Investigation of EscA as a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon component EseC

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Edwardsiella tarda is an important Gram-negative enteric pathogen affecting both animals and humans. It possesses a type III secretion system (T3SS) essential for pathogenesis. EseB, EseC and EseD have been shown to form a translocon complex after secretion, while EscC functions as a T3SS chaperone for EseB and EseD. In this paper we identify EscA, a protein required for accumulation and proper secretion of another translocon component, EseC. The *escA* gene is located upstream of *eseC* and the EscA protein has the characteristics of T3SS chaperones. Cell fractionation experiments indicated that EscA is located in the cytoplasm and on the cytoplasmic membrane. Mutation with in-frame deletion of *escA* greatly decreased the secretion of EseC, while complementation of *escA* restored the wild-type secretion phenotype. The stabilization and accumulation of EseC in the cytoplasm were also affected in the absence of EscA. Mutation of *escA* did not affect the transcription of *eseC* but reduced the accumulation level of EseC as measured by using an EseC-LacZ fusion protein in *Ed. tarda*. Co-purification and co-immunoprecipitation studies demonstrated a specific interaction between EscA and EseC. Further analysis showed that residues 31–137 of EseC are required for EseC-EscA interaction. Mutation of EseC residues 31–137 reduced the secretion and accumulation of EseC in *Ed. tarda*. Finally, infection experiments showed that mutations of EscA and residues 31–137 of EseC increased the LD₅₀ by approximately 10-fold in blue gourami fish. These results indicated that EscA functions as a specific chaperone for EseC and contributes to the virulence of *Ed. tarda*.

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INTRODUCTION

Edwardsiella tarda is a facultative aerobic Gram-negative pathogen with a wide host range, infecting animals including fish (Sae-Oui *et al.*, 1984), amphibians (Kourany *et al.*, 1977), reptiles (Goldstein *et al.*, 1981), birds (Cook & Tappe, 1985) and mammals (including humans) (Janda & Abbott, 1993). Edwardsiellosis, caused by *Ed. tarda*, has been found in many commercially important cultured fish, and leads to extensive losses in both freshwater and marine aquaculture (Thune *et al.*, 1993). In humans, *Ed. tarda* can cause gastrointestinal (Janda & Abbott, 1993) and extra-intestinal infections (Yang & Wang, 1999). The pathogenesis of *Ed. tarda* is multifactorial, including factors that

enable the bacteria to invade non-phagocytic cells (Kourany *et al.*, 1977; Ling *et al.*, 2000), survive in phagocytes (Srinivasa Rao *et al.*, 2001, 2003), and produce virulence factors such as haemolysins (Janda & Abbott, 1993; Chen *et al.*, 1996) and catalases (Srinivasa Rao *et al.*, 2003). Recently, two important protein secretion systems, a type III secretion system (T3SS) (Tan *et al.*, 2005; Zheng *et al.*, 2007) and a type VI secretion system (T6SS) (Zheng & Leung, 2007), have been demonstrated to be virulence-associated.

Flagellar and non-flagellar systems (Saier, 2004; Pallen *et al.*, 2005) constitute two broad classes of T3SSs. Flagellar type III secretion systems (F-T3SSs) are responsible for the export of flagellum components (Macnab, 2003), while non-flagellar type III secretion systems (NF-T3SSs) translocate proteins across both the bacterial envelope and the eukaryotic plasma membrane in an ATPase-dependent fashion (Pallen *et al.*, 2005). Two kinds of proteins secreted

Abbreviations: ECP, extracellular protein; EPEC, enteropathogenic *E. coli*; ICP, intracellular protein; T3SS, type III secretion system (F-T3SS and NF-T3SS, flagellar and non-flagellar T3SS).

include the anti-host effectors, which are translocated into the cytosol of the target cells, where they subvert a variety of cellular processes (Waterman & Holden, 2003; Ghosh, 2004), and the translocon proteins, which form pores in a host cell's plasma membrane through which the effectors can enter the infected cell (Büttner & Bonas, 2002).

Efficient secretion of proteins through any T3SS usually depends upon the presence of chaperones that bind to the T3SS-secreted proteins (Parsot *et al.*, 2003). Chaperones stabilize newly synthesized proteins, protecting them from aggregation and proteolysis in the cytoplasm, and then deliver proteins to the secretion apparatus (Parsot *et al.*, 2003; Ghosh, 2004). In addition, some T3SS chaperones also function as regulators for T3SS gene expression and/or protein secretion (Darwin & Miller, 2001; Francis *et al.*, 2002). Two distinct functional classes of chaperones exist for NF-T3SSs: class I chaperones, which bind to effectors, and class II chaperones, which bind to translocons (Page & Parsot, 2002; Parsot *et al.*, 2003). Class I chaperones share a moderate degree of sequence homology, with a common mixed α/β -helical fold (Parsot *et al.*, 2003; Pallen *et al.*, 2005), while some class II chaperones have been recently reported to share TPR-like motifs (Bröms *et al.*, 2006; Edqvist *et al.*, 2006; Büttner *et al.*, 2008). Interactions of class II chaperones and their substrates are necessary for functions involving regulatory, structural and effector mechanisms (Delahay & Frankel, 2002; Olsson *et al.*, 2004), while some class II chaperones are reported to have defined regions that interact with their cognate substrates (Daniell *et al.*, 2003; Edqvist *et al.*, 2006).

In pathogenic *Ed. tarda*, an identified T3SS contains 35 open reading frames. EseB, EseC and EseD have been shown to be major components of the extracellular proteins secreted by the T3SS (Tan *et al.*, 2005), and these three proteins form a protein complex after secretion (Zheng *et al.*, 2007). EseB, EseC and EseD are respectively homologous to translocon components EspA, EspD and EspB of enteropathogenic *Escherichia coli* (EPEC) (Ide *et al.*, 2001), and to the translocon components SseB, SseC and SseD of *Salmonella* spp. (Nikolaus *et al.*, 2001). These findings suggest that EseB, EseC and EseD may function as translocon components, facilitating the translocation of effectors. These three Ese proteins contribute to *Ed. tarda* pathogenesis, as mutation of *eseB*, *eseC* or *eseD* led to a 10-fold decrease in virulence compared to the wild-type (Tan *et al.*, 2005).

As chaperones are key mediators of the T3SS virulence strategy, previous research was designed to identify the chaperones of *Ed. tarda* T3SS. One chaperone, EscC, was shown to function as a T3SS chaperone for the putative translocon components EseB and EseD (Zheng *et al.*, 2007). However, no chaperone has hitherto been identified for another putative translocon component, EseC. EseC is homologous to proteins belonging to the EspD family (YopB from *Yersinia* and IpaB from *Shigella*), which require chaperones in order to properly perform their functions (Cornelis & Van Gijsegem, 2000; Büttner &

Bonas, 2002). Analysis of the secondary structure indicated that the EseC protein contains two predicted hydrophobic transmembrane domains and two coiled-coil domains (Fig. 1B), similar to its homologues, such as EspD and YopB. These observations suggested that EseC may have functions similar to those of homologues involved in the pathogenesis process of cell attachment, pore formation and translation of effectors (Cornelis & Van Gijsegem, 2000; Büttner & Bonas, 2002). The *escA* gene is located upstream of *eseC* (Fig. 1A) and the EscA protein is homologous to the class II chaperones in other bacterial species such as CesD (38 % identity and 57 % similarity) in EPEC (Wainwright & Kaper, 1998), LcrH/SycD (24 % identity and 48 % similarity) in *Yersinia* spp. (Neyt & Cornelis, 1999), PcrH (23 % identity and 42 % similarity) in *Pseudomonas* spp. (Bröms *et al.*, 2003) and IpgC (23 % identity and 42 % similarity) in *Shigella* spp. (Ménard *et al.*, 1994). Interestingly, CesD was shown to interact with EspD but not EspB; this interaction is required in order to ensure proper secretion of EspD and EspB. However, IpgC was shown to bind directly to two translocon proteins (IpaB and IpaC) in the cytoplasm (Ménard *et al.*, 1994). Given the similarity of EseC and EspD, EscA could play a role similar to that of CesD and may be required for proper secretion of EseC. Here we present evidence that EscA functions as a specific chaperone responsible for the stabilization and efficient secretion of EseC in *Ed. tarda*. In addition, a defined domain in EseC is demonstrated to be required for the interaction between EseC and EscA to occur. This domain also plays a role in the pathogenesis of *Ed. tarda*.

METHODS

Bacteria strains, plasmids, culture media and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown at 37 °C on LB-agar plates or with shaking in LB-broth (Difco). *Ed. tarda* strains were grown at 25 °C on tryptic soy agar (TSA) plates or with shaking in tryptic soy broth (TSB, Difco). For the induction of T3SS expression, *Ed. tarda* strains were grown without shaking in a 5 % (v/v) CO₂ incubator at 25 °C in Dulbecco's modified Eagle medium (DMEM, Invitrogen). When required, culture media were supplemented with appropriate antibiotics: ampicillin (Amp, 50 µg ml⁻¹), colistin (Col, 12.5 µg ml⁻¹), kanamycin (Km, 50 µg ml⁻¹), chloramphenicol (Cm, 34 µg ml⁻¹) and tetracycline (Tet, 50 µg ml⁻¹).

Construction of in-frame deletion mutants of *Ed. tarda* PPD130/91. Overlap PCR was used to generate in-frame deletion fragments. The unmarked in-frame deletion mutants in *Ed. tarda* PPD130/91 were constructed according to a previously described method (Mo *et al.*, 2007). The Δ escA mutant removed codons 2–130 of EscA, whereas the Δ escC_{31–137} mutant removed an internal fragment of codons 31–137 of EseC.

To create a complementing plasmid for the Δ escA mutant, the *escA* gene was amplified and introduced into plasmid pACYC184. The resulting plasmid, pACYC184-*escA*, was transformed into the Δ escA mutant to produce Δ escA + *escA*.

Construction of the recombinant EscA protein and generation of polyclonal antibody. The polyclonal anti-EscA antibody was

Fig. 1. Effect of EscA on the secretion and accumulation of EseC. (A) Schematic representation of the *escA*–*eseC* region of *Ed. tarda*. (B) Schematic representation of the secondary structure of the EseC protein, indicating the predicted transmembrane (T) and coiled-coil (C) regions. (C) 2D PAGE analysis of the supernatant proteins of the *Ed. tarda* wild-type strain and Δ *escA* mutant cultured in DMEM at 25 °C with 5% (v/v) CO₂ for 24 h. Supernatant proteins of each strain were separated on Immobiline DryStrips (pH 3–10) and adjusted according to the relative amount of proteins secreted by each strain: wild-type (10 µg) and Δ *escA* (5 µg). Gels (12.5% polyacrylamide) were silver-stained. Translocon proteins EseB, EseC and EseD are indicated. (D) Western blotting analysis of the extracellular proteins (ECPs) and intracellular proteins (ICPs) of *Ed. tarda* wild-type, Δ *escA* and Δ *escA*+*escA* strains. Bacteria were adjusted to an equivalent amount of cells (OD₅₄₀ 0.5). The ECPs and ICPs of each strain were extracted from equal amounts of cells and analysed by Western blotting using anti-EscA, -EseB, -EseC and -EseD polyclonal antibodies. Relative units of the EseC proteins in (D), as measured with the Fluor-S Multi-imager (Bio-Rad), were as follows: wild-type ECP (lane 1), 100.0; Δ *escA* mutant ECP (lane 2), 1.5 ± 0.5 ; Δ *escA*+*escA* ECP (lane 3), 95.2 ± 3.0 ; wild-type ICP (lane 4), 36.6 ± 3.4 ; Δ *escA* mutant ICP (lane 5), 9.7 ± 1.2 ; and Δ *escA*+*escA* ICP (lane 6), 35.4 ± 4.2 .

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description or genotype	Reference or source
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169 (ϕ80 <i>lacZ</i>AM15) <i>hsdR17 recA1 gyrA96 thi-1 relA1</i></i>	Hanahan (1983)
MC1061 (λ <i>pir</i>)	<i>thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara14 xyl5 supE44 λpir</i>	Rubir�s <i>et al.</i> (1997)
SM10 (λ <i>pir</i>)	<i>thi thr leu tonA lacY supE recA-RP4-2-Tc-Mu Km^r λpir</i>	Miller & Mekalanos (1988)
BL21(DE3)/pLysS	F [−] <i>ompT hsdS (r_B[−] m_B[−]) gal dcm (DE3) tonA pLysS(Cm^r)</i>	Tan <i>et al.</i> (2005)
SY327 (λ <i>pir</i>)	Δ (<i>lac, pro</i>) <i>argE</i> (Am) <i>rif malA recA56 λpir</i>	Miller & Mekalanos (1988)
S17-1 (λ <i>pir</i>)	Tp ^r Sm ^r <i>recA thi pro hsdR[−] M⁺</i> RP4:2-Tc:Mu:Km Tn7 λ <i>pir</i>	Simon <i>et al.</i> (1983)
<i>Ed. tarda</i>		
PPD130/91	Wild-type, Col ^r Km ^s Amp ^s	Ling <i>et al.</i> (2000)
Δ <i>escA</i>	PPD130/91, <i>escA</i> in-frame deletion of codons 2–130	This study
Δ <i>eseC</i> _{31–137}	PPD130/91, <i>eseC</i> in-frame deletion of codons 31–137	This study
Δ <i>escA</i> + <i>escA</i>	PPD130/91, Δ <i>escA</i> carrying pACYC184- <i>escA</i>	This study
<i>eseC</i> ::pVIK111	PPD130/91, in-frame <i>eseC-lacZ</i> translational fusion	This study
<i>eseC</i> ::pVIK112	PPD130/91, in-frame <i>eseC-lacZ</i> transcriptional fusion	This study
Δ <i>escA</i> <i>eseC</i> ::pVIK111	Δ <i>escA</i> ; in-frame <i>eseC-lacZ</i> translational fusion	This study
Δ <i>escA</i> <i>eseC</i> ::pVIK112	Δ <i>escA</i> ; in-frame <i>eseC-lacZ</i> transcriptional fusion	This study
Plasmids		
pGEMT-easy	Cloning vector; Amp ^r	Promega
pRE112	pGP704 suicide plasmid, <i>pir</i> dependent, Cm ^r <i>oriT oriV sacB</i>	Edwards <i>et al.</i> (1998)
pETDuet-1	Expression vector, Amp ^r	Novagen
pACYC184	Cloning vector, Cm ^r Tc ^r	Fermentas Life Sciences
pA1	pETDuet-1 with <i>escA</i> in MCS1 (encodes His ₆ -EscA)	This study
pC1	pETDuet-1 with <i>eseC</i> in MCS1 (encodes His ₆ -EseC)	This study
pC2	pETDuet-1 with <i>eseC</i> in MCS2 (encodes EseC)	This study
pAC	pETDuet-1 with <i>escA</i> in MCS1 and <i>eseC</i> in MCS2 (encodes His ₆ -EscA + EseC)	This study
pAC _{Δ1–30}	<i>eseC</i> _{Δ1–30} fragment introduced into MCS2 of pA1	This study
pAC _{Δ31–137}	<i>eseC</i> _{Δ31–137} fragment introduced into MCS2 of pA1	This study
pAC _{Δ138–193}	<i>eseC</i> _{Δ138–193} fragment introduced into MCS2 of pA1	This study
pAC _{Δ194–243}	<i>eseC</i> _{Δ194–243} fragment introduced into MCS2 of pA1	This study
pAC _{Δ255–318}	<i>eseC</i> _{Δ255–318} fragment introduced into MCS2 of pA1	This study
pAC _{Δ319–402}	<i>eseC</i> _{Δ319–402} fragment introduced into MCS2 of pA1	This study
pAC _{Δ403–429}	<i>eseC</i> _{Δ403–429} fragment introduced into MCS2 of pA1	This study
pAC _{Δ430–506}	<i>eseC</i> _{Δ430–506} fragment introduced into MCS2 of pA1	This study
pVIK111	<i>lacZY</i> for translation fusions, Km ^r	Kalogeraki & Winans (1997)
pVIK112	<i>lacZY</i> for transcription fusions, Km ^r	Kalogeraki & Winans (1997)

generated according to a previously established method (Zheng *et al.*, 2007). For generation of the EscA recombinant protein, the *escA* gene was amplified by PCR and cloned into the MCS1 (multiple cloning site 1) of pETDuet-1, yielding plasmid pA1 with an N-terminal His₆-tag. The recombinant EscA was expressed in *E. coli* BL21(DE3)/pLysS and purified with Ni²⁺-NTA agarose beads under conditions recommended by the manufacturer (Qiagen). The purified His₆-EscA protein was used to immunize New Zealand White rabbits.

Construction of recombinant plasmids encoding His₆-EscA+ EseC and His₆-EscA+EseCA. For construction of the recombinant plasmid encoding His₆-EscA and EseC, the *eseC* gene was amplified and introduced into MCS2 (multiple cloning site 2) of pA1, yielding the plasmid pAC. For comparison, the *eseC* gene was then introduced into MCS1 and MCS2 of pETDuet-1 to produce plasmids pC1 and pC2, respectively.

The in-frame deletion fragments of *eseC* were constructed following the overlap extension PCR procedure described above. The resulting fragments were introduced into MCS2 of pA1, giving plasmids pAC _{Δ 1–30}, pAC _{Δ 31–137}, pAC _{Δ 138–193}, pAC _{Δ 194–243}, pAC _{Δ 255–318}, pAC _{Δ 319–402}, pAC _{Δ 403–429} and pAC _{Δ 430–506} (Table 1). These plasmids

encode various His₆-EscA + EseCA proteins. The recombinant plasmids were transformed into *E. coli* BL21(DE3)/pLysS.

Preparation of proteins of cell fractions. To prepare each protein fraction, *Ed. tarda* was cultured in DMEM to induce expression of the T3SS, and the equivalent of OD₅₄₀=0.5 bacterial cultures were analysed. The supernatant proteins, cell surface proteins and intracellular proteins were collected according to the method described by Beuzn *et al.* (1999) with some modification. Briefly, the bacterial cells were separated from 5 ml culture by centrifugation at 5000 *g* for 5 min at 4 C; the culture supernatant was filtered through a 0.22 m Millipore membrane filter and subsequently precipitated with 10% (w/v) trichloroacetic acid (TCA). The cell pellet was resuspended with 0.3 ml PBS (pH 7.4). The suspension was then mixed with 0.2 ml *p*-xylene for 5 min at room temperature and centrifuged at 2500 *g* for 10 min at 4 C. The organic layer was discarded and the aqueous layer was mixed with 1.2 ml acetone and maintained at –20 C to precipitate the secreted surface proteins. The cell pellet was collected and used for preparation of intracellular proteins (ICPs). The extracellular proteins (ECPs) consisted of the fraction of detached surface proteins plus the fraction of supernatant proteins.

For subcellular fractionation, the cytoplasmic membrane and the cytoplasm proteins were prepared using the method described by Neyt & Cornelis (1999). Briefly, *Ed. tarda* bacteria cultured in DMEM were harvested from the supernatant, and the supernatant proteins were separated by precipitation with 10% TCA as described above. The bacterial cells were resuspended in 10 mM cold Tris/HCl (pH 8.0) and 5 mM MgCl₂, and sonicated. The cellular debris was precipitated by centrifugation at 10 000 g for 5 min, and the supernatant was centrifuged for 30 min at 100 000 g to separate the membrane (insoluble) fraction from the cytosolic (soluble) fraction.

Each protein sample was dissolved in 50 µl ReadyPrep reagent 3 (Bio-Rad), and an equal volume (10 µl per lane) of each fraction was loaded on the SDS-PAGE gel for Western blotting.

2D PAGE and Western blotting. 2D PAGE was performed as previously described (Srinivasa Rao *et al.*, 2004). For Western blotting, protein fractions were resolved by 12% SDS-PAGE and electrotransferred onto nitrocellulose membranes according to the method described by Towbin *et al.* (1979). EscA and EseC were detected by incubation overnight at 4 °C with a 1:1000 dilution of anti-EscA and anti-EseC rabbit polyclonal antibodies, respectively, followed by a 1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). Antibody complexes were detected by development in 3,3'-diaminobenzidine tetrahydrochloride (DAB). Anti-DnaK monoclonal antibody (Merck) was used at a dilution of 1:2000 for detection of cell fractions.

The relative concentration of EseC in cell fractions was estimated by refractive densitometry using a Fluor-S Multi-imager (Bio-Rad) in the reflective mode. Values were calculated from optical density units (ODu) adjusted for band volume (ODu × mm²) using the Quantity One 4.3.0 software package (Bio-Rad). Wild-type EseC protein in the ECP fraction was used as an internal quantity standard. Three individual repetitions were analysed for each experiment.

Construction of transcriptional and translational fusions, and β-galactosidase assay. Plasmids pVIK112 and pVIK111 have been used to study the regulation of genes at the transcriptional and the translational levels (Kalogeraki & Winans, 1997). For the construction of LacZ transcriptional or translational strains, a 486 bp internal fragment (corresponding to positions 902–1387 bp) of *eseC* was amplified from PPD130/91 genomic DNA and introduced into the *XbaI/EcoRI*-digested sites of pVIK112 or pVIK111, respectively. The resulting plasmids were transferred from S17-1 *λpir* into wild-type *Ed. tarda* or the *ΔescA* mutant strain via conjugation and integrated into the chromosome by homologous recombination. The resulting strains (*eseC*::pVIK111, *eseC*::pVIK112, *ΔescA eseC*::pVIK111 and *ΔescA eseC*::pVIK112) were grown in DMEM for 24 h at 25 °C with 5% (v/v) CO₂. Bacterial cells were harvested and β-galactosidase activity was measured as described by Zheng *et al.* (2007). Each measurement was performed with triplicate samples.

Analysis of EseC stabilization. *Ed. tarda* wild-type and *ΔescA* strains were grown in DMEM at 25 °C with 5% (v/v) CO₂ to an OD₅₄₀ of 0.5. Chloramphenicol was then added to a final concentration of 200 µg ml⁻¹ to inhibit protein synthesis. Bacterial cultures (1.5 ml) were sampled at 15 min intervals from time 0 to 120 min. *Ed. tarda* cells were collected by centrifugation (12 000 g, 4 °C, 2 min), and the bacterial samples were boiled in the SDS-PAGE loading buffer (Fermentas) for 5 min and analysed by Western blotting using anti-EseC sera.

Co-immunoprecipitation of EscA and EseC. An experiment for co-immunoprecipitation of EscA and EseC was performed using the Seize primary immunoprecipitation kit (Pierce). A 200 µl volume of the beads was mixed with 100 µg of the purified anti-EscA antibody, and the mixture was incubated with gentle shaking at room temperature for 4 h. After washing, the mixture was incubated with

the ECPs or ICPs overnight at 4 °C with shaking. After washing the mixture three times with washing buffer, the complexes were recovered from the beads with elution buffer. The purified proteins were then analysed by SDS-PAGE followed by Western blotting with anti-EseC and anti-EscA sera.

Co-purification assay. The interactions of EscA-EseC and EscA-EseCA were determined by co-purification of the His₆-EscA + EseC and His₆-EscA + EseCA complexes in a Ni²⁺-NTA column. After induction with 1 mM IPTG, bacteria containing the corresponding recombinant plasmids were harvested and resuspended in NTM buffer [300 mM NaCl, 50 mM Tris/HCl (pH 8.0), 10 mM β-mercaptoethanol] supplemented with 20 mM imidazole before being lysed by sonication. Then the lysates were cleared by centrifugation and incubated at 4 °C for 1 h with 50 µl Ni²⁺-NTA agarose beads, with gentle mixing. The suspensions were successively transferred into a column and washed five times with NTM buffer supplemented with 30 mM imidazole. The bound proteins were eluted from the column with 50 µl NTM buffer supplemented with 200 mM imidazole.

Virulence of mutant *Ed. tarda* in fish. Healthy naïve blue gourami fish (*Trichogaster trichopterus* Pallas) were infected with *Ed. tarda* wild-type and mutant strains as previously described (Ling *et al.*, 2000). The mortality of the fish was recorded over a period of 7 days after intramuscular injection. The LD₅₀ values were calculated according to the method developed by Reed & Muench (1938).

RESULTS

Influence of EscA on the accumulation and secretion of EseC

Chaperones for the type III secretion translocon components and effectors often affect the secretion and/or stabilization of the target proteins (Page & Parsot, 2002). To ascertain the role of *escA*, a non-polar in-frame deletion mutant of *ΔescA* was constructed. The *ΔescA* mutant and the wild-type strain were grown in DMEM at 25 °C in a 5% (v/v) CO₂ incubator to induce the expression of the T3SS proteins. The supernatant of each strain was collected and analysed by 2D PAGE (Fig. 1C). Significantly, the *ΔescA* mutant was determined to have lost the EseC protein in the supernatant, yet still displayed the characteristic EseB and EseD protein spots.

ECPs containing the proteins secreted on the cell surface and into the culture medium were collected from the wild-type, *ΔescA* and *ΔescA* + *escA* strains. Samples representing the equivalent number of bacteria were loaded and analysed by Western blotting. As illustrated in Fig. 1(D), EscA was not detected in ECPs, and the secretion of EseC was greatly decreased (1.5 ± 0.5 relative units) (lane 2) in the *ΔescA* mutant compared to that in the wild-type strain (100 relative units) (lane 1). The phenotype of decreased secretion of EseC was due to the mutation of *escA*, as complementing the *ΔescA* mutant with *escA* restored the secretion of EseC (95.2 ± 3.0 relative units) (lane 3) to almost the wild-type level. As calculated, the wild-type strain produced about 136.6 relative units of EseC (100.0 in ECP and 36.6 ± 3.4 in ICP), but the *ΔescA* mutant produced about 11.2 relative units of EseC (1.5 ± 0.5 in

Fig. 2. Effect of EscA on the stabilization of EseC. *Ed. tarda* wild-type and $\Delta escA$ strains were grown in DMEM at 25 °C with 5% (v/v) CO₂ to an OD₅₄₀ of 0.5. Chloramphenicol (200 μ g ml⁻¹) was then added to the medium. Bacterial cultures were sampled at 15 min intervals and equal amounts of cells (OD₅₄₀ 0.5) were analysed by Western blotting using anti-EseC polyclonal antibody.

ECP and 9.7 ± 1.2 in ICP). Thus, only 13.4% of EseC was secreted in the absence of *escA*, while 73.2% of EseC was secreted in the wild-type strain. The accumulation and secretion of EseB and EseD were not obviously affected in the $\Delta escA$ mutant and were comparable to those of the wild-type strain and the complemented strain (Fig. 1D and data not shown). This finding suggested that *escA* affects the accumulation and secretion of EseC in *Ed. tarda*.

Influence of EscA on the stabilization of EseC in *Ed. tarda*

To determine whether the reduced amount of accumulation or secretion of EseC was due to the reduced stabilization of this protein in the absence of *escA*, the amount of EseC in the *Ed. tarda* culture was monitored after adding chloramphenicol to inhibit bacterial protein synthesis. As shown in Fig. 2, in the presence of chloramphenicol the amount of EseC in the $\Delta escA$ mutant gradually reduced over time, and was practically undetectable after 90 min. However, the amounts of EseC in the wild-type and complemented strains were not affected during the monitoring period. This result demonstrated that EscA may affect the stabilization of EseC.

Lack of influence of EscA on the transcription of *eseC*

Some chaperones reportedly affect the transcription and even the translation of their cognate substrates (Tucker & Galán, 2000; Darwin & Miller, 2001). Therefore, the effect of $\Delta escA$ on EseC could be partially explained by EscA's alteration of the transcription and/or translation of the *eseC* gene. To investigate this possibility, we examined the effect of a deletion mutation in *escA* on EseC at the transcription and translation levels. Following the method described in Methods, a 486 bp fragment of *eseC* (positions 902–1387 bp) was transferred into plasmids pVIK112 and pVIK111, creating β -galactosidase transcriptional and translational fusions, respectively. The resultant plasmids were then integrated into the chromosomes of *Ed. tarda* wild-type strain and $\Delta escA$ mutant, creating the transcriptional fusion strains *eseC*::pVIK112 and $\Delta escA$ *eseC*::pVIK112 and the translational fusion strains *eseC*::pVIK111 and $\Delta escA$ *eseC*::pVIK111, respectively.

The resultant strains, containing the intact N-terminus of EseC (462 residues), were selected by PCR. Cells of the fused strains were collected and used for measuring β -galactosidase activities. No significant difference in β -galactosidase activity was observed between *eseC*::pVIK112 (55 ± 12 Miller units) and $\Delta escA$ *eseC*::pVIK112 (58 ± 5 Miller units) ($P > 0.05$), suggesting that the deletion of *escA* did not affect the transcription of EseC-LacZ in the bacteria. In contrast, the β -galactosidase activity in $\Delta escA$ *eseC*::pVIK111 (6 ± 4 Miller units) was reduced approximately 5–10-fold compared to that in *eseC*::pVIK111 (48 ± 10 Miller units), demonstrating that *escA* affected the EseC-LacZ level in the bacteria ($P < 0.05$). Because the $\Delta escA$ *eseC*::pVIK111 strain detected contained the intact N-terminus of EseC, it is impossible that the decrease in β -galactosidase activity was due to the absence of the interaction region of EseC-EscA, which was located at residues 31–137 of EseC (see below). Furthermore, the results supported the conclusion that deletion of *escA* might affect the stabilization of EseC in the cytoplasm due to the fact that $\Delta escA$ did not affect the transcription of *eseC*. However, the possibility cannot be excluded that EscA could play a role in the translation of EseC.

Localization of EscA to the membrane and the cytoplasm

Based on the lack of any recognizable signal peptide sequence in EscA, it was hypothesized that EscA would be localized in the bacterial cytosol, like its homologues (Neyt & Cornelis, 1999). To examine the cellular location of EscA, protein fractions of supernatant, membrane (insoluble) and cytoplasm (soluble) from wild-type, $\Delta escA$ and complemented strains were analysed by Western blotting. As illustrated in Fig. 3, EscA was detected in both the soluble and insoluble fractions in the wild-type and complemented strains. However, it was not detected in the supernatant fractions or in the $\Delta escA$ background. DnaK, a cytosolic protein, was found only in the soluble fractions and was absent in the ECPs and insoluble fractions, indicating that the membrane fractions were not contaminated by the bacterial cytoplasm. These results suggested that EscA is not a secreted protein but a protein located in the cytoplasm and on the bacterial membrane.

Fig. 3. Localization of EscA within *Ed. tarda* cells. Wild-type, Δ escA and Δ escA+escA bacteria were cultured in DMEM at 25 °C with 5% (v/v) CO₂ for 24 h. The supernatant, insoluble (membrane proteins), and soluble (cytoplasm proteins) fractions were extracted and analysed by Western blotting using the anti-EscA polyclonal antibody. Anti-DnaK was used to confirm the effective fractionation.

However, it is possible that EscA formed small insoluble aggregates and was fractionated with the insoluble fractions, and was therefore detected in these fractions.

Association of EscA and EseC in *E. coli* and *Ed. tarda*

Chaperones often perform their function by associating specifically with their cognate substrates (Daniell *et al.*, 2001; Zheng *et al.*, 2007). We hypothesized that the influence of EscA on EseC is mediated via protein–protein interaction. To investigate the potential interaction between EscA and EseC, the pETDuet-1 co-expression system and the His₆-tag purification system were exploited in the sense that only proteins linked with His₆ were eluted from Ni²⁺-NTA agarose beads. A plasmid (pAC) that encodes both His₆-EscA and EseC was generated, and the proteins were produced simultaneously in *E. coli* BL21(DE3)/pLysS. To serve as controls, plasmids encoding His₆-EscA (pA1), His₆-EseC (pC1, with *eseC* in MCS1), and EseC (pC2, with *eseC* in MCS2) were constructed. After overproduction of these proteins in *E. coli*, clear extracts were mixed with Ni²⁺-NTA agarose beads and proteins adsorbed on the beads were analysed by Western blotting. The result (Fig. 4A) clearly indicated that EseC was co-purified with His₆-EscA (pAC). As negative controls, only EseC or EscA alone was detected on the Ni²⁺-NTA beads incubated with extracts from bacteria expressing His₆-EseC (pC1) or His₆-EscA (pA1), respectively. These results demonstrated that EseC and EscA specifically interact with each other *in vitro*.

Overproduction of EseC (which reacts with anti-EseC antibody) in *E. coli* produced multiple lower bands, while His₆-EseC (pC1) produced more bands than His₆-EscA-EseC (pAC), suggesting that the interaction between EscA and EseC may also be important for the stabilization of EseC *in vitro*. To investigate this effect in detail, the EseC patterns expressed in *E. coli* were analysed without

co-purification in the absence and in the presence of EscA. As shown in Fig. 4(B), the reaction between His₆-EseC (pC1) and anti-EseC resulted in a greater number of low bands than His₆-EscA-EseC (pAC), regardless of the ITPG induction time (1, 2, 3 or 4 h). Thus the data showed that the presence of EscA causes the EseC protein to be more stable in *E. coli*, supporting the finding that EscA is required for the stabilization of EseC.

The fractionation experiments which showed that EscA and EseC were both found in the cytoplasm of *Ed. tarda* suggested that the two proteins may interact with each other *in vivo*. To demonstrate such an interaction, experiments were conducted to co-immunoprecipitate this putative complex. Synthesis of T3SS proteins was induced in the wild-type, Δ escA and complemented strains. The cell pellets were collected and washed with *p*-xylene to eliminate secreted T3SS proteins that could be absorbed to the outer membrane. After sonication, the clear extracts were incubated with anti-EscA polyclonal antibody and the complex was recovered on Protein A-Sepharose beads. The purified proteins were analysed by Western blotting after washing and eluting. As shown in Fig. 4(C), EscA and EseC were recovered from the extracts of the wild-type and complemented strains, but not from the Δ escA mutant, indicating that cytoplasmic EseC is physically associated with EscA in bacterial cells. These results provided strong evidence that EscA and EseC are associated in *Ed. tarda*.

Localization of the binding region in EseC

Having determined that EscA and EseC interact with each other *in vitro* and *in vivo*, we attempted to identify the interaction site that contributes to the formation of the EscA-EseC complex. A series of eight in-frame deletion mutations spanning the entire *eseC* gene were constructed to determine a discrete binding site in EseC (Table 1). These deletions removed all of the characteristic regions of the protein, including two hypothetical transmembrane helices spanning residues 194–243 and 255–318, and two coiled-coils spanning residues 138–193 and 430–506. All of the truncated proteins were individually co-expressed with EscA in *E. coli* BL21(DE3)/pLysS. The bacterial lysate, not mixed with the Ni²⁺-NTA agarose beads, was used as the input fraction to confirm protein production in the co-expression system by Western blotting. As illustrated in Fig. 5(A), all of the proteins with the expected size were produced in the co-expression system. Subsequently, the clear extracts were mixed with Ni²⁺-NTA agarose beads, and proteins adsorbed on the beads were analysed by Western blotting. As shown in Fig. 5(B), truncated proteins expressed from mutations of *eseC*_{Δ1–30}, *eseC*_{Δ138–193}, *eseC*_{Δ194–243}, *eseC*_{Δ255–318}, *eseC*_{Δ319–402}, *eseC*_{Δ403–429} and *eseC*_{Δ430–506} were co-purified with EscA. However, protein expressed from the mutation of *eseC*_{Δ31–137} failed to be co-purified with EscA, implying that residues 31–137 of EseC (EseC_{31–137}) are likely to be involved in the binding site for EscA.

Fig. 4. Interaction between EscA and EseC. (A) Co-purification of EseC with EscA in *E. coli*. *E. coli* BL21(DE3)/pLysS bacteria harbouring plasmids encoding His₆-EscA (pA1), His₆-EseC (pC1), EseC (pC2) or His₆-EscA+EseC (pAC) were cultured in LB medium at 37 °C. Cultures induced with 1 mM IPTG were collected at 4 h and adjusted to OD₅₄₀ 0.5. The clear extracts of equal amounts of bacterial cells were incubated with Ni²⁺-NTA agarose beads and the proteins binding to the beads were analysed by Western blotting using anti-EscA and anti-EseC polyclonal antibodies. (B) Expression patterns of EseC in the presence and absence of EscA in *E. coli*. Cultures of *E. coli* BL21(DE3)/pLysS harbouring plasmids encoding His₆-EseC (pC1) or His₆-EscA+EseC (pAC) were collected 1, 2, 3 and 4 h after the addition of 1 mM IPTG. The clear extracts of equal amounts of bacterial cells (OD₅₄₀ 0.5) were analysed by Western blotting using anti-EseC polyclonal antibody. The lower bands detected with anti-EseC are degradation products of the hybrid proteins. (C) Co-immunoprecipitation of EseC with anti-EscA antibody. *Ed. tarda* wild-type, ΔescA, ΔescA+escA and ΔescC₃₁₋₁₃₇ strains were cultured in DMEM at 25 °C with 5 % (v/v) CO₂ for 24 h. Co-immunoprecipitates were prepared from ECPs (lanes 1–4) and the soluble ICPs (lanes 5–8) of each strain as described in Methods, and analysed by Western blotting using anti-EscA and anti-EseC polyclonal antibodies.

Interaction of EseC₃₁₋₁₃₇ with EscA in *Ed. tarda*

Because EseC₃₁₋₁₃₇ was demonstrated to be the binding region with EscA *in vitro*, experiments were conducted to determine whether this region interacts with EscA *in vivo*. *Ed. tarda* mutant ΔescC₃₁₋₁₃₇, with codons 31–137 of *escC* deleted, was constructed. Co-immunoprecipitation and Western blotting were performed with the cell pellets of the

wild-type strain and ΔescC₃₁₋₁₃₇. The results showed that the protein complex of EscA and the truncated EseC was not detected in the ICP of ΔescC₃₁₋₁₃₇ (Fig. 4C, lane 8), while the protein complex of EscA and EseC was detected in the wild-type and *escA* complemented strains (Fig. 4C, lanes 5 and 7). As a negative control, this protein complex was not detected in the ECPs of these bacteria. The failure of EseC_{Δ31-137} to

Fig. 5. Localization of the binding segment in EseC. (A) Expression analysis of His₆-EscA + EseCΔ. *E. coli* BL21(DE3)/pLysS strains harbouring plasmids encoding His₆-EscA + EseC (pAC), His₆-EscA + EseC_{Δ1-30} (pAC_{Δ1-30}), His₆-EscA + EseC_{Δ31-137} (pAC_{Δ31-137}), His₆-EscA + EseC_{Δ138-193} (pAC_{Δ138-193}), His₆-EscA + EseC_{Δ194-243} (pAC_{Δ194-243}), His₆-EscA + EseC_{Δ255-318} (pAC_{Δ255-318}), His₆-EscA + EseC_{Δ319-402} (pAC_{Δ319-402}), His₆-EscA + EseC_{Δ403-429} (pAC_{Δ403-429}) or His₆-EscA + EseC_{Δ430-506} (pAC_{Δ430-506}) were cultured in LB medium at 37 °C. Bacterial cultures induced by 1 mM IPTG were collected at 4 h. The clear extracts of equivalent amounts of bacterial cells (OD₅₄₀ 0.5) were analysed by Western blotting using anti-EscA and anti-EseC polyclonal antibodies. (B) Localization of the EseC binding segment with EscA. Upon confirmation of the production of recombinant EscA and EseC_Δ proteins in the co-expressed system in (A), the clear extracts in (A) were incubated with Ni²⁺-NTA agarose beads and the proteins binding to the beads were analysed by Western blotting using anti-EscA and anti-EseC polyclonal antibodies.

bind to EscA suggested that residues 31–137 in EseC are required for the interaction with EscA in *Ed. tarda*.

To investigate the effect of EseC_{Δ31-137} on the secretion of EseC in *Ed. tarda*, ECP and ICP from the induced culture of ΔeseC₃₁₋₁₃₇ were collected and analysed by Western blotting. As shown in Fig. 6, accumulation of the smaller EseC_{Δ31-137} protein was observed in the ICP (12.3 ± 1.2 relative units), but not in the ECP (0.0 relative units) of the ΔeseC₃₁₋₁₃₇ mutant. The amount of EseC_{Δ31-137} in the ICP was comparable to the amount of EseC (12.1 ± 1.0 relative units) in the ICP of the ΔescA mutant, both of which accounted for about 31% of the EseC (38.7 ± 2.6 relative units) in the ICP of the wild-type strain. This finding indicated that EseC₃₁₋₁₃₇ is required for the accumulation and secretion of EseC by interacting with EscA in *Ed. tarda*.

Contribution of EscA and EseC_{Δ31-137} to the virulence of *Ed. tarda*

EseC has been reported to contribute to the pathogenesis of *Ed. tarda* (Tan *et al.*, 2005). To further investigate if this

contribution is related to the normal secretion of EseC, the LD₅₀ values of the ΔescA and ΔeseC₃₁₋₁₃₇ mutants in blue gourami fish were examined. The deletion of escA increased the LD₅₀ value by about 1 log ($10^{6.1}$) compared to the wild-type strain ($10^{5.1}$), while the complemented strain restored the LD₅₀ value ($10^{5.3}$) to approximately the same level as that of the wild-type strain. Similarly, ΔeseC₃₁₋₁₃₇ also resulted in approximately 1 log increase in the LD₅₀ value ($10^{5.9}$). These results indicated that EscA and residues 31–137 of EseC contribute to the pathogenesis of *Ed. tarda*.

DISCUSSION

Previous studies have shown that *Ed. tarda* utilizes a T3SS for its pathogenesis (Tan *et al.*, 2005). EseB, EseC and EseD are T3SS-dependent secreted proteins and form a translocon complex after their secretion (Zheng *et al.*, 2007). EscC is a specific chaperone for EseB and EseD (Zheng *et al.*, 2007), while no chaperone for EseC has hitherto been identified. In this study, escA was demonstrated to function as a specific chaperone for EseC. EscA possesses the

Fig. 6. Effect of the EseC_{31–137} segment on the accumulation and secretion of EseC. *Ed. tarda* wild-type, Δ escA mutant, Δ escA+escA and Δ escC_{31–137} strains were cultured in DMEM at 25 °C with 5 % (v/v) CO₂ for 24 h. The ECPs and ICPs of each strain were extracted from equivalent amounts of cells (OD₅₄₀ 0.5) and analysed by Western blotting using anti-EscA and anti-EseC polyclonal antibodies. Relative units of EseC protein, as measured with the Fluor-S Multi-imager (Bio-Rad), were as follows: wild-type ECP, 100.0; Δ escA mutant ECP, 1.8 ± 0.4 ; Δ escA+escA ECP, 100.3 ± 2.1 ; Δ escC_{31–137} ECP, 0; wild-type ICP, 38.7 ± 2.6 ; Δ escA mutant ICP, 12.1 ± 1.0 ; Δ escA+escA ICP, 36.9 ± 1.9 ; and Δ escC_{31–137} ICP, 12.3 ± 1.2 .

common characteristics of a T3SS chaperone, including a low molecular mass (17.5 kDa), an acidic pI (4.79) and a proximate location of the encoding gene to that of its cognate substrate EseC. The data showed that EscA affects the accumulation and stability of cytoplasmic EseC, as well as its secretion into the culture medium, and the effect of EscA on EseC was demonstrated to be post-transcriptional. As an ICP, EscA interacts with EseC *in vitro* and *in vivo*, and a defined region of residues 31–137 in EseC (EseC_{31–137}) is necessary for this interaction. Moreover, EseC_{31–137} affects the accumulation and secretion of EseC in *Ed. tarda*. Finally, challenge experiments with blue gourami fish demonstrated that both EscA and EseC_{31–137} contribute to the virulence of *Ed. tarda*.

Mutation in EscA reduces the accumulation and secretion of EseC, but has a less severe effect than mutations in some EscA homologues such as SycD/LcrH in *Yersinia*, CesD and CesD2 in EPEC and SseA in *Salmonella*, which almost abolish the accumulation and secretion of their cognate substrates (Neyt & Cornelis, 1999; Waterman & Holden, 2003; Zurawski & Stein, 2003; Edqvist *et al.*, 2006). EspD in EPEC requires two chaperones (CesD and CesD2) for complete secretion and accumulation activities (Neves *et al.*, 2003; Waterman & Holden, 2003). The similarity of EseC to EspD leads to the hypothesis that more than one chaperone may exist for EseC. Moreover, even in the presence of EscA, degradation of EseC can be found in the input fractions of His₆-EscA+EseC (pAC) (Fig. 4B), implying that EscA does not completely protect EseC from degradation. A putative additional chaperone may exist in native *Ed. tarda* that is necessary for the stability of EseC. Because EseC is not secreted in the absence of *eseE* (Zheng

et al., 2007), EseE may act as a second chaperone. Further study is needed to clarify this hypothesis.

Unlike most T3SS chaperones localized in the cytoplasm, EscA was found both on the cytoplasmic membrane and in the cytoplasm. A limited number of NF-T3SS chaperones are reported to be membrane-associated, such as CesD and CesT from EPEC (Elliott *et al.*, 1999; Wainwright & Kaper, 1998), YscB from *Yersinia* (Jackson *et al.*, 1998), SseA from *Salmonella* (Zurawski & Stein, 2003) and EscC from *Ed. tarda* (Zheng *et al.*, 2007). This finding is reminiscent of the function of the F-T3SS chaperone. During F-T3SS protein export, the chaperone–substrate complexes dock at the membrane ATPase (Gauthier & Finlay, 2003; Thomas *et al.*, 2004), which facilitates the release of the chaperone from the cognate secreted substrate in an ATP-dependent manner (Akedo & Galán, 2005). After release by an escort mechanism in F-T3SS protein export, free chaperones can be cycled (Evans *et al.*, 2006). Thus, chaperone transition between cytosol and membrane compartments facilitates F-T3SS export of the flagellar component and, by extension, facilitates secretion of NF-T3SS proteins. This view is supported by previous research relating to EPEC T3SS, which indicated that the chaperone CesT and its export substrate Tir can interact independently or collectively with ATPase EscN (Gauthier & Finlay, 2003); CesT recruits Tir and they co-localize to the EPEC inner membrane (Thomas *et al.*, 2005). Therefore, membrane association may be a necessary step for the interaction of the secreted protein-chaperone complex with T3SS ATPase. These findings may help to explain the dual location of EscA.

The fact that EseC requires residues 31–137 for binding EscA and for its accumulation and secretion is consistent with studies showing that most translocon components associate with their cognate chaperones via the N-terminal regions (Harrington *et al.*, 2003). Transmembrane and coiled-coil domains are found at high frequency among T3SS structural and secreted components and have been shown to play important roles in the function of translocons and effectors (Daniell *et al.*, 2001; Delahay & Frankel, 2002). In this research, however, the two transmembrane helices (EseC_{194–243} and EseC_{255–318}) and the two coiled-coil regions (EseC_{138–193} and EseC_{430–506}) in EseC were found not to be involved in the interaction. This finding is not exclusive to EseC. Residues 56–99 of YopB, which contain no typical secondary structure, are responsible for binding the cognate chaperone SycD (Neyt & Cornelis, 1999). The mechanism of this interaction is not clear. However, by using domain linker prediction (Miyazaki *et al.*, 2002), two domain linkers were predicted in EseC, spanning residues 59–84 and 117–122 (data not shown), implying that regions between these two domain linkers possibly possess a functional secondary structure. Supporting this idea, DNASIS analysis confirmed that many short hydrophobic segments were found dispersedly at residues 44–49, 70–76, 80–83, 86–87, 91–92 and 100–101, which may be important with regard to protein–protein interactions (Jones & Thornton, 1995). Whether these

hydrophobic segments located within the N-terminus of EseC have effects on the EseC-EscA interaction is yet to be investigated. We also tried to seek a specific binding region in EscA using the same procedure, but such a linear region responsible for binding to EseC was not detected (data not shown). Further study of the structural mechanism of the EscA-EseC interaction is still needed.

The observation that mutation of *escA* or *eseC* increases the LD₅₀ value by approximately 1 log unit provides evidence that an intact translocon is essential for the function of the T3SS in the pathogenesis of *Ed. tarda*. An understanding of this system will provide greater insight into the virulence mechanism of this pathogen. Our results will also aid in the development of new approaches to combating edwardsiellosis. In a previous study, we reported that an attenuated strain mutated in a T3SS gene elicited good protection against edwardsiellosis in fish (Mo *et al.*, 2007). The findings in this study will be very useful in the future for the development of effective attenuated live vaccines against edwardsiellosis. Furthermore, it is unclear at this point whether there is an additional chaperone for EseC, and EscA's regulatory role in the function of T3SS is likewise not understood. The mutants constructed in this research will also be very helpful in future studies attempting to identify the additional chaperone(s) of EseC and the possible regulatory role of EscA in T3SS function.

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