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 modeling 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-κB 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 signaling during *E. piscicida* infection.

KEY WORDS: *Edwardsiella piscicida*, T3SS effector, EseH, MAPK pathway

INTRODUCTION

Gram-negative bacterial pathogens often use type III secretion systems (T3SS) to inject effector proteins into host cells, to target host signaling 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) signaling 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 signaling pathways and their associated transcriptional networks of which the activation of MAPK signaling 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 signaling 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 signaling 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.*, 2009). *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 increases resistance to multiple host defenses and hence displays 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 remains 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-κB 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 signaling 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 hours 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 293T cells, we observed that EseH was targeted into the nucleus of host cells, compared with EseG that located into cell cytosol, as revealed by immuno-fluorescence 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 transfected-293T 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 modeling 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 *Pseudomonas 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 modeling 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 signaling pathways

Previous studies showed that phosphothreonine lyases regulate the dephosphorylation of components of the MAPK and NF-κB signaling 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-κB 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 normal levels in HeLa cells infected with a complemented *eseH* strain (Fig. 4). Notably, IκBα degradation was not affected by the absence of EseH (Fig. 4). To further clarifying whether the EseH inhibiting the MAPK signaling 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 were not affected by the absence of EseH (Fig 4). Collectively, these results indicate that EseH specifically regulates MAPK signaling in infected cells.

Phosphothreonine lyases activity of EseH

To confirm that EseH regulates MAPK signaling 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 293T 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 293T cells with TNF-α induced activating phosphorylation of ERK1/2, p38α and JNK MAPK pathways in untransfected 293T cells, but not in cells expressing wild-type EseH (Fig. 5). Importantly, activation of MAPKs induced by TNF-α was unimpaired in 293T cells expressing the EseH mutants (Fig. 5). The results suggest that the *E. piscicida* T3SS effector, EseH, regulates MAPK signaling cascades through its phosphothreonine lyase activity.

Role of EseH in *E. piscicida* infected zebrafish

The MAPK signaling cascades regulates 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. 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 hrs in the liver and kidney, but was undetectable by 72 hrs 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 hrs after infection (Fig. 6C). These results indicate that EseH is important for the virulence and colonization of *E. piscicida* in zebrafish.

DISCUSSION

The bacterial T3SS virulence factors are injected into eukaryotic cells and inhibit signaling 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 et al., 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 *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 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 signaling.

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-κB, 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 (Veronika 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 analyze the post-translational modifications that lead to unsaturated amino acids catalyzed 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; 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 *⊿eseH* mutant by overlapping PCR. The fragement 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 293T 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 hours 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 hours. 293T cells were transfected using standard calcium phosphate method, and the medium changed to SFM for 12-16 hours 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 hours 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 hours. 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 analyzed, 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

5,000 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). 150 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 HEK293T cells

293T cells were seeded at $6x10^5$ cells per well into 6-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 3,000 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 293T cells were seeded at $1.5x10^5$ cells per well into 24-well plates with coverslips at the bottom of the wells. After infection of HeLa cells, or transfection of 293T 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 analyzed 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. 10 μl 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 analyzed 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 IκBα, 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 center 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 photo-period 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 anesthetized, and the livers and kidneys were sampled from 5 fish and pooled for bacteria count.

Statistical analysis

Statistical analysis was performed using GraphPad Prism program (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 analyze differences among multiple groups. Differences in fish survival were assessed using the Long-rank (Mantel-Cox) test. Statistical significance was defined as * $p<0.05$.

AUTHORS 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. analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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CONFLICTS INTERESTS

The authors declare there are no conflicts interests.

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FIGURE LEGENDS

Figure 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 3 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 3 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 hrs, then incubated for an additional 4 hrs with fresh media. The infected cells were loaded with CCF4/AM, and the translocation of effectors were analyzed by fluorescence microscopy. Blue fluorescence indicates the positive translocation of T3SS- or T6SS-dependent effectors. Data are representative of at least 3 experiments, representative microscopic images are shown.bar=100 uM. * $p<0.05$.

Figure 2. EseH is translocated into the nucleus. (A) Immunofluorescence microscopy analysis of transient expression of EseH-HA or EseG-HA fusion protein in 293T 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 3 experiments. (B) Graph show mean ± SD of six fields. Data are representative of at least 3 experiments. * $p<0.05$. (C) EseH-HA-transfected 293T cells lysates were collected, and cell cytosol, membrane and nuclear fractions were separated by gradient centrifugation, and the EseH-HA expression was analyzed by western blot. Cytosol was probed with anti-tubulin antibody. Membrane fraction

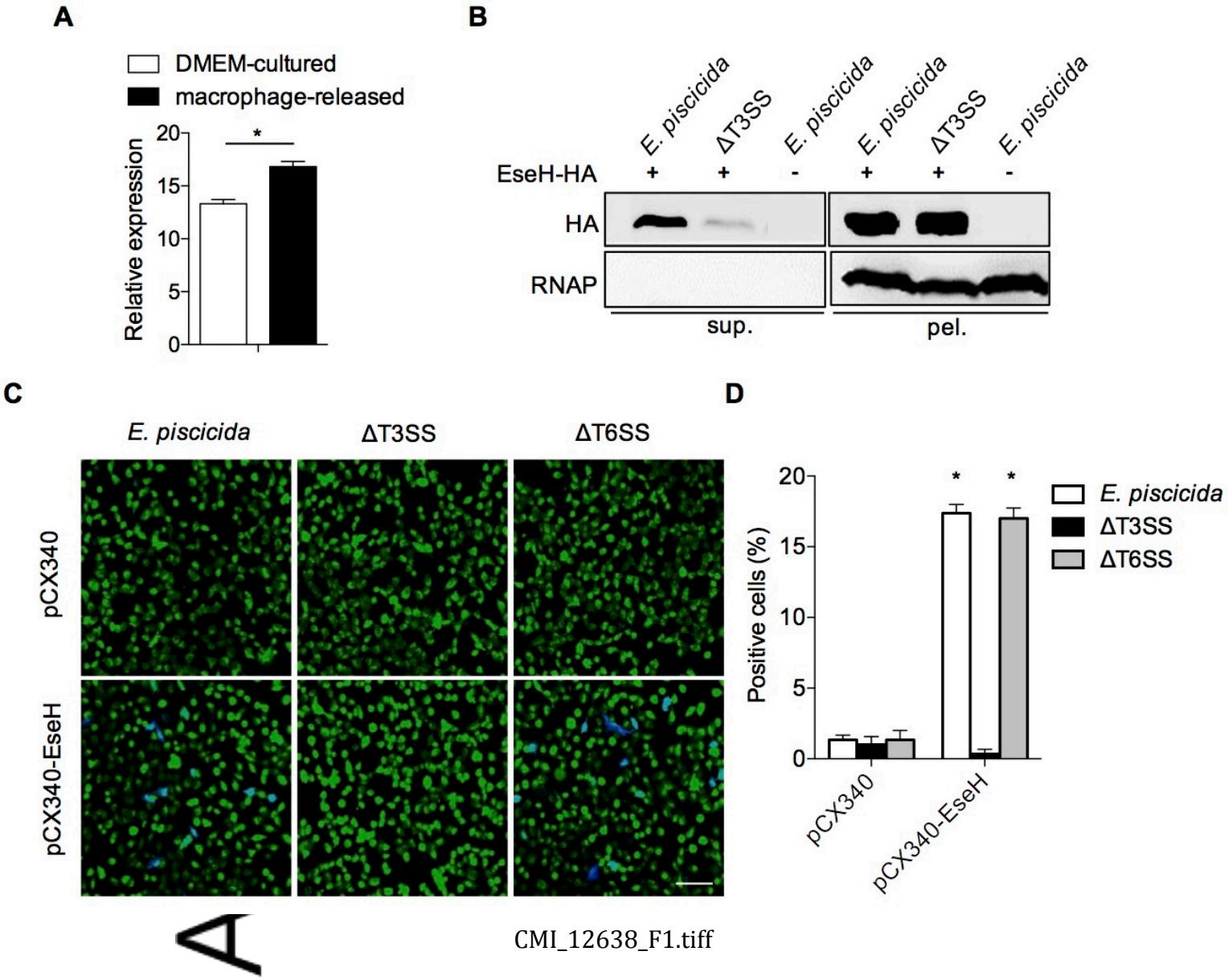
was probed with anti-calnexin antibody. Nuclear fraction was probed by anti-HDAC antibody. Data are representative of at least 3 experiments.

Figure 3. Homology modeling 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 modeling 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.

Figure 4. EseH regulates MAPK signaling in *E. piscicida* infected HeLa cells. HeLa cells were infected with *E. piscicida* strains for 2 hours, and the cell lysates were probed for IκBα, 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 analyzed by NIH ImageJ. Data are representative of at least 3 experiments.

Figure 5. Mutation of predicted lyase catalytic sites of EseH abolishes the MAPK inhibitory activity. Indicated EseH mutants transient expressed 293T cells were pretreated with tumor necrosis factor-α. Cell lysates were probed for IκBα, p-ERK1/2 and total ERK1/2, p-p38α and total p38α, p-JNK and total JNK. EseH-HA was probed to confirm the proteins expression. The signal intensities were quantitatively analyzed by NIH ImageJ. Data are representative of at least 3 experiments.

Figure 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, 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 3 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 3 experiments. * $p<0.05$.



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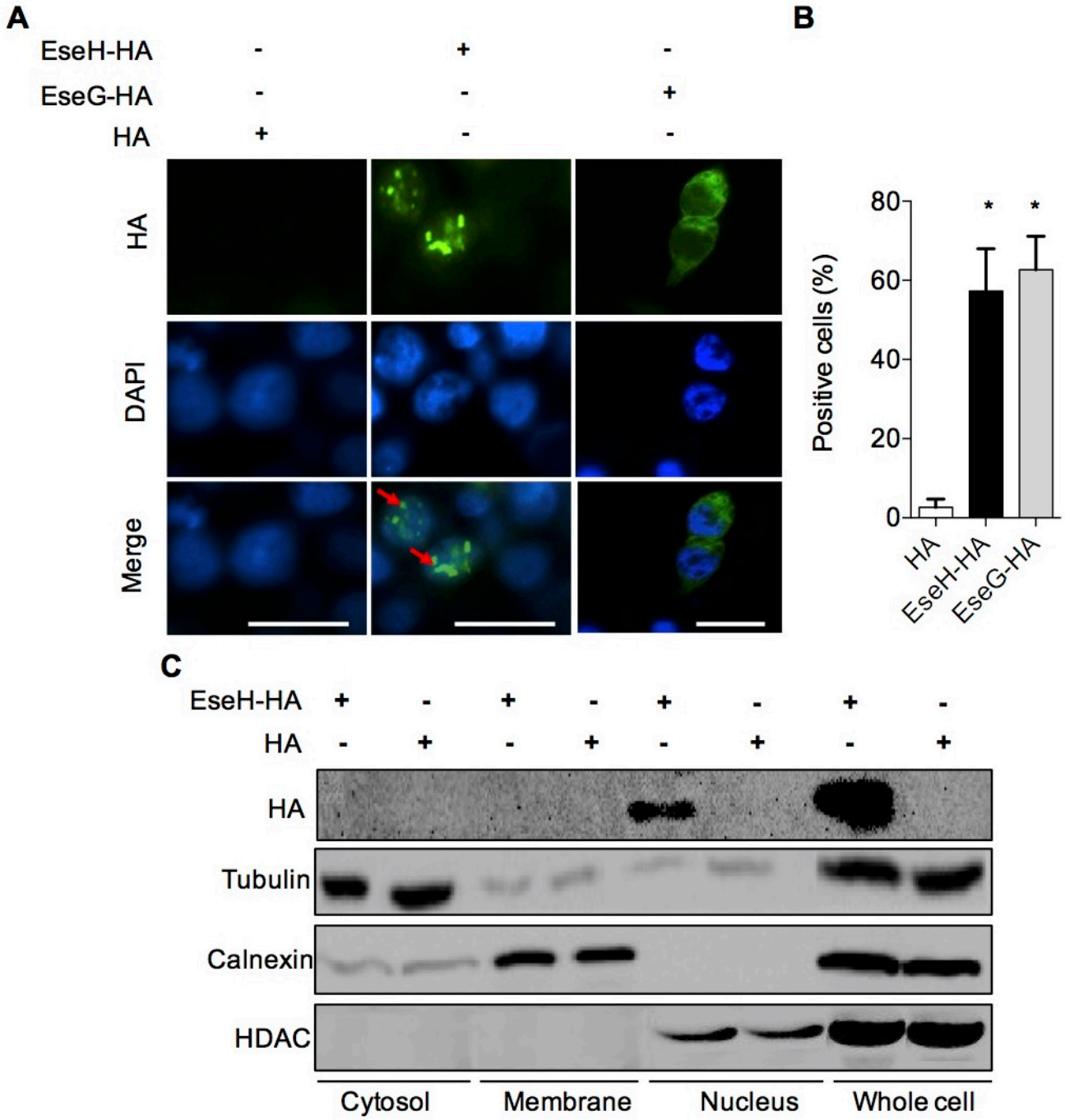

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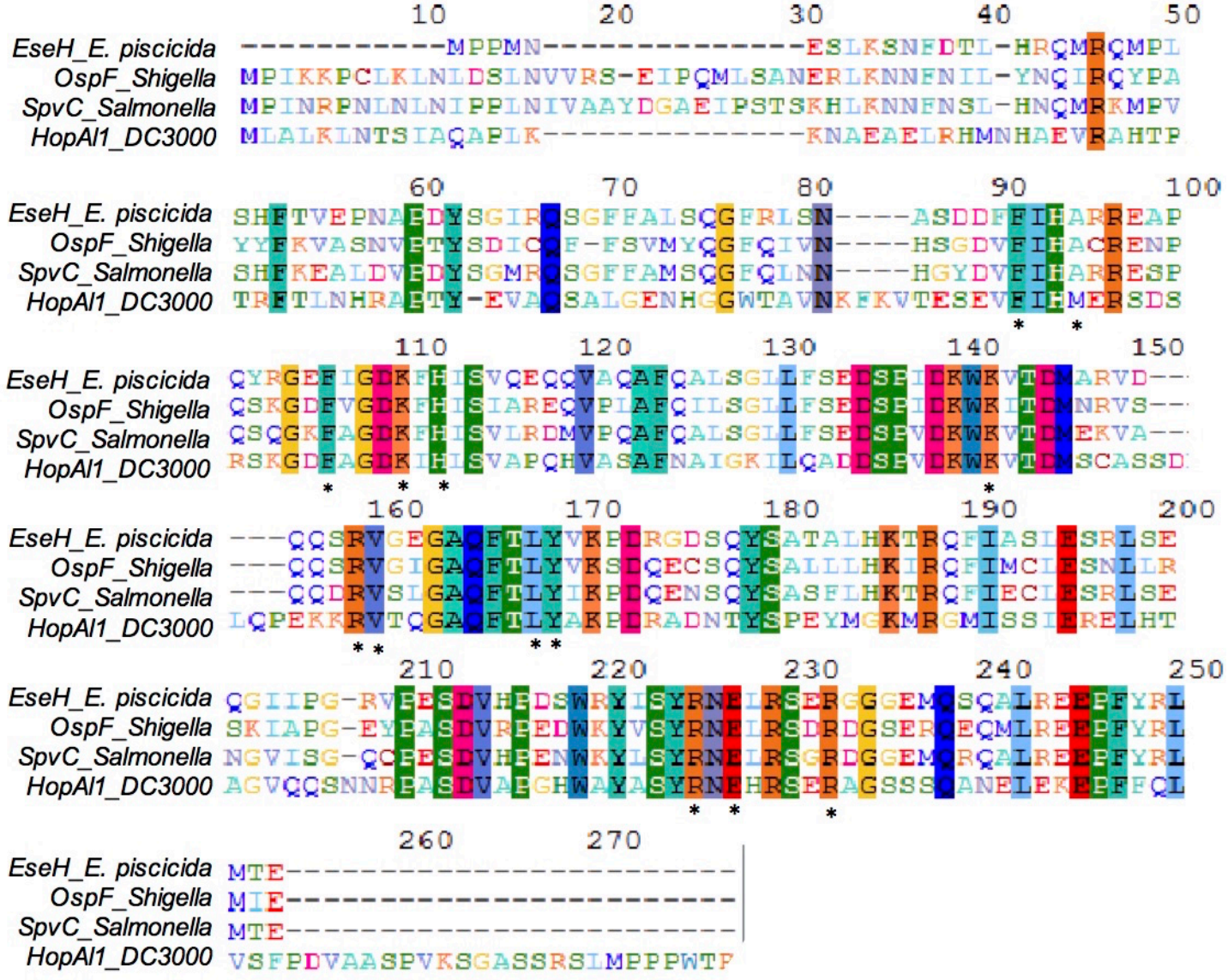

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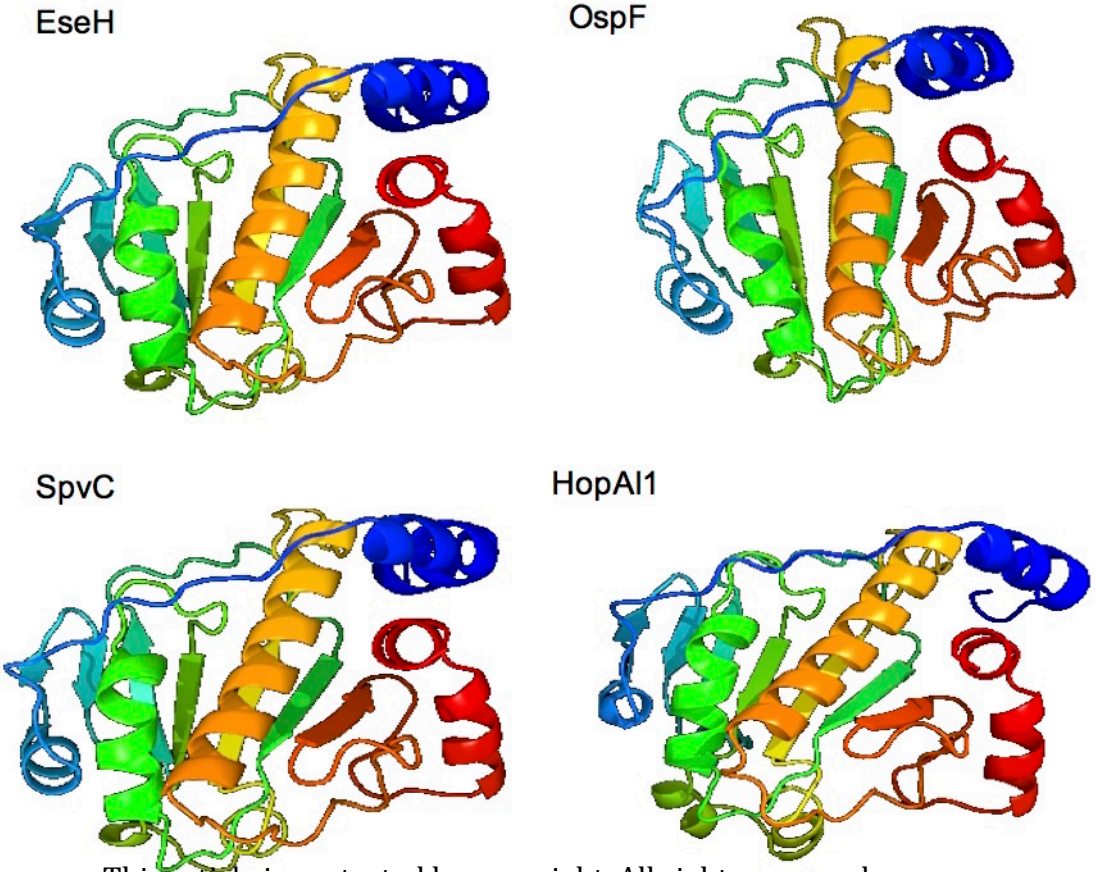

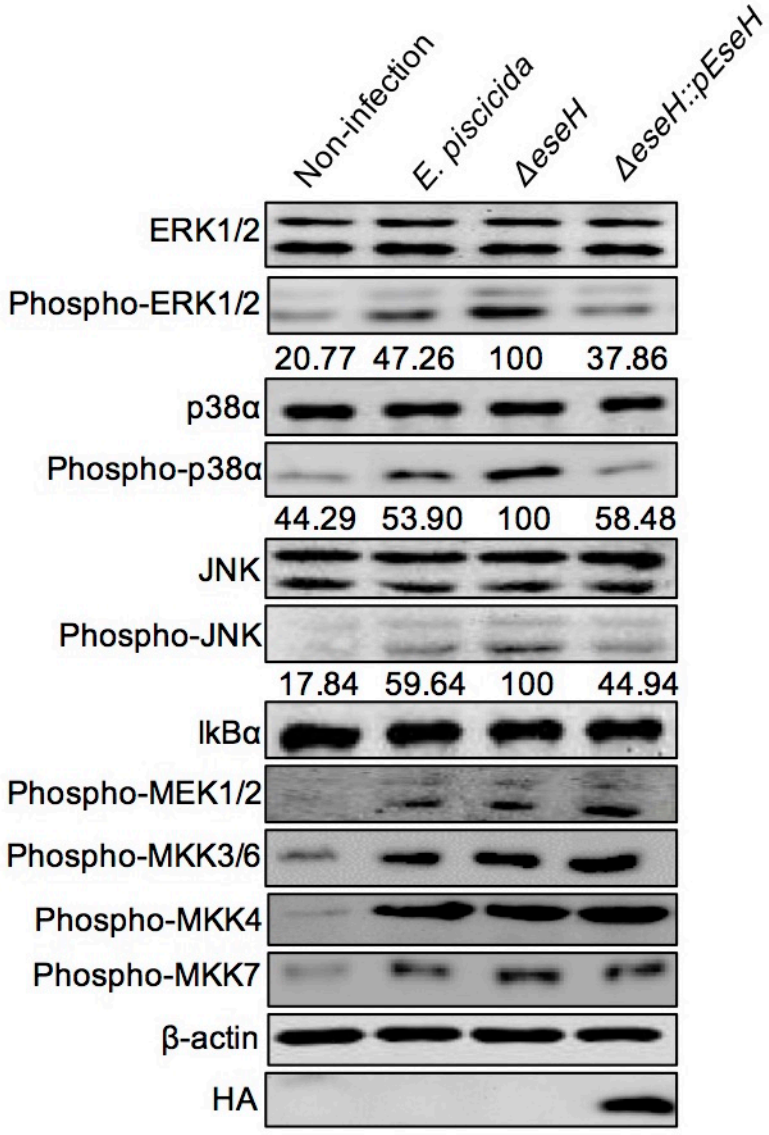

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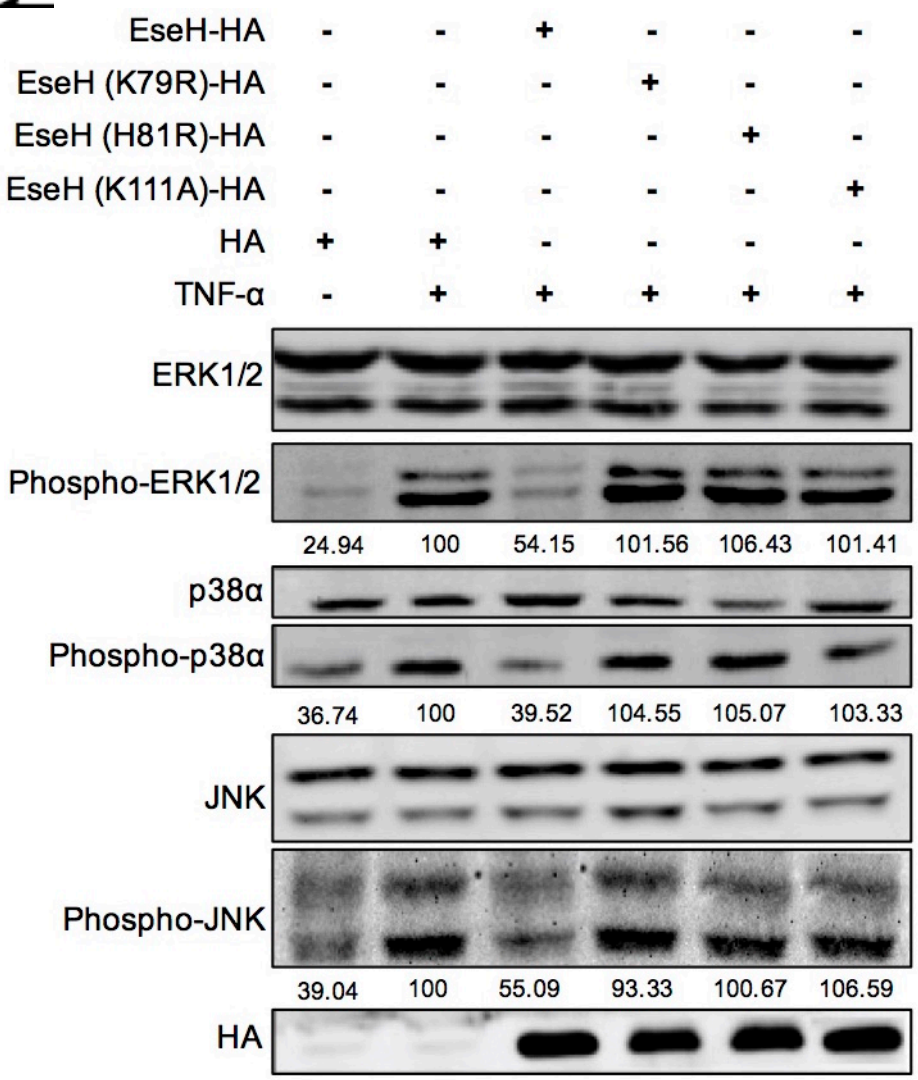

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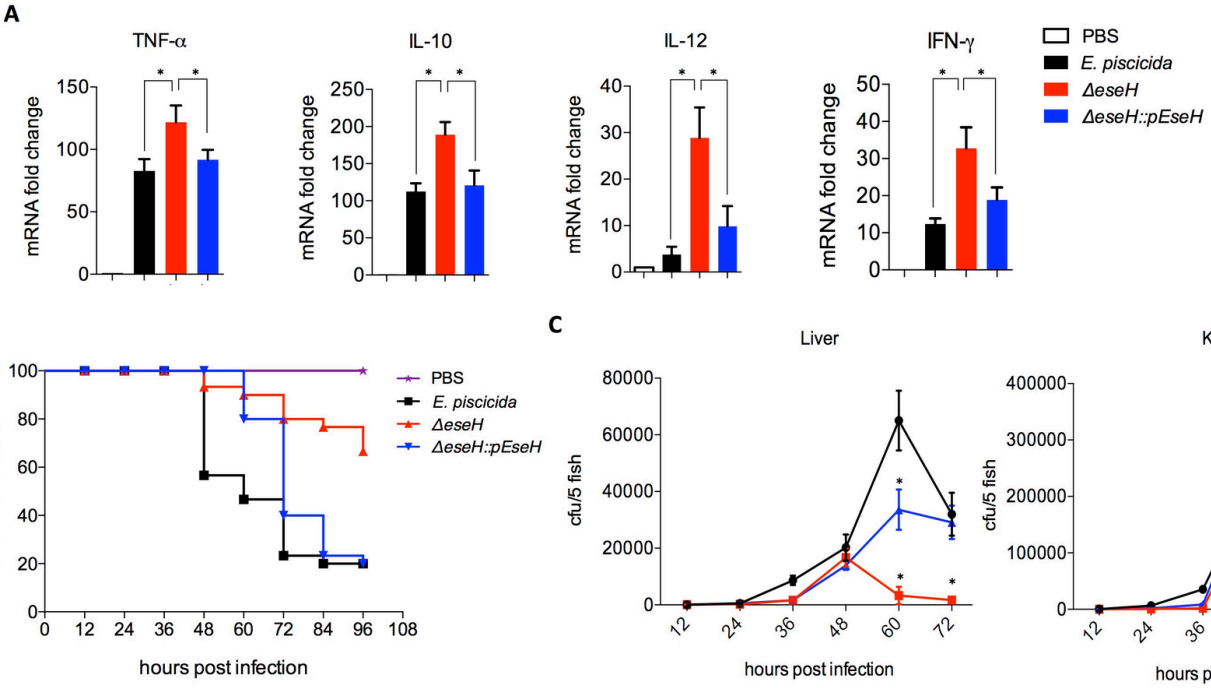

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