant of the wild-type *E. piscicida*, but not in the supernatant of T3SS mutant *E. piscicida* (Fig. 1B). Importantly, comparable production of EseH-HA was observed in the cell pellets of wild-type and T3SS mutant *E. piscicida* (Fig. 1B). To determine whether EseH was a substrate of *E. piscicida* T3SS or T6SS, the in-frame fusions of β-lactamase TEM-1 with *eseH* were transformed into wild type, T3SS mutant or T6SS mutant *E. piscicida*, respectively, then the intracellular translocation was assessed by confocal microscopy, as shown in Fig. 1C, the EseH translocation was impaired in T3SS mutant *E. piscicida* when compared with wild type and T6SS mutant *E. piscicida*, which suggested that EseH was traslocated into cells in a T3SS-dependent manner. Furthermore, the epithelial cell lines (HeLa cells) were infected with wild-type or T3SS *E. piscicida* carrying a plasmid expressing the EseH-HA fusion protein. After 8 h of infection, EseH was detected in nucleus of the cells infected with wild-type *E. piscicida,* but not with the T3SS mutant by immunofluorescence microscopy (Fig. S1). Collectively, these results suggest that EseH can function as a T3SS effector in *E. piscicida*.

EseH is targeted into the nucleus

Since *E. piscicida* EseH can be translocated into host cells via a T3SS-dependent mechanism, we investigated the localization of EseH in host cells. After transient expressing of EseH-HA in 293 T cells, we observed that EseH was targeted into the nucleus of host cells, compared with EseG that located into cell cytosol, as revealed by immunofluorescence microscopy (Fig. 2A and B, Fang *et al.,* 2016), while the cells morphology was not much changed in EseH-transfected cells, compared with control-transfected cells (Fig. S2). Moreover, subcellular fractionation showed that EseH localized to the nucleus of

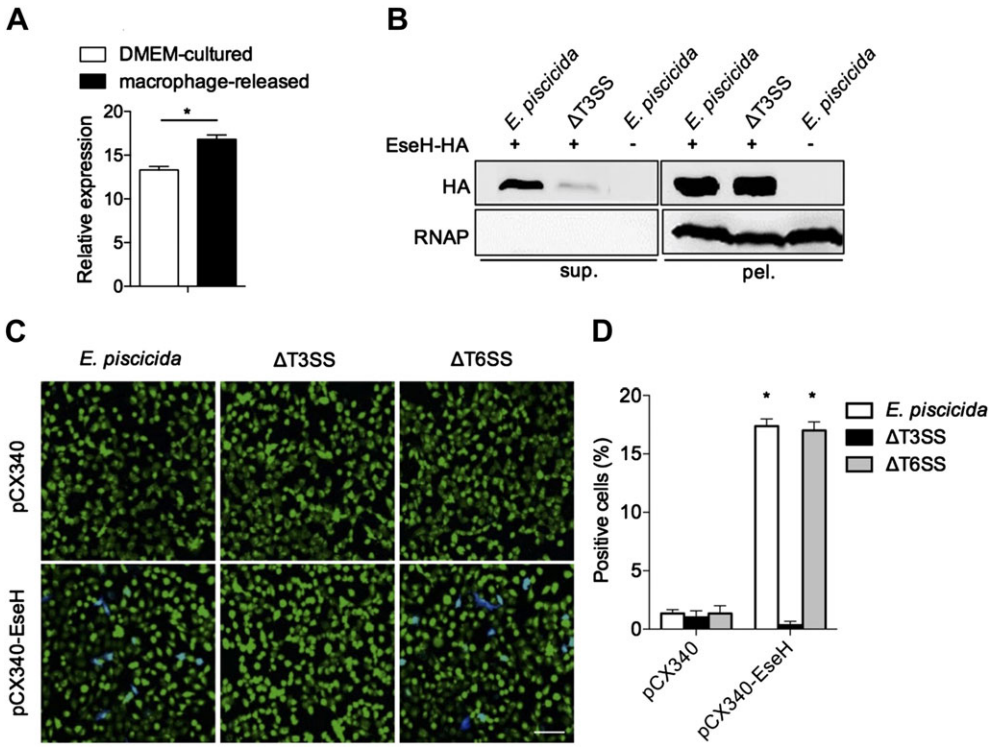

Fig. 1. Identification of EseH, a new *E. piscicida* T3SS effector.
A. Transcript profile analysis of ETAE_1757 by real-time PCR in macrophage-released *E. piscicida* or DMEM-cultured *E. piscicida*. Transcript expression of 16S RNA was used as internal control. Data are representative of at least three experiments. Graphs show mean ± SD of triplicate cultures, * $p < 0.05$.
B. Western blot analysis of EseH secreted into the culture supernatant. Anti-HA was used for EseH-HA fusion protein probing, and the antibody against RNAP was used as bacterial cytosolic marker. Data are representative of at least three experiments.
C. HeLa cells were infected with wild type *E. tarda*, ΔT3SS or ΔT6SS carrying constructs expressing fusion proteins of β-lactamase TEM-1 with EseH at a MOI of 100 for 3 h, then incubated for an additional 4 h with fresh media. The infected cells were loaded with CCF4/AM, and the translocation of effectors was analysed by fluorescence microscopy. Blue fluorescence indicates the positive translocation of T3SS- or T6SS-dependent effectors. Data are representative of at least three experiments, representative microscopic images are shown. bar = 100 μM.
D. Graph show mean ± SD of six fields. Data are representative of at least 3 experiments. * $p < 0.05$.

transfected-293 T cells, but not in the cytosolic or membrane fractions (Fig. 2C). Taken together, the results suggest that *E. piscicida* EseH can be translocated into host cells in a T3SS dependent manner, and targeted into the nucleus of host cells.

Homology modelling of E. piscicida *EseH*

We performed sequence based homology searches, motif analysis and PHYRE2 fold recognition that revealed that EseH contains phosphothreonine lyase activity sites with the critical amino acid sides that are conserved in orthologues in *Shigella* (OspF), *Salmonella* (SpvC) and *P. syringae* (HopAI1) (Fig. 3A). These results suggest that EseH is a member of the bacterial T3SS effector family with phosphothreonine lyase activity (Alfano and Collmer, 2004; Mazurkiewicz *et al.,* 2008; Haneda *et al.,* 2012; Brennan *et al.,* 2008). Furthermore, structure modelling revealed that EseH may share the same conserved domain with the T3SS effectors OspF, SpvC and HopAI1 (Fig. 3B). Taking together, these results suggest that *E. piscicida EseH* is a T3SS effector that belongs to the family of phosphothreonine lyases.

EseH inhibits MAPK signalling pathways

Previous studies showed that phosphothreonine lyases regulate the dephosphorylation of components of the MAPK and NF-kB signalling pathways (Kramer *et al.,* 2007; Mazurkiewicz *et al.,* 2008; Haneda *et al.,* 2012; Brennan *et al.,* 2008). For example, upon over expression, or *in vitro*, SpvC can target ERK1/2, p38α and JNK, but *in vivo*, under physiological conditions, only ERK1/2 are dephosphorylated (Haneda *et al.,* 2012; Brennan *et al.,* 2008; Mazurkiewicz *et al.,* 2008). Thus, we examined the effects of EseH on the regulation of the MAPK and NF-kB pathways. After infection of HeLa cells with wild-type or Δ*eseH E. piscicida*, we found that activation of ERK1/2, p38α and JNK was enhanced in Δ*eseH E. piscicida* infected cells compared to cells infected with the wild-type strain (Fig. 4). Importantly, the increased activation of MAPK kinases induced by Δ*eseH E. piscicida* was reduced to

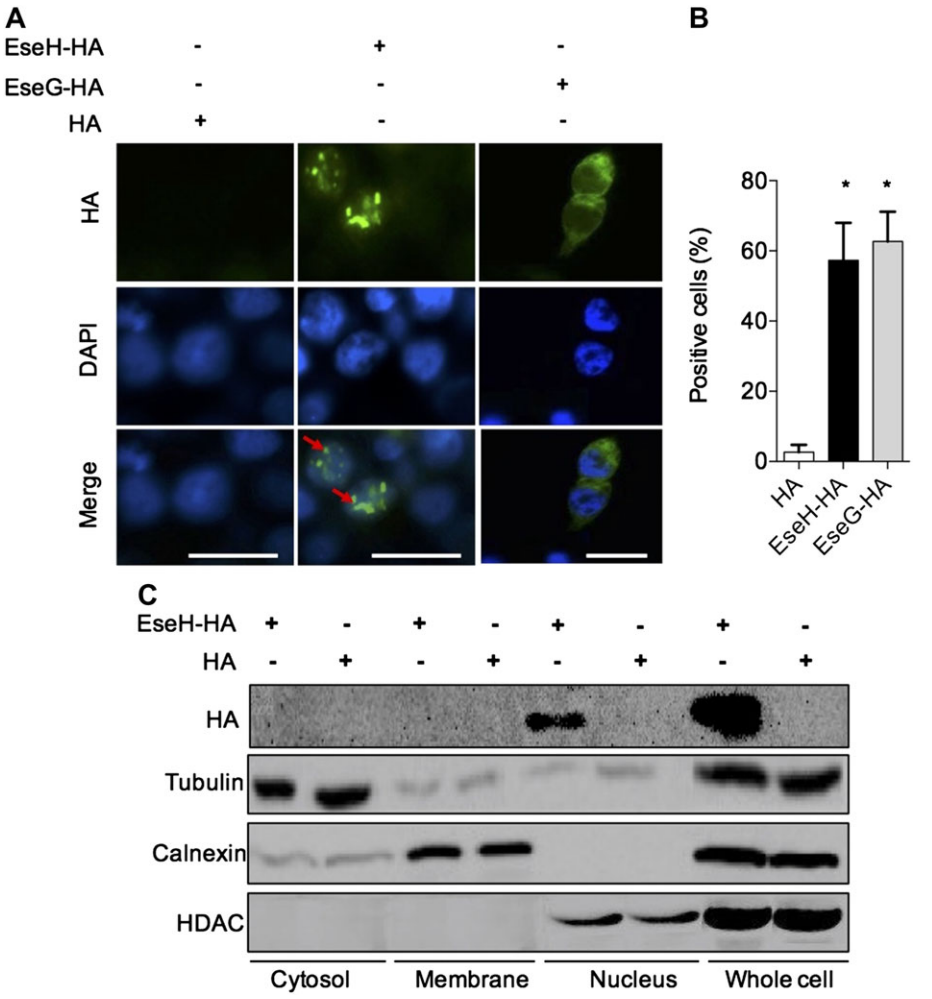

Fig. 2. EseH is translocated into the nucleus.
A. Immunofluorescence microscopy analysis of transient expression of EseH-HA or EseG-HA fusion protein in 293 T cells. Cells were fixed and labeled with anti-HA antibody (green) and nuclei were stained with DAPI (blue). Representative microscopic images are shown. Data are representative of at least three experiments.
B. Graph shows mean ± SD of six fields. Data are representative of at least three experiments. * $p < 0.05$.
C. EseH-HA-transfected 293 T cells lysates were collected, and cell cytosol, membrane and nuclear fractions were separated by gradient centrifugation, and the EseH-HA expression was analysed by western blot. Cytosol was probed with anti-tubulin antibody. Membrane fraction was probed with anti-calnexin antibody. Nuclear fraction was probed by anti-HDAC antibody. Data are representative of at least three experiments.

normal levels in HeLa cells infected with a complemented *eseH* strain (Fig. 4). Notably, IkBα degradation was not affected by the absence of EseH (Fig. 4). To further clarify whether the EseH inhibiting the MAPK signalling directly, or act on MAP2K, we examined the effects of EseH on regulation of the MAP2K pathways, and found that activation of MEK1/2, MKK3/6, MKK4 and MKK7 was not affected by the absence of EseH (Fig. 4). Collectively, these results indicate that EseH specifically regulates MAPK signalling in infected cells.

Phosphothreonine lyases activity of EseH

To confirm that EseH regulates MAPK signalling pathways through its putative phosphothreonine lyase activity, we generated EseH mutants in which the conserved lysine and histidine catalytic residues required for phosphothreonine lyase activity were mutated (Mazurkiewicz *et al.,* 2008). We transfected 293 T cells with plasmids producing wild-type and mutant ($K^{79} \rightarrow R^{79}$ (K79R), $H^{81} \rightarrow R^{81}$ (H81R) and $K^{111} \rightarrow A^{111}$ (K111A)) EseH and the transfected cells were stimulated with TNF-α. Stimulation of 293 T cells with TNF-α induced activating phosphorylation of ERK1/2, p38α and JNK MAPK pathways in untransfected 293 T cells, but not in cells expressing wild-type EseH (Fig. 5). Importantly, activation of MAPKs induced by TNF-α was unimpaired in 293 T cells expressing the EseH mutants (Fig. 5). The results suggest that the *E. piscicida* T3SS effector, EseH, regulates MAPK signalling cascades through its phosphothreonine lyase activity.

Role of EseH in E. piscicida *infected zebrafish*

The MAPK signalling cascades regulate the transcriptional activation of a wide array of pro-inflammatory genes (Akira *et al.,* 2006; Dong *et al.,* 2002). To assess the immunomodulatory activity of EseH, we assessed the expression of cytokines in zebrafish infected with wild-type and mutant *E. piscicida*. Infection with wild-type E. *piscicida* induced the expression of TNF-α, IL-12, IL-10 and IFN-γ transcripts, which was further enhanced in zebrafish infected with the *eseH* mutant (Fig. 6A). The enhancement of cytokine expression observed with *eseH* mutant was abolished when zebrafish were infected with the complemented *eseH E.*

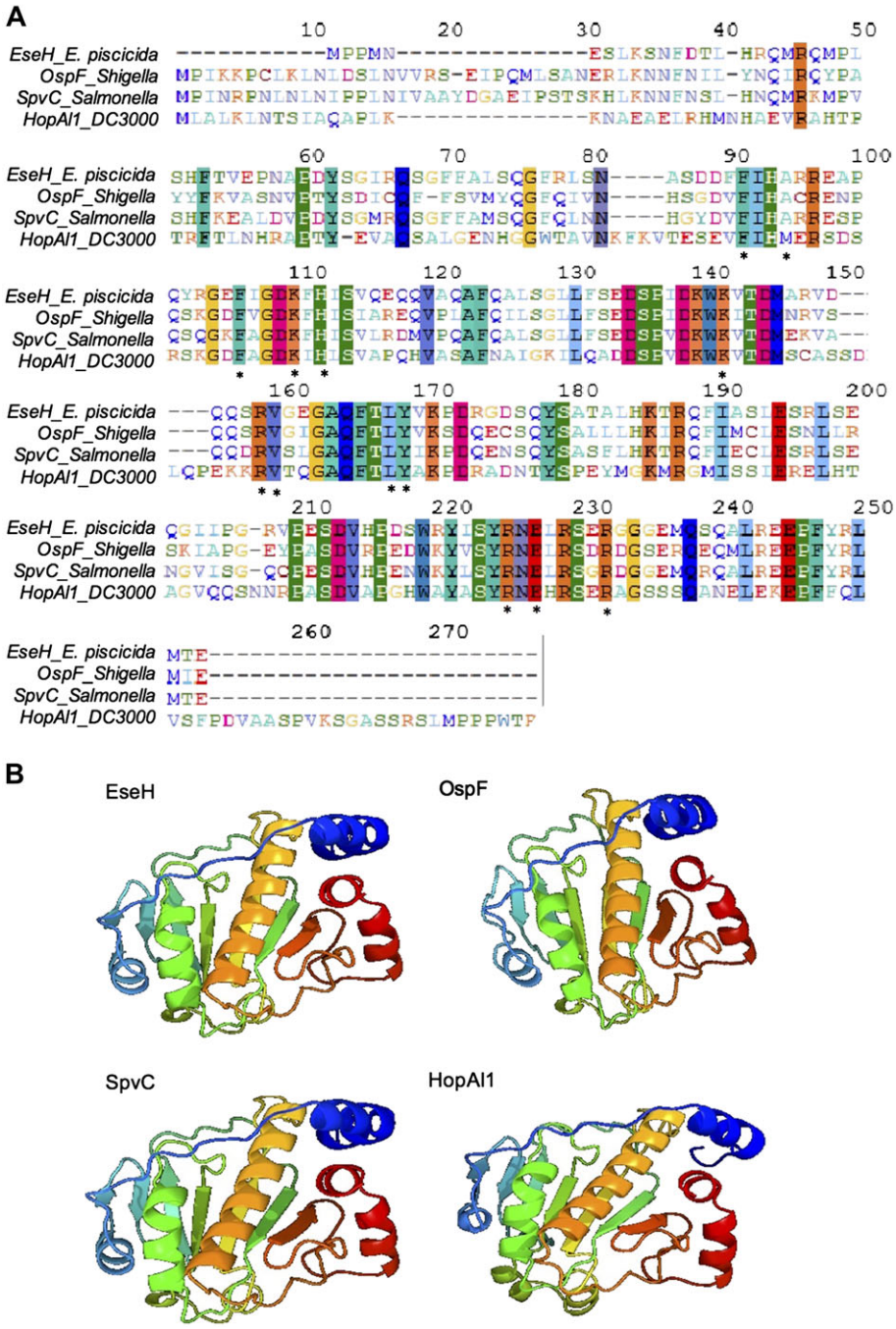

Fig. 3. Homology modelling analysis of EseH.
A. Sequence conservation of *E. piscicida* EseH. ClustalW multi-sequence alignment with phosphothreonine lyase family of OspF from *Shigella*, SpvC from non-typhoid *Salmonella* serotypes and HopAI1 from *Pseudomonas syringae pv. Tomato* DC3000. Aliphatic, hydrophobic amino acids (pink), hydrophobic amino acids (green), aliphatic hydrophilic amino acids (yellow), hydrophilic amino acids (orange) are highlighted, (*) indicates conserved functional sites of phosphothreonine lyase family.
B. Structure modelling of EseH and indicated phosphothreonine lyase family members was performed using the I-TASSER Web server. Images were colored by rainbow N to C terminus.

piscicida strain (Fig. 6A). To further assess whether EseH regulates the virulence of *E. piscicida in vivo*, we infected adult zebrafish with wild-type and *eseH* mutant and monitored fish survival after infection. Zebrafish infected with wild-type *E. piscicida* or complemented *eseH* strain showed marked mortality with ~80% of the animals succumbing by day 3–4 post-infection whereas only ~30% of fish succumbed to infection with the *eseH* mutant (Fig. 6B). To assess whether EseH regulates pathogen colonization in fish tissues, we determined the bacteria burden in the liver and kidney of fish infected intramuscularly with 100 wild-type, *eseH* mutant or complemented *eseH E. piscicida* strain. We found that colonization of the *eseH* mutant strain was comparable to the wild-type strain at 48 h in the liver and kidney, but was undetectable by 72 h post infection (Fig. 6C). In contrast, there was clear colonization of the wild-type and complemented *eseH E. piscicida* strains in the liver and kidney 72 h after infection (Fig. 6C). These results indicate that EseH is important for the virulence and colonization of *E. piscicida* in zebrafish.

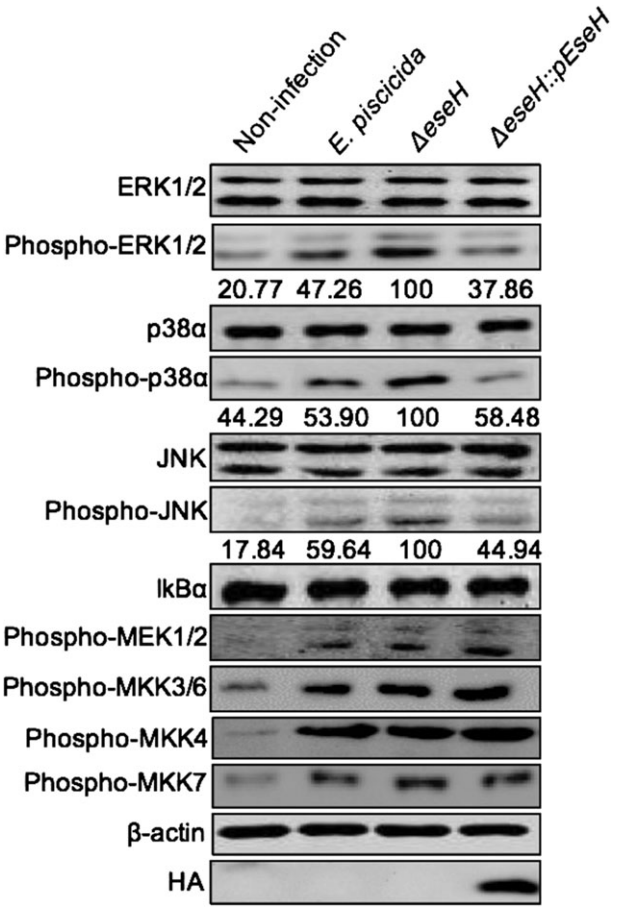

Fig. 4. EseH regulates MAPK signalling in *E. piscicida* infected HeLa cells. HeLa cells were infected with *E. piscicida* strains for 2 h, and the cell lysates were probed for IkBα, p-ERK1/2 and total ERK1/2, p-p38α and total p38α, p-JNK and total JNK. β-actin is shown as a loading control. EseH-HA was probed to confirm the protein expression. The signal intensities were quantitatively analysed by NIH ImageJ. Data are representative of at least three experiments.

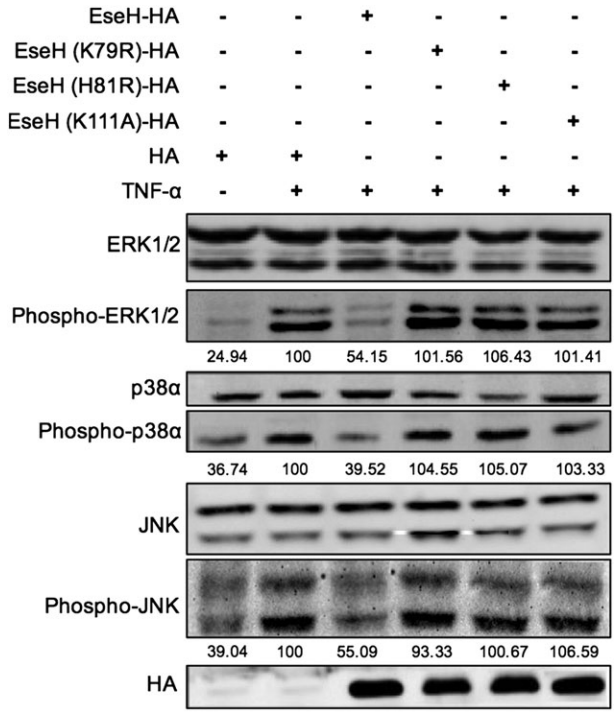

Fig. 5. Mutation of predicted lyase catalytic sites of EseH abolishes the MAPK inhibitory activity. Indicated EseH mutants transient expressed 293 T cells were pretreated with tumour necrosis factor-α. Cell lysates were probed for IkBα, p-ERK1/2 and total ERK1/2, p-p38α and total p38α, p-JNK and total JNK. EseH-HA was probed to confirm the protein expression. The signal intensities were quantitatively analysed by NIH ImageJ. Data are representative of at least three experiments.

Discussion

The bacterial T3SS virulence factors are injected into eukaryotic cells and inhibit signalling by irreversibly inactivating MAPKs (Shan *et al.,* 2007; Bhavsar *et al.,* 2007; Wei *et al.,* 2012). Previous study revealed that the *Salmonella* T3SS virulence factor, SpvC, is required for bacteria virulence in mice infection model (Gulig and Chiodo, 1990; Matsui *et al.,* 2001; Haneda *et al.,* 2012). *Shigella* OspF, which shares 71% sequence identity with SpvC, attenuates the recruitment of polymorphonuclear leukocytes in mouse lung infection model (Arbibe *et al.,* 2007). *E. piscicida* is an intracellular pathogen that uses T3SS to promote its lifestyle in both epithelial and phagocytic cells, which contributes to virulence upon contact with target cells (Tan *et al.,* 2005; Okuda *et al.,* 2006; Srinivasa Rao *et al.,* 2004; Xie *et al.,* 2010; Xie *et al.,* 2015). Previous works indicated that deletion of a single gene, such as *eseB*, *eseD*, *escA*, *eseC* or *esaN*, reduces virulence in blue gourami fish (Wang *et al.,* 2009; Zheng *et al.,* 2007). Mutation any of the Ese genes impairs T3SS function with the exception of EseG, the first characterized effector in *E. piscicida*, which has been shown to disassemble microtubule structures when overexpressed in mammalian cells, while deletion of *eseG* gene results in attenuated virulence in the blue gourami fish infection model (Xie *et al.,* 2010). Another identified *E. piscicida* effector, EseJ, contributes to virulence by reducing bacterial adhesion to EPC cells and contributes to the *E. piscicida* pathogenesis by infection with blue gourami fish *in vivo* (Xie *et al.,* 2015). Apart from these two effectors, little is known about the T3SS effectors of this important fish pathogen. Our results indicate that the newly identified T3SS effector, EseH, does contribute to virulence of *E. piscicida* in zebrafish infection model, which will shed light on better understanding of the complex role of bacterial T3SS effectors in regulation of host immune responses, and their contribution to systemic infection.

Localization of phosphothreonine lyases is an important factor for specificity because some physiological targets of MAPKs exist only in certain cellular compartments. There seem to be differences between phosphothreonine lyases even though the bacteria replicate in epithelial cells and macrophages. *Salmonella* SpvC is translocated into the

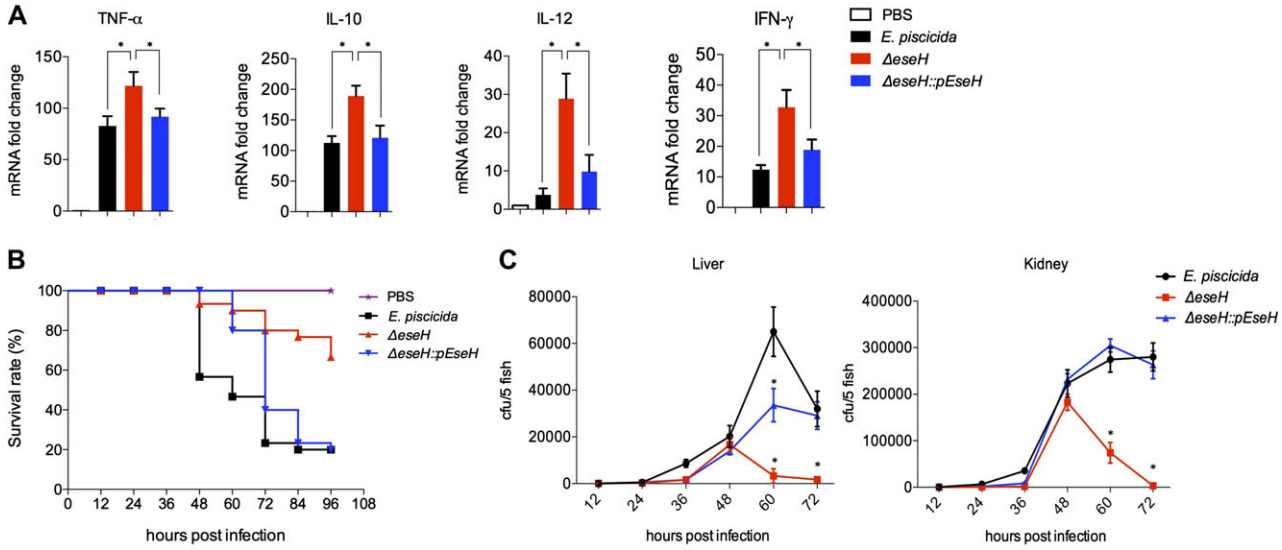

Fig. 6. Critical role of EseH in promoting virulence and colonization of *E. piscicida* in the zebrafish model.
A. Expression of cytokines of TNF-α, IL-10, IL-12 and IFN-γ in zebrafish tissue infected with wild-type, *eseH* and complemented *eseH E. piscicida*. PBS-treated fish were used as a control. The experiments were performed in triplicate with 15 fish in each group. Error bars indicate SD of technical replicates. * $p < 0.05$.
B. Survival of zebrafish infected with wild-type, *eseH* and complemented *eseH E. piscicida* (50 cfu/fish). $N = 30$ fish per group. Data shown are from at least three representative experiments.
C. Bacteria burden in zebrafish liver and kidney was measured at indicated time points after infection with wild-type, *eseH* and complemented *eseH E. piscicida*. $N = 5$ fish per group per time point. Data are representative of at least three experiments. * $p < 0.05$.

cytoplasm of infected macrophages and cervical epithelial (HeLa) cells, where it is susceptible to degradation (Mazurkiewicz *et al.,* 2008); however, in HeLa cells, *Shigella* OspF co-localizes with microtubules and accumulates in the nucleus despite lacking a nuclear-localization signal (Zurawski *et al.,* 2006). This leads to the dephosphorylation of nuclear ERK (Arbibe *et al.,* 2007). However, the localization of other phosphothreonine lyases remains unknown. In this study, our results provided the evidence that *E. piscicida* phosphothreonine lyase, EseH, was translocated into the nucleus of infected epithelial cells, and leads to the dephosphorylation of ERK, p38 and JNK signalling.

Phosphothreonine lyases are specific for pThr-Xaa-pTyr motifs present in MAPKs (Li *et al.,* 2007); however, the identity of the physiological substrates of these enzymes still need further clarification. In contrast to the activity of OspF- or SpvC-deficient mutants, wild type *Shigella* and *Salmonella* infections reduce JNK, p38 and ERK phosphorylation (Li *et al.,* 2007). However, Arbibe *et al.* did not observe reduced JNK phosphorylation in *Shigella* infected Caco-2 cells (Arbibe *et al.,* 2007). Furthermore, transient OspF expression in the SW480 colorectal cancer cell line reduces ERK phosphorylation (Kim *et al.,* 2008). Haneda *et al.* also suggested that SpvC only dephosphorylate ERK1/2 *in vivo* (Haneda *et al.,* 2012). Phosphothreonine-lyase-dependent dephosphorylation of recombinant p38, ERK and JNK is detected *in vitro*, although JNK is not dephosphorylated by *Shigella* under bacterial infection (Mazurkiewicz *et al.,* 2008; Arbibe *et al.,* 2007; Li *et al.,* 2007). However, during *Shigella flexneri* infection, OspF was proved to potentiate the activation of the JNK and NF-kB, within the phosphothereonine lyase activity on p38, and results from the disruption of a negative feedback loop regulation between p38 and TGF-beta activated kinase 1 (Reiterer *et al.* 2011). In this study, our results provide evidence that EseH from the pathogen *E. piscicida* as a new T3SS effector with phosphothreonine lyase activity, that specifically inhibits phosphorylation of ERK1/2, p38α and JNK, both under bacterial infection and transient expression in host cells. However, further comparative mass spectrometry analysis is required to analyse the post-translational modifications that lead to unsaturated amino acids catalysed by phosphothreonine lyases.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1. The *E. piscicida* wild-type strain used in this study was EIB202. *E. coli* DH5α, cc118 λ*pir* and SM10 λ*pir* were used as hosts for the construction of plasmids and for the conjugation of the pir-dependent suicide plasmid. *E. coli* were grown in Luria-Bertani (LB) broth or on LB agar at 37 °C, while *E. piscicida* strains were grown in

Trypticase Soy Broth (TSB), Dulbecco's modified essential medium (DMEM) or on tryptic soy agar (TSA) at 30 °C. Antibiotics were added to the media at the following concentrations: ampicillin (Amp), 100 μg/ml; kanamycin (Km), 50 μg/ml; colistin (Col), 16.7 μg/ml; and tetracycline (Tet), 12.5 μg/ml.

Construction of plasmids and mutant strains

To construct the *E. piscicida eseH* deletion strain, an in-frame deletion mutation of *eseH* was generated by *sacB*-based allelic exchange. Two PCR fragments were generated from EIB202 genomic DNA for the construction of the *ΔeseH* mutant by overlapping PCR. The fragment upstream were constructed using primer pairs deletion-*eseH*-P1 and -P2, and the fragment downstream were constructed using primers deletion-*eseH*-P3 and -P4. The PCR products containing a deletion from amino acid (aa) 9 to aa 211 of EseH, then cloned into the *sacB* suicide vector pDMK, linearized with BglII and SphI, and the resulting plasmids were used to transform *E. coli* CC118 λ*pir*. The correct plasmids were then used to transform *E. coli* SM10 λ*pir* and then conjugated into EIB202. Transconjugants with the plasmids integrated into the chromosome by homologous recombination were selected on tryptic soy agar (TSA) medium containing kanamycin or colistin. To complete the allelic exchange for in-frame deletions, double-crossover events were counter-selected on TSA plates containing 10% sucrose. All the mutants were confirmed by PCR amplification of the respective DNA loci, and subsequent DNA sequencing of each PCR product. The primers used in this study are listed in Table S2. We constructed puTt-pro0456-eseH-HA using one step cloning kit (Vazyme) to express constitutively EseH in *E. piscicida*. The plasmid was electroporated into the *ΔeseH* strain to complement the mutant strain, and into wild-type and ΔT3SS strains to generate strains over expressing EseH.

The DNA sequence of *eseH* gene was amplified with primers pCX340-*eseH*-F and pCX340-*eseH*-R (listed in Table S2) and ligated into the NdeI and KpnI restriction sites of pCX340 to create pCX340-*eseH*. And DNA sequence of *eseG* gene was amplified with primers pCX340-*eseG*-F and pCX340-*eseG*-R (listed in Table S2) and ligated into the EcoR and KpnI restriction sites of pCX340 to create pCX340-*eseG*.

To construct plasmids expressing eseH-HA, eseH (K79R)-HA, eseH (H81R)-HA and eseH (K111A)-HA in eukaryotic cells, plasmid pCDH was linearized with XbaI and BamHI and the PCR products of eseH-HA, eseH (K79R)-HA, eseH (H81R)-HA and eseH (K111A)-HA containing compatible ends were inserted into the linearized pCDH plasmid using one step cloning kit.

Cell cultures, infection and transfection

J774A.1, HeLa and 293 T cells were cultured in Dulbecco's modified essential medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS). J774A.1 was infected at a multiplicity of infection (MOI) of 10:1, in DMEM containing 10% (vol/vol) FBS (growth medium, GM) and gentamicin (100 μg/ml) was added 2 h after infection for 30 min to kill extracellular bacteria, followed by GM containing 10 μg/ml gentamicin for the remainder of the experiment. HeLa cells were infected at a MOI of 100. Before infection, the culture medium was changed to serum-free medium (SFM) for 12–16 h. 293 T cells were transfected using standard calcium phosphate method, and the medium changed to SFM for 12–16 h before TNF-α stimulation.

RNA extraction and quantitative real-time PCR

J774A.1 cells were infected as described above, and the extracellular bacteria were harvested at 8 h post infection. *E. piscicida* were also cultured in medium and harvested as DMEM-cultured samples. RNA of both samples was extracted using an RNA isolation kit (Tiangen, Beijing, China). One microgram of each RNA sample was used for cDNA synthesis with the MMLV reverse transcriptase (ToYoBo, Tsuruga, Japan). RT-PCR was carried out on a FTC-200 detector (Funglyn Biotech, Shanghai, China) using the SYBR green real-time PCR kit (ToYoBo). Total RNA from whole fish was prepared as described above. The expression of TNF-α, IL-12, IL-10 and IFN-γ was determined using real-time PCR (ABI Step One qPCR). Each primer pair (Table S2) was designed using NCBI/Primer-BLAST. The expression of each gene was normalized to that of the β-actin transcript and expressed as -fold change relative to the expression seen in PBS-injected zebrafish. All quantitative PCRs were performed for three biological replicates, and the data for each sample were expressed relative to the expression level of the β-actin gene by using the $2^{-\Delta\Delta CT}$ method.

Secretion assay

For analysis of protein secretion under T3SS inducing conditions, *E. piscicida* strains were grown overnight in TSB medium and then subcultured 1:100 in fresh DMEM and grow for an additional 12 h. L-arabinose was added to induce the expression of EseH-HA when OD_{600} was 0.6. To ensure that protein from equal numbers of cells was analysed, protein samples were adjusted to a volume in which 1 ml of culture corresponds to $OD_{600} = 1$. Bacteria were collected in 50 ml tubes, and centrifuged at 5000 *g* for 10 min at 4 °C. Extracellular proteins were obtained by ultrafiltration from supernatants, which were filtered through a 0. 22 μm filter membrane unit (Millipore, Darmstadt, Germany) with a 10 kD molecular weight cut-off Amicon Ultra-15 centrifugal filter device (Millipore). One hundred fifty micrograms of each protein was boiled for 15 min in SDS sample buffer, and stored at −20 °C before western blot analysis.

TEM1 protein translocation assay

The translocation of translational fusions between TEM1 and EseH was evaluated by detecting β-lactamase activity in infected HeLa cells as previously described (Sory *et al.,* 1995). Briefly, TEM1 fusions (pCX340-*eseH*, described in Tabel S1) were transformed into wild type and T3SS *E. pisicida* by electroporation. Bacteria were grown in TSB overnight at 30 °C, then diluted into DMEM and grown standing at 30 °C until OD_{600} reached to 0.8. HeLa cells were then infected with strains harbouring the TEM1 fusions at a MOI of 100. Infected cells were centrifuged at 400 *g* for 10 min to initiate bacterial-cell contact followed by incubation at 35 °C for 3 h after which the cells were washed 3 times and incubated with fresh

DMEM without serum for another 4 h. At this time point, cells were washed three times with DMEM and loaded with the fluorescent substrate CCF2/AM (LiveBLAzer-FRET B/G loading kit; Invitrogen) in the β-lactamase loading solution supplemented with 15 mM Probenecid (Invitrogen). Cells were incubated in dark for 120 min at room temperature and then observed under a Nikon A1R confocal microscope. At least 400 cells were counted in triplicate wells to determine the percentage of cells emitting a blue fluorescence (TEM1-positive).

Fractionation assay of transfected HEK293 T cells

293 T cells were seeded at 6×10^5 cells per well into six-well plates. After transfection, cells were lysed in HB buffer (250 mM sucrose, 150 mM NaCl, 3 mM imidazole, and 0.5 mM EDTA with pH 7.4), then centrifuged at 3000 *g* for 15 min at 4 °C. The precipitate was component of cell membrane, which was then resuspended with 240 μl HB buffer mixed with protein loading buffer, boiled and centrifuged. The supernatant was used to fractionate component of cytosol and nucleus using nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions.

Fluorescence microscopy

HeLa cells or 293 T cells were seeded at 1.5×10^5 cells per well into 24-well plates with coverslips at the bottom of the wells. After infection of HeLa cells, or transfection of 293 T cells, the cells were washed with PBS three times and then fixed with 4% (wt/vol) paraformaldehyde for 10 min at room temperature. Fixed cells were washed in PBS and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After washing with PBS, EseH-HA was stained with anti-HA for 30 min and nuclei were stained with DAPI (Sigma) for 10 min at room temperature respectively. Fixed samples were viewed on a Nikon A1R confocal microscope. Images were analysed using ImageJ (NIH).

Western blot and antibodies

Cells were lysed in 50 mM Tris, 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA with pH 7.4. Lysates were mixed with protein loading buffer, boiled and centrifuged. Ten microlitres of the cell lysate was separated by SDS-PAGE on a 12% gel and transferred to PVDF membrane (Millipore), then probed with specific antibodies, and antibody binding detected by chemiluminescence. The signal intensities were quantitatively analysed by NIH ImageJ.

Antibodies for HA and β-actin were purchased from Hua'an Bio. Antibody for RNAP was purchased from Santa Cruz. Antibodies for IkBα, ERK1/2, phospho-ERK1/2, p38α, phospho-p38α, JNK and phospho-JNK were purchased from Cell Signaling Technology. Antibodies for phospho-MEK1/MEK2 and phospho-MKK4 were purchased from R&D systems, and antibodies for phospho-MKK3/MKK6 and phospho-MKK7 were purchased from Cell Signaling Technology. HRP-conjugated anti-mouse or anti-rabbit antibodies were purchased from Beyotime.

Infection of E. piscicida *in zebrafish*

Zebrafish (*Danio rerio*) weighing 0.3 ± 0.1 g were obtained from the animal centre at East China University of Science and Technology (Shanghai, China). The fish were stocked in 10 l tanks and maintained at 22 ± 2 °C in a zebrafish cultivation system with a photoperiod of 12:12 h (light:dark) and recirculating fresh water within the fresh water aquarium pathogen containment facility. The fish were fed ad libitum daily with commercial pellets (Bloodworms, Rainone, Beijing) and acclimated to the tanks for at least 1 week prior to commencing the experiments. All of the zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (http://ZFIN.org).

Healthy zebrafish were infected with *E. piscicida* strains as described previously by Yang et al. (34) with slight modifications. *E. piscicida* were grown in Trypticase Soy Broth (TSB) at 30 °C before infection. Dose of 100 bacteria were injected intramuscularly near the dorsal fins, with 30 fish per infection. Fish mortalities were recorded over a period of 7 days. At indicated time points, the infected fish were anaesthetized, and the livers and kidneys were sampled from 5 fish and pooled for bacteria count.

Statistical analysis

Statistical analysis was performed using GraphPad Prism programme (GraphPad Software). All data were representative of at least three independent experiments and were presented as mean ± SD (standard deviation). Differences between two groups were evaluated using Student's *t* test. One-way ANOVA test was used to analyse differences among multiple groups. Differences in fish survival were assessed using the Long-rank (Mantel–Cox) test. Statistical significance was defined as * $p < 0.05$.

Author contributions

Q.L., D.Y. and M.H. conceived the study; M.H. performed the majority of experiments, R.C. and Z.W. did the zebrafish infection experiments; M.H., D.Y., Q.W., Y.Z., G.N. and Q.L. analysed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Acknowledgements

We thank Dr. Melody Y. Zeng and Dr. Yuan He from the University of Michigan Medical School for critical review of the manuscript. This work was supported by the National Natural Science Foundation of China (31430090 and 31472308) and the Chinese National High Technology Research and Development Program (2013AA093101).

Conflict of interests

The authors declare there are no conflicts interests.

References

Abramovitch, R.B., Anderson, J.C., and Martin, G.B. (2006) Bacterial elicitation and evasion of plant innate immunity. *Nat Rev Mol Cell Biol* **7**: 601–611.

Akira, S., Uematsu, S., and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124**: 783–801.

Alfano, J.R., and Collmer, A. (2004) Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu Rev Phytopathol* **42**: 385–414.

Arbibe, L., Kim, D.W., Batsche, E., Pedron, T., Mateescu, B., Muchardt, C., *et al.* (2007) An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. *Nat Immunol* **8**: 47–56.

Ausubel, F.M. (2005) Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol* **6**: 973–979.

Bhavsar, A.P., Guttman, J.A., and Fimlay, B.B. (2007) Manipulation of host-cell pathways by bacterial pathogens. *Nature* **449**: 827–834.

Brennan, D., Roe, S.M., and Barford, D. (2008) Structure and mechanism of the *Chromobacterium violaceum* VirA phosphothreonine lyase. *FEBS J* **275**: 268–268.

Dong, C., Davis, R.J., and Flavell, R.A. (2002) MAP kinases in the immune response. *Annu Rev Immunol* **20**: 55–72.

Escoll, P., Mondino, S., Rolando, M., and Buchrieser, C. (2016) Targeting of host organelles by pathogenic bacteria: a sophisticated subversion strategy. *Nat Rev Microbiol* **14**: 5–19.

Fang, S., Zhang, L., Lou, Y., Yang, D., Wang, Q., Zhang, Y., and Liu, Q. (2016) Intracellular translocation and localization of *Edwardsiella tarda* type III secretion system effector EseG in host cells. *Microb Pathog*. DOI: 10.1016/j.micpath.2016.05.008.

Galán, J.E., and Collmer, A. (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**: 1322–1328.

Galan, J.E., and Wolf-Watz, H. (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444**: 567–573.

Gulig, P.A., and Chiodo, V.A. (1990) Genetic and DNA sequence analysis of the *Salmonella typhimurium* virulence plasmid gene encoding the 28,000-molecular-weight protein. *Infect Immun* **58**: 2651–2658.

Ham, H., Sreelatha, A., and Orth, K. (2011) Manipulation of host membranes by bacterial effectors. *Nat Rev Microbiol* **9**: 635–646.

Haneda, T., Ishii, Y., Shimizu, H., Ohshima, K., Lida, N., Danbara, H., and Okada, N. (2012) *Salmonella* type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection. *Cell Microbiol* **14**: 485–499.

He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nurnberger, T., and Sheen, J. (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. *Cell* **125**: 563–575.

Jie, Z., Feng, S., Yan, L., Haitao, C., Linjie, C., Hongtao, L., *et al.* (2007) A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host Microbe* **3**: 175–185.

Kim, D.W., *et al.* (2008) OspF directly attenuates the activity of extracellular signal-regulated kinase during invasion by *Shigella flexneri* in human dendritic cells. *Mol Immunol* **45**: 3295–3301.

Kramer, R.W., Slagowski, N.L., Eze, N.A., Giddings, K.S., Morrison, M.F., Siggers, K.A., *et al.* (2007) Yeast functional genomic screens lead to identification of a role for a bacterial effector in innate immunity regulation. *PLoS Pathog* **3**: e21.

Leung, K.Y., Siame, B.A., Tenkink, B.J., Noort, R.J., and Mok, Y.K. (2012) *Edwardsiella tarda*—virulence mechanisms of an emerging gastroenteritis pathogen. *Microbes Infect* **14**: 26–34.

Li, H., Xu, H., Zhou, Y., Zhang, J., Long, C., Li, S., *et al.* (2007) The phosphothreonine lyase activity of a bacterial type III effector family. *Science* **315**: 1000–1003.

Matsui, H., *et al.* (2001) Virulence plasmid-borne spvB and spvC genes can replace the 90-kilobase plasmid in conferring virulence to *Salmonella enterica* serovar Typhimurium in subcutaneously inoculated mice. *J Bacteriol* **183**: 4652–4658.

Mazurkiewicz, P., Thomas, J., Thompson, J.A., Liu, M., Arbibe, L., Sansonetti, P., and Holden, D.W. (2008) SpvC is a *Salmonella* effector with phosphothreonine lyase activity on host mitogen-activated protein konases. *Mol Microbiol* **67**: 1371–1383.

Mohanty, B.R., and Sahoo, P.K. (2007) *Edwardsiellosis* in fish: a brief review. *J Bioscience* **32**: 1331–1344.

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev* **198**: 249–266.17.

Okuda, J., Arikawa, Y., Takeuchi, Y., Mahmoud, M.M., Suzaki, E., Kataoka, K., *et al.* (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-kappaB target genes protecting the macrophage from staurosporine-induced apoptosis. *Microb Pathog* **41**: 226–240.

Okuda, J., Kiriyama, M., Yamanoi, E., and Nakai, T. (2008) The type III secretion system-dependent repression of NF-kappaB activation to the intracellular growth of *Edwardsiella tarda* in human epithelial cells. *Fems Microbiol Lett* **283**: 9–14.

Osuna, A., Ortega, G., Gamarro, F., Castanys, S., and Mascaro, M.C. (1984) Some factors affecting the in vitro invasion of HeLa cells by *Trypanosoma cruzi*. *Int J Parasitol* **14**: 253–257.

Reiterer, V., Grossniklaus, L., Tschon, T., Kasper, CA. Sorg, I. and Arrieumeriou, C.(2011) Shigella flexneri type III secreted effector OspF reveals new crosstalks of proinflammatory signaling pathways during bacterial infection. *Cell Signal* **23**(7): 1188–1196.

Saijo, Y., and Schulza-Lefert, P. (2008) Manipulation of the eukaryotic transcriptional machinery by bacterial pathogens. *Cell Host Microbe* **2**: 96–99.

Schlenker, C., and Surawicz, C.M. (2009) Emerging infections of the gastrointestinal tract. *Best Pract Res Clin Gastroenterol* **23**: 89–99.

Shan, L., He, P., and Sheen, J. (2007) Intercepting host MAPK signaling cascades by bacterial type III effectors. *Cell Host Microbe* **1**: 167–174.

Shao, S., Lai, Q., Liu, Q., Wu, H., Xiao, J., Shao, Z., *et al.* (2015) Phylogenomics characterization of a highly virulent

Edwardsiella strain ET080813T encoding two distinct T3SS and three T6SS gene clusters: propose a novel species as *Edwardsiella anguillarum sp. nov. Syst Appl Microbiol* **38**: 36–47.

Sory, M.P., Boland, A., Lambermont, I., and Cornelis, G.R. (1995) Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. *Proc Natl Acad Sci U S A* **92**(26): 11998–2002.

Srinivasa Rao, P.S., Yamada, Y., Tan, Y.P., and Leung, K.Y. (2004) Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. *Mol Microbiol* **53**: 573–586.

Tan, Y.P., Zheng, J., Tung, S.L., Rosenshine, I., and Leung, K. Y. (2005) Role of type III secretion in *Edwardsiella tarda* virulence. *Microbiology* **151**: 2301–2313.

Wang, B., Mo, Z.L., Mao, Z.X., Zou, Y.X., Xiao, P., Li, J., *et al.* (2009) Investigation of EscA as a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon component EseC. *Microbiology* **155**: 1260–1271.

Wang, B., Yu, T., Dong, X., Zhang, Z., Song, L., Xu, Y., and Zhang, X.H. (2013) *Edwardsiella tarda* invasion of fish cell lines and the activation of divergent cell death pathways. *Vet Microbiol* **163**: 282–289.

Wei, P., Wong, W.W., Park, J.S., Corcoran, E.E., Peisajovich, S.G., Onuffer, J.J., *et al.* (2012) Bacterial virulence proteins as tools to rewire kinase pathways in yeast and immune cells. *Nature* **488**: 384–388.

Xie, H.X., Lu, J.F., Zhou, Y., Yi, J., Yu, X.J., Leung, K.Y., and Nie, P. (2015) Identification and functional characterization of the novel *Edwardsiella tarda* effector EseJ. *Infect Immun* **83**: 1650–1660.

Xie, H.X., Yu, H.B., Zheng, J., Nie, P., Foster, L.J., Mok, Y.K., *et al.* (2010) EseG, an effector of the type III secretion system of *Edwardsiella tarda*, triggers microtubule destabilization. *Infect Immun* **78**: 5011–5021.

Zhang, L.Z., Ni, C.S., Xu, W.T., Dai, T.C., Yang, D.H., Wang, Q. Y., *et al.* (2016) Intra-macrophage infection reinforces the virulence of *Edwardsiella tarda*. *J Bacteriol* **198**: 1534–1542.

Zheng, J., Li, N., Tan, Y.P., Sivaraman, J., Mok, Y.K., Mo, Z.L., and Leung, K.Y. (2007) EscC is a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon components EseB and EseD. *Microbiology* **153**: 1953–1962.

Zurawski, D.V., Mitsuhata, C., Mumy, K.L., McCormick, B.A., and Maurelli, A.T. (2006) OspF and OspC1 are *Shigella flexneri* type III secretion system effectors that are required for postinvasion aspects of virulence. *Infect Immun* **74**: 5964–5976.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Strains and plasmids used in this study.
Table S2. Primers used in this study.
Fig S1. Immunofluorescence microscopy analysis of HeLa cells stimulated for 8 h with wild-type or ΔT3SS *E. piscicida*, carrying a plasmid expressing EseH-HA fusion protein. (Left) PFA-fixed samples were labeled with antibody to detect the EseH-HA (green), and nuclei were stained with DAPI (blue). Representative microscopic images are shown. Data are representative of at least three experiments. (Right) Graph show mean ± SD of six fields. Data are representative of at least three experiments. * $p < 0.05$.
Fig. S2. Confocal analysis of transient expression of EseH-HA fusion protein in 293T cells. Cells were fixed and labeled with anti-HA antibody (green), nuclei were stained with DAPI (blue) and F-actin were stained with anti-actin antibody (red). Representative microscopic images are shown. Data are representative of at least three experiments.