Secreted in a Type III Secretion System-Dependent Manner, EsaH and EscE Are the Cochaperones of the T3SS Needle Protein EsaG of Edwardsiella piscicida

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ABSTRACT The intracellular EscE protein tightly controls the secretion of the type III secretion system (T3SS) middle and late substrates in Edwardsiella piscicida. However, the regulation of secretion by EscE is incompletely understood. In this work, we reveal that EscE interacts with EsaH and EsaG. The crystal structures of the EscE-EsaH complex and EscE-EsaG-EsaH complex were resolved at resolutions of 1.4 Å and 1.8 Å, respectively. EscE and EsaH form a hydrophobic groove to engulf the C-terminal region of EsaG (56 to 73 amino acids [aa]), serving as the cochaperones of T3SS needle protein EsaG in E. piscicida. V61, K62, M64, and M65 of EsaG play a pivotal role in maintaining the conformation of the ternary complex of EscE-EsaG-EsaH, thereby maintaining the stability of EsaG. An in vivo experiment revealed that EscE and EsaH stabilize each other, and both of them stabilize EsaG. Meanwhile, either EscE or EsaH can be secreted through the T3SS. The secondary structure of EsaH lacks the fourth and fifth α helices presented in its homologs PscG, YscG, and AscG. Insertion of the α4 and α5 helices of PscG or swapping the N-terminal 25 aa of PscG with those of EsaH starkly decreases the protein level of the chimeric EsaH, resulting in instability of EsaG and deactivation of the T3SS. To the best of our knowledge, these data represent the first reported structure of the T3SS needle complex of pathogens from Enterobacteriaceae and the first evidence for the secretion of T3SS needle chaperones.

IMPORTANCE Edwardsiella piscicida causes severe hemorrhagic septicemia in fish. Inactivation of the type III secretion system (T3SS) increases its 50% lethal dose (LD50) by ~10 times. The secretion of T3SS middle and late substrates in E. piscicida is tightly controlled by the intracellular steady-state protein level of EscE, but the mechanism is incompletely understood. In this study, EscE was found to interact with and stabilize EsaH in E. piscicida. The EscE-EsaH complex is structurally analogous to T3SS needle chaperones. Further study revealed that EscE and EsaH form a hydrophobic groove to engulf the C-terminal region of EsaG, serving as the cochaperones stabilizing the T3SS needle protein EsaG. Interestingly, both EscE and EsaH are secreted. Our study reveals that the EscE-EsaH complex controls T3SS protein secretion by stabilizing EsaG, whose secretion in turn leads to the secretion of the middle and late T3SS substrates.

KEYWORDS cochaperone, secretion, T3SS needle protein, Edwardsiella piscicida
into the host cell cytoplasm. The injectisome is composed of membrane-embedded protein rings extended by a hollow needle formed by a single polymerized shaft, through which effectors are secreted or translocated to aid infection.

*Edwardsiella piscicida*, previously known as *Edwardsiella tarda* (1), is a Gram-negative intracellular pathogen and one of the causes of hemorrhagic septicemia in fish. It is also an emerging agent for human infection through wound contact with ill fish (2). The T3SS of *E. piscicida* is encoded by its chromosome and is required for its survival and replication in epithelial cells or macrophage cells (3–5). Of the substrates secreted by the T3SS, EseB, EseC, and EseD function as the translocon, forming pores on host membranes through which effectors are translocated (6). EseG, EseJ, EseH, and EseK have been identified to be the T3SS effectors of *E. piscicida* (7–10). Besides the translocon and effectors, needle protein is also secreted through the T3SS basal body. The T3SS needle of *Yersinia*, *Pseudomonas*, *Aeromonas*, enteropathogenic *Escherichia coli* (EPEC), *Shigella*, *Salmonella* pathogenicity island 2 (SPI2), *Salmonella* SPI1, *Burkholderia*, or *Chlamydia trachomatis* is composed of a single protein: YscF, PscF, AscF, EscF, MxiH, SsaG, Prgl, BsaL, or CdsF, respectively (11–21). Chaperones are required to bind to newly synthesized needle protein and to keep it in a secretion-competent state (22, 23). The sequence homologies between type III needle chaperones are notably low among different bacterial pathogens; however, they share a similar structure and possess common features, such as their small size and an often acidic pl. Before secretion, chaperones bind to the C-terminal region of needle protein to maintain its soluble state and to prevent it from undergoing self-assembly or degradation by YscE-YscG in *Yersinia* spp., PscE-PscG in *Pseudomonas aeruginosa*, AscE-AscG in *Aeromonas hydrophila*, EscE-EscG in EPEC, and CdsE-CdsG in *C. trachomatis* (12, 16, 18, 21, 24–26). Among them, PscE-PscG was first discovered and characterized in *P. aeruginosa*: PscE and PscG were shown to form a stable, soluble complex with PscF in the cytoplasm at a 1:1:1 ratio, thus blocking premature polymerization of PscF (24, 27).

Chaperones for the type III needle of *E. piscicida* remain unidentified. Our recent study demonstrated that the type III protein EscE (Orf13 protein) shares sequence homology with SsaE in *Salmonella enterica*, YscE in *Yersinia enterocolitica*, and CV2595 in *Chromobacterium violaceum* (28). YscE and YscG are the cochaperones of T3SS needle YscF. However, we failed to find a homolog of YscG in the *E. piscicida* T3SS gene cluster through a primary sequence homology search. In this study, the proteins in the EscE-EsaH complex are proven to be the cochaperones of T3SS needle protein EsaG, as revealed by its crystal structure. Moreover, it is demonstrated that both EscE and EsaH are secreted in a T3SS-dependent manner.

**RESULTS**

**Sequence analysis of EsaG, EsaH, and EscE.** The *esaG* gene is located upstream of *esaH* and downstream of *esrC* in the T3SS gene cluster of *E. piscicida* (Fig. 1A). It encodes a peptide of 73 amino acids (aa) with a predicted molecular mass of 8.0 kDa and a pl of 6.5. Using CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and InterPro (http://www.ebi.ac.uk/interpro/search/sequence) for informatic analysis, EsaG was found to belong to the T3SS needle_F superfamily and shares homology with the type III needle protein MxiH of *Shigella*, YscF of *Yersinia pestis*, SsaG of *Salmonella enterica*, PscF of *P. aeruginosa*, and EscF of EPEC. Notably, EsaG shares 61.8% and 64.4% similarity with SsaG and EscF, respectively, and 42.6%, 43.0%, and 45.3% similarity with YscF, AscF, and PscF, respectively (Table 1).

The *esaH* gene is located between *esaG* and *esaI* in the *esrC-esaL* transcriptional unit in the T3SS gene cluster of *E. piscicida* (Fig. 1A). The *esaH* gene encodes a peptide of 88 amino acids, with a predicted molecular mass of 9.6 kDa and a pl of 4.9. Using CDD and InterPro, EsaH was found to be in the same family as the *Salmonella* SPI2 protein SsaH, which recently has been reported to be one of the chaperones of the *Salmonella* SPI2 needle protein SsaG, as revealed by a coimmunoprecipitation assay (17). EsaH shares 46.4% similarity with *S. enterica* SsaH, 35.5% similarity with EPEC/enterohemorrhagic...
E. coli (EHEC) EscG, 25% similarity with Y. enterocolitica YscG, 16.8% similarity with A. hydrophila AscG, and 7.7% similarity with P. aeruginosa PscG (Table 1).

The escE gene (previously known as orf13) is located upstream of esaG, spaced by esrC in the T3SS gene cluster of E. piscicida (Fig. 1A). It encodes an 8.7-kDa protein with a pI of 7.9. EscE belongs to the T3SS YscE superfamily. When domains were searched using Delta-BLAST, EscE was found to share 36.6%, 35.1%, 39.2%, and 42.9% similarity with SsaE of Salmonella spp., PscE of P. aeruginosa, YscE of Y. pestis, and AscE of A. hydrophila, respectively (Table 1). Unexpectedly, our previous study revealed that EscE can be secreted and translocated in a T3SS-dependent manner, and it regulates the secretion of the T3SS proteins according to its own steady-state protein level inside E. piscicida (28). Accordingly, it was verified that EscE (Orf13) is secreted and directly interacts with mammalian factor Cugbp2 upon its translocation (29).

EscE interacts with EsaH and EsaK. To search for the interacting partner of EscE, we cloned escE into the yeast two-hybrid system vector pGBKT7 and explored the pairwise

![Image](FIG 1 Interaction of EscE with EsaH. (A) Schematic representation of the escE-esaL region of the E. piscicida T3SS. Arrows represent each of the open reading frames. (B) Interaction of EscE with EsaH and EsaK as revealed by yeast two-hybrid assay results. Yeast two-hybrid results were obtained using high-stringency Sabouraud dextrose lacking Trp, Leu, His, and Ade (SD/Trp−Leu−His−Ade) (top) and medium-stringency SD/−Trp−Leu (bottom). Yeast strains transformed with pGADT7-esaH plus pGBK7-escE or pGADT7-esaK plus pGBK7-escE were able to grow on the selective high-stringency plate as blue colonies like the positive control (transformed with pGADT7-T plus pGBK7-S3). Yeast strains transformed with pGADT7-T plus pGBK7-Lam were the negative control.

\[ \text{EscE and EsaH Are the Secreted T3SS Needle Chaperones} \]

### TABLE 1 Similarity matrix for EscE, EsaG, and EsaH homologs

| E. piscicida protein | Homologous protein (% similarity)a |
|---------------------|----------------------------------|
|                     | S. enterica (SPI-2) | EPEC/eHEC | Y. enterocolitica | A. hydrophila | P. aeruginosa |
| EscE                | SsaE (36.6)         | EscE− (41.9) | YscE (39.2)    | AscE (42.9)  | PscE (35.1)  |
| EsaG                | SsaG (61.8)         | EscF (64.4) | YscF (42.6)    | AscF (43.0)  | PscF (45.3)  |
| EsaH                | SsaH (46.4)         | EscG (35.5) | YscG (25.0)    | AscG (16.8)  | PscG (7.7)   |

*Protein sequences of EscE, EsaG, and EsaH and their homologs in other bacteria were compared. The percentage of similar amino acid residues was calculated using EMB0SS Needle protein alignment (www.ebi.ac.uk/Tools/pfa/emboss_needle/). The corresponding protein sequences were retrieved through the GenBank accession numbers for the proteins: EscE (WP_012847727.1), SsaE (NP_460361.1), EscE− (WP_000638726.1), YscE (CAF25424.1), AscE (ANTF5727.1), PscE (NP_250409.1), EsaH (ACY83705.1), SsaG (NP_460371.1), EscF (NP_312579.1), YscF (CAF25425.1), AscF (WP_021229954.1), PscF (NP_250410.1), EsaH (ADM40922.1), SsaH (BCH84983.1), EscG (NP_312578.1), YscG (WP_011901822.1), AscG (ABF70178.1), and PscG (NP_250411.1).
interaction between EscE and 23 proteins of the *E. piscicida* T3SS, as described by Liu et al. (30). The results showed that yeast strains transformed only with pGBKT7-escE plus pGADT7-esaK or pGADT7-esaH were able to grow in the selective medium as blue colonies, similar to the positive control, in which the yeast was transformed with pGBKT7-53 plus pGADT7-T. The yeast strain transformed with pGBKT7-Lam and pGADT7-T was used as the negative control, which failed to grow in the selective medium (Fig. 1B). Yeast strains transformed with pGBKT7-escE and the other 21 recombinant plasmids did not grow (see Fig. S1 in the supplemental material). This result indicates that either EsaH or EsaK may interact with EscE.

**Crystal structure of the EscE-EsaH complex.** When expressed in *E. coli* BL21(DE3), EscE is soluble and stable, while EsaH is insoluble and unstable. By coexpression and copurification, we obtained the EscE-EsaH complex. The crystal structure of the EscE-EsaH complex was solved by single-wavelength anomalous diffraction (SAD) at a resolution of 1.8 Å, and it has been refined to an Rwork/Rfree of 20%/22.8% with good geometry (Table 2). The final structure of EscE-EsaH consists of residues 5 to 73 of EscE and residues 2 to 68 of EsaH. The C-terminal region of EsaH (residues 69 to 88) is invisible, and no electron density could be traced in our structure, indicating that this region is flexible in this binary complex. However, EscE and EsaH form a compact fold, and the first helix of EsaH docked deeply into the concave surface formed by the two antiparallel helices (Hb and Hc) and the short helix (Ha) of EscE (Fig. 2A and B). Despite the low sequence homology, the triple-helix structure of EscE resembles that of AscE and PscE, with root mean square deviations (RMSDs) of 0.9 Å and 1.6 Å, respectively. As AscE-AscG and PscE-PscG are cochaperones, the EscE-EsaH complex was superimposed with AscE-AscG and PscE-PscG, and their structural similarity was revealed with RMSDs of 2.2 Å and 2.4 Å, respectively (Fig. 2C). Further

| Parameter | EscE-EsaH (Se) | EscE-EsaH-EsaG |
|-----------|---------------|----------------|
| PDB ID    | 7Y6B          | 7Y6C           |
| Wavelength (Å) | 0.9785        | 0.9791         |
| Space group | P21           | C2             |
| a, b, c (Å) | 55.5, 52.75, 65.51 | 123.85, 57.75, 54.8 |
| a, b, c (%) | 90.0, 114.0, 90.0 | 90.0, 98.8, 90.0 |
| Resolution (Å) | 1.8           | 1.4            |
| Rmerge (%) | 6.7 (38.0)    | 5.3 (27.5)     |
| I/σ(I) (%) | 9.0 (2.8)     | 14.8 (4.6)     |
| Completeness (%) | 97.6 (98.0) | 98.1 (98.3) |

Crystal data collection and refinement statistics

**TABLE 2**

| Parameter         | EscE-EsaH (Se) | EscE-EsaH-EsaG |
|-------------------|---------------|----------------|
| PDB ID            | 7Y6B          | 7Y6C           |
| Wavelength (Å)    | 0.9785        | 0.9791         |
| Space group       | P21           | C2             |
| a, b, c (Å)       | 55.5, 52.75, 65.51 | 123.85, 57.75, 54.8 |
| a, b, c (%)       | 90.0, 114.0, 90.0 | 90.0, 98.8, 90.0 |
| Resolution (Å)    | 1.8           | 1.4            |
| Rmerge (%)        | 6.7 (38.0)    | 5.3 (27.5)     |
| I/σ(I) (%)        | 9.0 (2.8)     | 14.8 (4.6)     |
| Completeness (%)  | 97.6 (98.0)   | 98.1 (98.3)    |

**Refinement statistics**

| Parameter         | EscE-EsaH (Se) | EscE-EsaH-EsaG |
|-------------------|---------------|----------------|
| Resolution (Å)    | 39.6–1.8      | 37.8–1.4       |
| No. of reflections| 30,376        | 73,628         |
| Rwork/Rfree (%)   | 20.0/22.8     | 20.4/23.2      |
| No. of atoms      |               |                |
| Protein           | 2,244         | 2,703          |
| Water             | 114           | 277            |
| B-factors (Å²)    |               |                |
| Protein           | 20.9          | 19.2           |
| Water             | 19.3          | 28.4           |
| RMSD              |               |                |
| Bond length (Å)   | 0.007         | 0.005          |
| Bond angle (°)    | 0.804         | 0.779          |
| Ramachandran plot (%) | 99.28       | 100            |

The highest-resolution shell is shown in parentheses.

**Crystal structure of the EscE-EsaH complex.** When expressed in *E. coli* BL21(DE3), EscE is soluble and stable, while EsaH is insoluble and unstable. By coexpression and copurification, we obtained the EscE-EsaH complex. The crystal structure of the EscE-EsaH complex was solved by single-wavelength anomalous diffraction (SAD) at a resolution of 1.8 Å, and it has been refined to an Rwork/Rfree of 20%/22.8% with good geometry (Table 2). The final structure of EscE-EsaH consists of residues 5 to 73 of EscE and residues 2 to 68 of EsaH. The C-terminal region of EsaH (residues 69 to 88) is invisible, and no electron density could be traced in our structure, indicating that this region is flexible in this binary complex. However, EscE and EsaH form a compact fold, and the first helix of EsaH docked deeply into the concave surface formed by the two antiparallel helices (Hb and Hc) and the short helix (Ha) of EscE (Fig. 2A and B). Despite the low sequence homology, the triple-helix structure of EscE resembles that of AscE and PscE, with root mean square deviations (RMSDs) of 0.9 Å and 1.6 Å, respectively. As AscE-AscG and PscE-PscG are cochaperones, the EscE-EsaH complex was superimposed with AscE-AscG and PscE-PscG, and their structural similarity was revealed with RMSDs of 2.2 Å and 2.4 Å, respectively (Fig. 2C). Further
inspection showed that Leu9 and Ala13 of EsaH and Leu7, Leu23, Leu27, V63, and Ile64 of EscE form a hydrophobic interface (Fig. 2D). Besides, the extensive hydrogen bonds (EsaH-Glu12-EscE-Leu7, EsaH-Glu25-EscE-Arg62, EsaH-Asp28-EscE-Arg62, EsaH-Asp35-EscE-Ser56, and EsaH-Asp35-EscE-Arg62) mediate the interactions between EsaH and EscE (Fig. 2D). The extensive interaction interface between EsaH and EscE makes EsaH stable in solution.

Both EscE and EsaH are required for the presence of EsaG. EscE-EsaH resembles AscE-AscG and PscE-PscG, which are cochaperones of the T3SS needle protein. The T3SS needle protein in E. piscicida is EsaG (Table 1). Needle protein is the early
substrate of the T3SS, and its failure in secretion always blocks secretion of the middle (translocon) and late (effector) substrates, as is the case for SPI2 of S. enterica (17) and LEE of EPEC (31). To investigate this in E. piscicida, the extracellular protein profiles of wild-type (WT) and ΔesaG, ΔescE, and ΔesaH mutant strains were compared. It was observed that disruption of EsaH, EscE, or EsaG obstructed the secretion of the translocon proteins EseB, EseC, and EseD and effector protein EseJ, as revealed by SDS-PAGE and Coomassie blue staining. Complementation of the ΔesaG or ΔesaH strain restored their secretion profile to that of the WT strain (Fig. 3A). This indicates that EsaG shares the same phenotype as EscE or EsaH, and each is essential for the secretion of T3SS middle and late substrates.

But how do these three proteins influence each other? To investigate, the intracellular steady-state protein level of EsaG, EsaH, or EscE was examined in the WT and ΔesaG, ΔescE, and ΔesaH mutant strains. The EsaH antibody detected a band of ~10 kDa from the WT strain or ΔesaG strain and failed to detect a band from the ΔescE strain or ΔesaH strain. This indicates that EsaH can be specifically probed with the EsaH antibody, and expression of EscE is the premise for a successful detection of EsaH. The EscE antibody detected a band of ~10 kDa from the WT strain or ΔesaG strain and failed to detect a band from the ΔescE strain, indicating its specificity. EscE was barely detected from the ΔesaH strain, which indicates that the steady-state protein level of EscE is largely dependent on the intracellular EsaH. The EsaG antibody detected a band (~10 kDa) only in the cell lysates of the WT strain, but not in those from the ΔesaH, ΔescE, or ΔesaG strain (Fig. 3B). This indicates that both EscE and EsaH are necessary for the steady-state EsaG in E. piscicida. The possibility of in-trans transcription links among escE, esaH, and esaG was excluded by a real-time PCR assay (data not shown). Therefore, the posttranslational links among them might be involved.

EsaH and EscE are mutually required for their stability and that of EsaG. The stabilities of EsaG, EsaH, and EscE in the absence of one another were determined. Given the low efficiency of the EsaG antibody in probing the intracellular EsaG, we incorporated a 3×FLAG tag at the N terminus of chromosomal esaG, obtaining WT 3×FLAG:esaG, ΔescE 3×FLAG:esaG, and ΔesaH 3×FLAG:esaG strains. The three strains were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with chloramphenicol (Cm) to inhibit protein synthesis, and the steady-state protein levels of EscE, EsaH, and EsaG were examined at 60-min intervals until 360 min after chloramphenicol treatment. As shown in Fig. 3C, while the EscE, EsaH, and EsaG protein levels remained unchanged in the WT strain after chloramphenicol treatment, the steady-state protein levels of EsaH and EsaG in the ΔescE strain sharply decreased, as did the steady-state protein levels of EsaG and EsaH in the ΔesaH strain. These results indicate that both EscE and EsaH are essential for maintaining the stability of EsaG, while EscE and EsaH are mutually required for their stability.

Crystal structure of the EscE-EsaG-EsaH complex. To understand how EscE collaborates with EsaH to stabilize EsaG and to ensure the secretion of EsaG, we determined the crystal structure of the EscE-EsaG-EsaH complex by molecular replacement at a resolution of 1.8 Å, which has been refined to an Rwork/Rfree of 20.4%/23.2% with good geometry (Table 2). The calculated electron density map allowed the unambiguous tracing of most of the EscE (2 to 72 aa) and EsaH (4 to 80 aa). However, only the C-terminal region of EsaG (56 to 73 aa) could be observed in the electron density map, and it adopts an α-helical conformation that complexes with EsaH and EscE (Fig. 4A). Notably, H5, an additional helix, was brought out at the C-terminal region of EsaH, and the five-helix bundle formed a groove to grasp EsaG56–73 (Fig. 4B). Additionally, the helix Ha of EscE also interacted with EsaG66–73. Upon close inspection of these ternary complex interactions, the EsaH residues Val11, Phe15, and Val18 of H1, Ile49 and Phe52 of H3, Leu75 and Leu78 of H5, and Leu4 of EscE were observed to form a hydrophobic pocket, which stabilized the EsaG anchor residues Val61, Met64, Met65, Ile68, Ile69, and Ile72 (Fig. 4C). Moreover, the hydrogen bonds between Lys62 of EsaG and Thr3/Asp9 of EscE anchored the EsaG to EscE, and the hydrogen bonds formed by Ile68 and Ile69 of EsaG together with Gln42 and His8 of EsaH anchored the EsaG to EsaH (Fig. 4D). Taken together, the helices H1 to H5 of EsaH adopt a
superhelical scaffold, creating a concave surface and a highly hydrophobic platform that "grasps" the C-terminal helix of EsaG. Both EscE and EsaH maintain the carboxyl terminus of their cognate fiber-forming partner EsaG in a coiled-coil interaction.

**Key amino acids related to the stability of EsaG.** Hydrophobic interaction plays a key role in the maintenance of the mechanical infrastructure. The hydrophobic residues aligned along the polymerization domain of the type III secretion needle-forming protein are responsible for its structural integrity and provide key interactions that ensure its robust nature (26). Hydrophobic interaction also plays an important role in maintaining the conformation of the heterotrimer structure. According to the interaction information obtained from the structure of the EscE-EsaG-EsaH complex, individual amino acid substitutions in EsaG were constructed, and their effect on the stability of EsaG was investigated. Targeting the C-terminal region of EsaG by alanine replacement influenced the stability of EsaG. Substitution of M64, V61/K62, or M64/M65 led to a stark degradation of EsaG. Among them, M64 played a pivotal role in stabilizing EsaG, as the protein level of EsaG M64 was the lowest among all the EsaG single-site mutation strains. Interestingly, the protein level of EsaG partially decreased upon point mutation of either V61 or K62, while it sharply decreased upon their double mutation, indicating a cooperative effect of these two sites. Besides, M65, I68, and I72 moderately influenced the stability of EsaG. However, the steady-state levels of EsaG M64/M65/I68/I69/I69

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**FIG 3** Importance of EscE, EsaH, and EsaG for the activity of the T3SS. (A) Protein secretion profiles of the *E. piscicida* wild-type (WT) and ΔescE, ΔesaH, ΔesaG, ΔesaH/pIN-esaH-HA, and ΔesaG/pIN-esaG-HA strains. Proteins in supernatants were concentrated and analyzed by Coomassie staining of 12% SDS-PAGE gel. (B) Steady-state protein levels of EscE, EsaH, and EsaG examined in the cell lysate of *E. piscicida* strains. Proteins on PVDF membranes were probed with anti-EscE, anti-EsaH, anti-EsaG, and anti-EvpC antibodies. EvpC, a protein secreted by the type VI secretion system (T6SS) but not by the T3SS, was used as a loading control. (C) EscE and EsaH stabilize each other, and both are required for maintaining the stability of EsaG. The *E. piscicida* WT 3×FLAG-esaG strain, ΔescE 3×FLAG-esaG strain, and ΔesaH 3×FLAG-esaG strain were cultured in the presence of 200 μg/mL Cm for different times, as indicated, and the bacterial pellets sampled were subjected to immunoblotting with FLAG (EsaG), EsaH, EscE, and DnaK antibodies. DnaK, a chaperone protein of *E. piscicida*, was used as a loading control. The immunoblotting data shown are representative images from three independent experiments.
or EsaG K62/M64/M65/I68/I69 were higher than that of EsaG M64/M65, EsaG I68/I69, or EsaG K62 (Fig. 4E). It is speculated that the simultaneous mutation of these multiple sites might have changed the conformation of the ternary complex, thus strengthening the interaction among the other amino acids, which resulted in a new balance.

**Both EsaH and Esc are secreted in a T3SS-dependent manner.** EscE is required to stabilize EsaH, and it can be secreted through the T3SS (28, 29). But will EsaH also be secreted upon secretion of EscE? To investigate, similar amounts of extracellular proteins (ECPs) and total bacterial proteins (TBPs) of wild-type and ΔesaN strains were probed with anti-EsaH, anti-EscE, anti-EseG and anti-EvpC antibodies. EsaN is an ATPase, energizing the secretion of T3SS substrates (4). EvpC, a type VI protein, was used as a loading control. Similar amounts of EsaH, EscE, and EseG were detected in the TBPs of the WT or ΔesaN strain; however, the three were only probed from the
ECPs of the WT strain, and not from that of the ΔesaN strain (Fig. 5A). This indicates that EsaH can also be secreted through the active T3SS.

EscE is translocated into host cells in a T3SS-dependent manner (28, 29), but can EsaH also be translocated? CyaA fusions have been used in many bacteria, including Edwardsiella, Yersinia, and Xanthomonas, to successfully monitor the translocation of

**FIG 4** Structure of the EsaG-EsaH-EscE complex. (A) Stereo view of the EsaG-EsaH-EscE complex (cartoon), with the secondary structures labeled. EsaG, EscE, and EsaH are colored in gray, green, and cyan, respectively. (B) Electrostatic surface potential of the EsaG binding site of the EsaH-EscE complex, which is shown on the electrostatic surface, with EsaG shown as sticks, colored as in panel A; (C) 2Fo − Fc electron density map (contoured at a level of 1.0 o) for EsaG in the EsaG-EsaH-EscE complex. The refined model of EsaG is superimposed on the electron density map and shown as the gray stick. The residues are labeled. (D) Closeup view of the EsaG-EsaH-EscE interaction interface, with the hydrogen bonding interactions denoted by yellow dashed lines. Six of the seven hydrophobic residues of EsaG (gray) that were mutated to Ala in this study are shown. (E) Screening of amino acids related to the stability of EsaG. Shown are the results from analysis of the effect of EsaG mutations on its stability in E. piscicida from which esaG was chromosomally deleted. Pellets from equal amounts of each strain were probed with HA and DnaK antibodies. DnaK, a chaperone protein of E. piscicida, and EvpC, a protein secreted through the T6SS, were used as loading controls.

ECPs of the WT strain, and not from that of the ΔesaN strain (Fig. 5A). This indicates that EsaH can also be secreted through the active T3SS.

EscE is translocated into host cells in a T3SS-dependent manner (28, 29), but can EsaH also be translocated? CyaA fusions have been used in many bacteria, including Edwardsiella, Yersinia, and Xanthomonas, to successfully monitor the translocation of
Secretion of EsaH is related to the \( \alpha_4 \) and \( \alpha_5 \) helices it lacks and also to its N-terminal sequence. (A) EsaH is secreted in a T3SS-dependent manner. Total bacterial proteins (TBPs) and extracellular proteins (ECPs) from similar amounts of the wild-type strain and T3SS mutant strain (\( \Delta \)esaN strain) were probed with EsaH, EscE, EseG, and EvpC antibodies. EvpC, a protein secreted by the type VI secretion system but not by T3SS, was used as a loading control. (B) EsaH cannot be translocated into host cells. J774A.1 cells were infected with the indicated \( E. piscicida \) WT strain or \( \Delta \)esaN strain carrying pACYC-esaH::cyaA, pACYC-escE::cyaA, and pACYC-esaE::cyaA, and intracellular cAMP levels were determined at 4 h postinfection, as described in Materials and Methods. Means \( \pm \) SD from one representative experiment are shown. **, \( P < 0.01 \). Inset: EsaH::CyaA can be secreted in a T3SS-dependent manner. Secretion of EsaH::CyaA from the WT/pACYC-esaH::cyaA strain and \( \Delta \)esaN/pACYC-esaH::cyaA strain was probed with anti-EsaH and anti-EvpC antibodies. EvpC was used as a loading control. (C) Multiple alignment of \( E. piscicida \) EsaH (GenBank no. ADM40922.1) with its homologs—SsaH from \( S. enterica \) subsp. \( enterica \) (GenBank no. BCH84983.1), PscG from \( P. aeruginosa \) (GenBank no. NP_250411.1), YscG from \( Y. pestis \) (GenBank no. WP_011901822.1), and AscG from \( A. hydrophila \) (GenBank no. ABF70178.1). Seven \( \alpha \) helices were found in PscG, YscG, or AscG, and \( \alpha \)-helix 4 and \( \alpha \)-helix 5 were absent in EsaH or SsaH. The secondary structures of EsaH and PscG are shown at the top and bottom of the graph, respectively. The conserved residues are boxed. (D) Expression and secretion of EsaH, EscE, EsaG, EseB, and EseG from \( \Delta \)esaH strain overexpressing EsaHmut1 or EsaHmut2. Total bacterial pellets or extracellular proteins from the indicated \( E. piscicida \) strains were immunoblotted for EsaH, EscE, EsaG, EseB, EseG, and EvpC. The immunoblotting data shown are a representative image from three independent experiments.
bacterial T3SS effectors (28, 32–35). To investigate, pACYC-esaH::cyaA was constructed, which expresses a chimeric protein, EsaH::CyaA. If the fusion protein is translocated into the host cell, CyaA will convert ATP into cAMP in the presence of the eukaryotic cell cytoplasmic protein calmodulin. J774A.1 cells were infected with the WT/pACYC-esaH::cyaA strain, the ΔesaN/pACYC-esaH::cyaA strain, the WT/pACYC-escE::cyaA strain, the ΔesaN/pACYC-escE::cyaA strain, the WT/pACYC-eseG::cyaA strain, and the ΔesaN/pACYC-eseG::cyaA strain. The intracellular cAMP levels at 4 h postinfection (hpi) were measured as the readout of the translocation of EsaH::CyaA, EscE::CyaA, or EseG::CyaA. As shown in Fig. 5B, the cAMP levels in both WT-infected and ΔesaN mutant-infected cells were similar to that of the uninfected control for EsaH::CyaA. The cAMP levels in WT-infected cells were 202.99 ± 63.57 fmol/mg of host protein for EseG::CyaA (positive control) and 76.89 ± 14.91 fmol/mg of host protein when the cAMP level of EseG::CyaA or EscE::CyaA was less than 6.0 fmol/mg of host protein in cells infected with the ΔesaN strain (negative controls). These results indicate that, different from EscE, which is translocated into host cells in a T3SS-dependent manner, EsaH is not translocated into J774A.1 cells. Next, we investigated whether the failure in EsaH::CyaA translocation is due to its incapability of being secreted. To investigate, extracellular proteins (ECPs) of the WT/pACYC-esaH::cyaA or ΔesaN/pACYC-esaH::cyaA strain were harvested before being probed with anti-EsaH and anti-EvpC.
antibodies. EvpC was used as a loading control. It was found that EsaH-CyaA was secreted from the WT but not the ΔesaN background (Fig. 5B, inset).

Taken together, both EsaH and EscE are secreted in a T3SS-dependent manner, while different from EscE, EsaH fails to be translocated.

**Secretion of EsaH is related either to its N-terminal sequence or the α4 and α5 helices it lacks.** Secondary structure alignment of the T3SS needle chaperones found seven helices from YscG of *Y. pestis*, AscG of *A. hydrophila*, and PscG of *P. aeruginosa*, but only five from EsaH of *E. piscicida* and SsaH of *S. enterica*: i.e., both EsaH and SsaH lack the α4 and α5 helices (Fig. 5C). To learn whether the absence of these two α helices accounts for the secretion of EsaH, PscG5–25 was inserted into the esaH gene, obtaining pJN-esaH1–4(pscG5–25)-esaH4–5-HA (pJN-esaHmut1-HA). Moreover, as the secretion signal of T3SS substrates is always located in their N-terminal regions, 1 to 25 aa of EsaH were swapped with 1 to 25 aa of PscG, obtaining pJN-pscG1–25 α4esaH26–88 α5-HA (pJN-esaHmut2-HA), pJN-esaHHA, pJN-esaHmut1-HA, and pJN-esaHmut2-HA were introduced into the ΔesaH strain, and their expression was induced with L-arabinose. It was observed that insertion of the α4 and α5 helices of PscG into EsaH moderately decreased the steady-state protein level of EscE, while swapping the N-terminal 25 aa with that of PscG starkly decreased EscE, meanwhile, the intracellular EsaG was undetectable under both conditions. The steady-state protein level of EseG, EseB, or EvpC in each strain did not change with the expression of the chimeric EsaH (Fig. 5D, left panel). Secretion of EsaH, EsaG, EscE, EseG, and EseB was detected from strains expressing any of the chimeric EsaH, when equal amounts of proteins were loaded per lane, as evidenced by EvpC (Fig. 5D, right panel). These data indicate that incorporation of the α4 and α5 helices of PscG into EsaH or swapping the N-terminal portion of EsaH might have interfered with its interaction with EscE or EsaG, therefore crippling the secretion of EsaH, EsaG, EscE, the T3SS translocon, and effectors.

**DISCUSSION**

In the absence of EscE, the secretion of EseB, EseC, EseD, or EseJ was obstructed. EscE tightly controls the secretion of T3SS middle and late substrates according to its steady-state protein level inside *E. piscicida* (28), but how EscE controls T3SS secretion in *E. piscicida* remains an enigma. In this article, we provide the first structural evidence that EscE and EsaH are the cochaperones of the T3SS needle protein EsaG. EscE delicately controls the secretion of middle and late substrates of the T3SS through stabilizing EsaG. Moreover, for the first time, we provide evidence that T3SS needle chaperones can be secreted.

Secretion of the T3SS translocon or effectors is obstructed when the T3SS needle chaperones are disrupted in EPEC, *Y. pestis*, or *S. enterica* serovar Typhimurium (16, 36, 37). Consistently, in the absence of the T3SS needle protein, secretion of the T3SS translocon and effectors is also obstructed (26, 38, 39). Recently, SsaE was reported to regulate cellular SsaH levels to control the secretion of early substrates of the T3SS needle SsaG and rod SsaI before promoting the secretion of the translocon, thereby demonstrating a chaperone-mediated switch in secretion from early to middle substrates of *Salmonella* SPI2 (17). In *E. piscicida*, EscE binds with EsaH and forms an EscE-EsaH complex. In the absence of EscE, the transcriptional level of *esaH* remains unchanged (data not shown), while no EsaH can be detected, emphasizing the fact that EscE is stabilizing EsaH. Moreover, in this study, we demonstrated that EsaH is the main chaperone that binds and stabilizes EsaG. Five helices of EsaH create a concave surface and a highly hydrophobic platform that “grasps” the C-terminal helix of EsaG46–73. The EscE-EsaH complex maintains the carboxyl terminus of the proteins’ cognate fiber-forming partner EsaG in a coiled-coil interaction, thereby stabilizing EsaG and ensuring its proper secretion. Secretion of EsaG leads to the secretion of T3SS middle and late substrates. In this study, we revealed that EscE regulates the secretion of T3SS substrates through controlling the steady-state protein level of EsaG, whose secretion initiates the secretion of T3SS middle and late substrates.

Seven helices were detected from PscG, YscG, and AscG by secondary structure analysis, and five from EsaH and SsaH, which lacked the fourth and fifth α helices of...
their homologs. EsaH can be secreted through the T3SS. Inserting the α4 and α5 helices of PscG between the third and fourth α helices of EsaH decreases the steady-state protein level of the chimeric EsaH inside *E. piscicida* and abolishes the secretion of EsaG or EscE. It is speculated that inserting the α4 and α5 helices into EsaH may destroy the interaction between EsaH and EscE or EsaH and EsaG, thereby impeding the secretion of T3SS middle and late substrates. Moreover, the RMSDs of EsaH/PscG and EsaH/YscG were 3.7 Å and 3.1 Å, respectively, indicating that, unlike PscG and YscG, EsaH may be a novel chaperone. The tetratricopeptide repeat region (TPR) is a structural motif of T3SS needle chaperones, and EsaH may form a partial TPR fold, which enables its secretion.

The EscE of *E. piscicida* can be translocated into host cells, where it interacts directly with mammalian factor Cugbp2 (28, 29). Although secreted, EsaH failed to be translocated. At present, only the chaperone Spa15 of *S. flexneri* has been reported to be secreted through the T3SS (40). Spa15 is associated in the bacterial cytoplasm with LpaA, LipG1, and OspC3, and it is necessary for the stability of the T3SS effector LipG1 and for the secretion of the T3SS effector LpaA (40, 41). Moreover, Spa15 is also translocated through the T3SS and contributes to the intracellular survival of *Shigella* by blocking apoptosis in the infected host cells (42). Whether EscE, EsaH, and Spa15 share some common characteristics in their structures that enable their secretion awaits further study.

The structure of the EscE-EsaG-EsaH complex shows that EsaH is the major binding partner of EsaG, EsaH and EscE form a hydrophobic pocket to bind the C-terminal region of EsaG, and this companionship prevents the degradation of EsaG. The hydrophobic pocket formed by EsaH and EscE mainly stabilizes the EsaG anchor residues Val61 and Met64. M64 plays a pivotal role in stabilizing EsaG, and V61 and K62 coefficiently stabilize EsaG to a similar extent to M64. Besides EsaH, EscE also interacts with EsaK, as revealed by the yeast two-hybrid assay results. In the absence of EsaK, the steady-state protein levels of EscE, EsaH, or EsaG are not disrupted, while the translocon or effectors fail to be secreted (unpublished data). Hence, we speculate that EsaK might interact with the EscE-EsaG-EsaH complex and contribute to the secretion of EsaG. Moreover, EsaK shares 19% similarity with SsaK (26). SsaK is the homolog of OrgB, and OrgB is one of the components of the sorting platform in *Salmonella* SPI1-T3SS (9). It is possible that the EscE-EsaG-EsaH complex might be docked at the sorting platform of *E. piscicida* T3SS through the interaction between EscE and EsaK, facilitating the secretion of EsaG, EscE, and EsaH.

Type III secretion occurs in a hierarchical manner (43): proteins involved in the assembly of the needle complex are secreted first, followed by the translocon and effectors. Early substrates are not injected into host cells (44, 45). In this work, we have proven that EsaH can be secreted through the T3SS but failed to be translocated; thereby, EsaH could be an early T3SS substrate of *E. piscicida*.

*Salmonella* stimulates gastroenteritis in humans and typhoid-like fever in mice. *Salmonella* SPI2 is switched on in response to the acidic pH inside the *Salmonella*-containing vacuole and is required for the establishment of systemic infection (46). The T3SS of *E. piscicida* has almost a full set of genes that are homologous to *Salmonella* SPI2 (47). By resolving the crystal structure of EscE-EsaG-EsaH, our study also sheds light on the needle assembly of *Salmonella* SPI2.

**MATERIALS AND METHODS**

**Bacterial strains.** The *Edwardsiella piscicida* PPD130/91 strain used in this study was previously known as *Edwardsiella tarda* PPD130/91 (1, 48). The *E. piscicida* strains and plasmids used in this study are described in Table 3. *E. piscicida* strains were grown at 25°C in tryptic soy broth (TSB) (BD Biosciences) or in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) under a 5% (vol/vol) CO2 atmosphere to activate T3SS. When required, appropriate antibiotics were supplemented at the concentrations of 12.5 μg/mL colistin (Col) (Sigma), 34 μg/mL chloramphenicol (Cm) (Amresco), 15 μg/mL tetracycline (Tet) (Amresco), and 50 μg/mL gentamicin (Gm) (Amresco).

**Construction of knockout mutants and plasmids.** The *esaH* or *esaG* gene was deleted from the chromosome of *E. piscicida* PPD130/91 by sacB-based allelic exchange using a method previously described (49, 50). The mutants obtained were verified by sequencing and immunoblotting. All primers used are listed in Table 4. The complementing plasmids pJN-esaH-HA and pJN-esaG-HA are derivatives...
of pJN105 (51), which carries the esaH or esaG gene under the control of 1-arabinose-inducible araBAD promoter. The fourth and fifth α-helix sequences of the pscG were inserted into esaH to obtain pJN-esaH_{1-2}\_pscG_{4-5}\_esaH_{14-15}\_HA (pJN-esaHmut1-HA). In a similar way, pJN-pscG_{1-25\_14-15}\_esaH_{14-15}\_HA (pJN-esaHmut2-HA) was constructed. The plasmids acquired were verified by DNA sequencing before being transferred into E. piscicida strains and their expression was verified by immunoblotting.

Immunoblotting analysis. E. piscicida extracellular proteins (ECPs) and bacterial lysates (total bacterial proteins [TPBs]) were prepared as described by Zheng and Leung (52). They were loaded onto a 12% NuPAGE gel for electrophoresis in morpholineethanesulfonic acid (MES) running buffer (Invitrogen) or loaded onto 16% Tricine SDS-PAGE as described by H. Schägger (53). For immunoblotting analysis, proteins transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore) were probed with rabbit antibodies against HA (13,000) (Sigma), EsaG (1,000) (7), EsaG (1,500) (52), EscE (1,1000) (28), EsaB (1,300) (54), EsaA (1,000), or EsaH (1,1000). The EsaH antibody was raised in mice against EsaH protein purified from E. coli BL21(DE3), the EsaG antibody was raised in rabbit against aa 18 to 31 (SNDH5SIT5GNW), which was conjugated with keyhole limpet hemocyanin, and the antisera obtained

of E. piscicida. which was conjugated with keyhole limpet hemocyanin, and the antisera obtained

### TABLE 3

| Strain or plasmid | Description and/or genotype* | Reference or source |
|-------------------|------------------------------|---------------------|
| **E. piscicida**  |                              |                     |
| PPD130/91        | Wild type, Km\(^{r}\) Col\(^{r}\) Amp\(^{r}\) (LD\(_{50}\) 10\(^{15.0}\)) | 48                  |
| ΔesaH mutant     | PPD130/91, in-frame deletion of esaH\(_{1-2}\) | This study          |
| ΔesaG mutant     | PPD130/91, in-frame deletion of esaG\(_{1-2}\) | This study          |
| ΔescE mutant     | PPD130/91, in-frame deletion of escE\(_{1-2}\) | 28                  |
| WT \(3\times\)FLAG:esaG | PPD130/91, chromosomal expression of \(3\times\)FLAG:esaG, Amp\(^{r}\) Km\(^{r}\) | This study          |
| ΔesaH \(3\times\)FLAG:esaG mutant | ΔesaH, chromosomal expression of \(3\times\)FLAG:esaG, Amp\(^{r}\) Km\(^{r}\) | This study          |
| ΔescE \(3\times\)FLAG:esaG mutant | ΔescE, chromosomal expression of \(3\times\)FLAG:esaG, Amp\(^{r}\) Km\(^{r}\) | 28                  |
| WT/pACYC-esaG::cyoA | PPD130/91 with pACYC-esaG::cyoA | 28                  |
| ΔesaH/pACYC-esaG::cyoA mutant | ΔesaH with pACYC-esaG::cyoA | 28                  |
| WT/pACYC-escE::cyoA | PPD130/91 with pACYC-escE::cyoA | 28                  |
| ΔesaH/pACYC-escE::cyoA mutant | ΔesaH with pACYC-escE::cyoA | 28                  |
| WT/pACYC-esaH::cyoA | PPD130/91 with pACYC-esaH::cyoA | This study          |
| ΔesaH/pACYC-esaH::cyoA mutant | ΔesaH with pACYC-esaH::cyoA | This study          |
| ΔesaH/pJN-esaH-HA mutant | ΔesaH with pJN-esaH-HA | This study          |
| ΔesaH/pJN-esaHmut1-HA mutant | ΔesaH with pJN-esaHmut1-HA | This study          |
| ΔesaH/pJN-esaHmut2-HA mutant | ΔesaH with pJN-esaHmut2-HA | This study          |
| **E. coli**      |                              |                     |
| DH5α             | α complementation            | Stratagene          |
| BL21(DE3)/pLysS  | F\(^{-}\) ompT hsdS (\(_{68}\) \(_{mb}\)) gal dcm(DE3)tonA pLysS(Cm\(^{r}\)) | Invitrogen          |

Plasmids

| Plasmids          | Description                                      | Reference or source |
|-------------------|--------------------------------------------------|---------------------|
| pMD18-T           | Cloning vector, Amp\(^{r}\)                     | TaKaRa              |
| pRE112            | Suicide plasmid, pir dependent, Cm\(^{r}\) oriT orI V sacB | 49                  |
| pRE-ΔesaH         | pRE112 with esaH flanking fragments              | This study          |
| pRE-ΔesaG         | pRE112 with esaG flanking fragments              | This study          |
| pET-21a           | Red helper plasmid, Amp\(^{r}\)                 | 60                  |
| pKD4              | Template plasmid with FLP recognition target site, Km\(^{r}\) | 60                  |
| pJN-esaH-HA       | pJN-105 with esaH-HA                            | This study          |
| pJN-esaHmut1-HA   | pJN-105 expressing EsaH with site mutation of V61A | This study          |
| pJN-esaK62-HA     | pJN-105 expressing EsaH with site mutation of K62A | This study          |
| pJN-esaM64-HA     | pJN-105 expressing EsaH with site mutation of M64A | This study          |
| pJN-esaM65-HA     | pJN-105 expressing EsaH with site mutation of M65A | This study          |
| pJN-esaG68-HA     | pJN-105 expressing EsaH with site mutation of I68A | This study          |
| pJN-esaG69-HA     | pJN-105 expressing EsaH with site mutation of I69A | This study          |
| pJN-esaG72-HA     | pJN-105 expressing EsaH with site mutation of I72A | This study          |
| pJN-esaG61 K62-HA | pJN-105 expressing EsaH with site mutations of V61A and K62A | This study          |
| pJN-esaG64 M65-HA | pJN-105 expressing EsaH with site mutations of M64A and M65A | This study          |
| pJN-esaG68 I69-HA | pJN-105 expressing EsaH with site mutations of I68A and I69A | This study          |
| pJN-esaG62 M64 M65 I69-HA | pJN-105 expressing EsaH with site mutations of K62A, M64A, M65A, I68A, and I69A | This study          |

*Col, colistin; Amp, ampicillin; Tet, tetracycline; Cm, chloramphenicol. Superscripts: r, resistance; s, sensitivity.
TABLE 4 Oligonucleotides used in this study

| Designation | Nucleotide sequence |
|-------------|---------------------|
| esag-for    | ATGGTACCGCTTATCCGGTGAAGTGTGGTTGA |
| esag-int-rev| CAGCGTGATAGCTATGGATACGATGTCTCT |
| esag-int-for| ATAGCCTACTCGGGTTCTATGTCGTCAGTCATATGCAATATGCTCCTTA |
| esag-check-for| ATGTACCCCATAGCTAAGGCAAGCATCGATAATCAGGAACATCATACGGATATCGCGTTTTCTCCTGAGTGTG |
| esag-check-rev| TCGTGTGTCGTATCTGCAGGAGGTG |
| esah-for | ATGTACCCCATAGCTAAGGCAAGCATCGATAATCAGGAACATCATACGGATATCGCGTTTTCTCCTGAGTG |
| esah-int-rev| CAAAATTAGGTAATGCGGACGACGCTTGGCTGGCT |
| esah-int-for| GGCATTACACTTCTGGCATGATC |
| esah-esah-com-for | CCGGATACCGTGGGTATGCCCACCCTAACGCATCT |
| esah-esah-com-rev | TCGTCTAGAAGGCTTACAGGTCTACGTAATCAGGAAACATCATACGGATATCGCGTTTTCTCCTGAGTGTG |
| esah-esah-com-for | CCGGATACCGTGGGTATGCCCACCCTAACGCATCT |
| esah-esah-com-rev | TCGTCTAGAAGGCTTACAGGTCTACGTAATCAGGAAACATCATACGGATATCGCGTTTTCTCCTGAGTGTG |
| Esag-2F | GGATCCCAACTTGGATATGTCTCGCA |
| Esag-33F | GGATCCGATCCGGCATGATGATACGCTGTCAGTTCC |
| 6His-EsaG-33F | CCAATGGTGATGCCCACCCTAACGCATCT |
| Esag-73R | CTCAGGAATCTCGGTTCGCTGCTAGT |
| EsaH-2F | CATGGGATTACGCTTATCGGCTGGAATGTTGTTGTA |
| EsaH-88R | CTCAGGAATCTCGGTTCGCTGCTAGT |
| EscE-1F | CATGGGATTACGCTTATCGGCTGGAATGTTGTTGTA |
| EscE-73R | CTCAGGAATCTCGGTTCGCTGCTAGT |
| esah-cyaA-F | GGGGTACCCCGTATCGATCGGCCATC |
| esah-cyaA-R | GGAAAGATCTTCGGGATCTTCTCCTGAGT |
| 3×FLAG-esag-for | GCCAGCCTTCAACACCGCGGCGCTAAACACGGTAAGGACGCTATGTGTAGGCTGGAGCTGCTTC |
| 3×FLAG-esag-rev | GCCAGGAAACCTGATGCGGCAGCAAGACGATATCGCTTCATGTTTATTGGTGATCGCAGTTC |

were purified using the specific peptide as the ligand (Genscript, China). To probe EscE, EsaH, or Esag, proteins transferred onto PVDF membrane (0.22-μm pore) were fixed in 2.5% glutaraldehyde for 1 h before being subjected to blocking and immunoblotting.

Yeast two-hybrid assay. The yeast two-hybrid assay was carried out using a GAL4 DNA-binding domain-encoding bait vector (pGBK7T7) and a GAL4 activation domain-encoding prey vector (pGADT7) in the yeast strain AH109, as described by Liu et al. (30). All fusion constructs were made as full-length fusions. The interaction between human lamin C bait fusion (pGBK7T7-Lam) and simian virus 40 (SV40) prey fusion (pGADT7-Tclontech) was used as a negative control, and the interaction between murine p53 bait fusion (pGBK7T7-p53) and pGADT7-Tclontech as a positive control.

Protein expression, purification, and gel filtration chromatography. The escE gene was cloned into a glutathione S-transferase (GST) fusion protein expression vector pGEX-6P-1 (GE Healthcare) and esah into pET-28b. The two plasmids obtained were cotransformed into E. coli BL21(DE3), and cultures were grown at 37°C to an optical density at 600 nm (OD600) of 0.6 to 0.8, before induction with 0.1 mM isopropyl-β-d-thiogalactopyranoside ( IPTG) (Sigma, USA) for 16 h at 20°C. The harvested cells were sonicated in buffer A (400 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10% glycerol). Cell lysate was centrifuged at 16,000 g for 1 h at 4°C to collect supernatant, which was incubated with glutathione Sepharose 4 FastFlow beads (GE Healthcare) at 4°C for 3 h. The proteins on beads were digested with 3C protease. The protein complex was further purified by size exclusion chromatography (HiLoad 16/600 Superdex 75 prep grade) in buffer B (25 mM Tris-HCl [pH 8.0], 150 mM NaCl) as the running buffer. Purified protein was analyzed by SDS-PAGE. The peak fraction was pooled and concentrated to 10 mg/mL.

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SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, DOC file, 2.1 MB.

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Z.X.Z., L.Y.L., S.B.X., J.F.L., Y.L.L., D.Y.L., and Y.Z. performed the experiments and prepared materials for the manuscript. J.L., Y.Z.Z., and L.J.L. analyzed the crystal structure data. H.X.X., Z.X.Z., and P.N. designed and supervised the experiments and interpreted the data. H.X.X. and Z.X.Z. conceptualized the study and wrote the manuscript.

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