EscC is a chaperone for the *Edwardsiella tarda* type III secretion system putative translocon components EseB and EseD

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Edwardsiella tarda is a Gram-negative enteric pathogen that causes disease in both humans and animals. Recently, a type III secretion system (T3SS) has been found to contribute to *Ed. tarda* pathogenesis. EseB, EseC and EseD were shown to be secreted by the T3SS and to be the major components of the extracellular proteins (ECPs). Based on sequence similarity, they have been proposed to function as the 'translocon' of the T3SS needle structure. In this study, it was shown that EseB, EseC and EseD formed a protein complex after secretion, which is consistent with their possible roles as translocon components. The secretion of EseB and EseD was dependent on EscC (previously named Orf2). EscC has the characteristics of a chaperone; it is a small protein (13 kDa), located next to the translocators in the T3SS gene cluster, and has a coiled-coil structure at the N-terminal region as predicted by COILS. An in-frame deletion of *escC* abolished the secretion of EseB and EseD, and complementation of Δ *escC* restored the export of EseB and EseD into the culture supernatant. Further studies showed that EscC is not a secreted protein and is located on the membrane and in the cytoplasm. Mutation of *escC* did not affect the transcription of *eseB* but reduced the amount of EseB as measured by using an EseB–LacZ fusion protein in *Ed. tarda*. Co-purification studies demonstrated that EscC formed complexes with EseB and EseD. The results suggest that EscC functions as a T3SS chaperone for the putative translocon components EseB and EseD in *Ed. tarda*.

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INTRODUCTION

Edwardsiella tarda is a Gram-negative bacillus of the family *Enterobacteriaceae*. It is a ubiquitous organism with a broad host range and wide geographical distribution. *Ed. tarda* is found in freshwater and marine environments and is known to colonize animals residing in these ecosystems (Plumb, 1993). In humans, *Ed. tarda* can cause gastro- and extra-intestinal infections, including myonecrosis (Slaven *et al.*, 2001), septic arthritis (Osiri *et al.*, 1997), bacteraemia (Yang & Wang, 1999), wound infections (Banks, 1992) and peritonitis (Clarridge *et al.*, 1980). *Ed. tarda* also causes haemorrhagic septicaemia in many farmed and feral fish and other aquatic animals, and leads to great losses in aquaculture industries (Thune *et al.*, 1993). Therefore, it is important to study the pathogenesis of *Ed. tarda* and find suitable strategies to prevent these diseases.

Many factors are reported to contribute to the pathogenesis of *Ed. tarda*. These include the abilities to adhere to, invade and replicate within epithelial cells (Janda *et al.*, 1991; Ling *et al.*, 2000) and fish tissues (Ling *et al.*, 2001) and to resist serum- (Janda *et al.*, 1991; Ling *et al.*, 2000) as well as phagocyte-mediated killing (Srinivasa Rao *et al.*, 2001). *Ed. tarda* also produces toxins and exoenzymes such as haemolysins (Hirono *et al.*, 1997), catalases (Srinivasa Rao *et al.*, 2003b) and dermatotoxins (Ullah & Arai, 1983) for disseminating infection. Recently, we have identified two important virulence mechanisms that are related to secretion systems, namely, a type III secretion system (T3SS) (Tan *et al.*, 2005) and a novel secretion system, EVP (*Ed. tarda* virulence protein) (Srinivasa Rao *et al.*, 2004). These two secretion systems cross-talk through a two-component system EsrA–EsrB and a regulator EsrC (Zheng *et al.*, 2005). Disruption of the T3SS or EVP gene cluster resulted in ~1–2 log increase in LD₅₀ in the blue gourami (*Trichogaster trichopterus* Pallas) host.

The T3SS is a contact-dependent protein secretion system and it is generally composed of about 20 proteins, including

Abbreviations: 2-DE, two-dimensional electrophoresis; ECP, extracellular protein; EPEC, enteropathogenic *Escherichia coli*; EVP, *Edwardsiella tarda* virulence protein; GST, glutathione S-transferase; T3SS, type III secretion system.

apparatus, effectors, regulators and chaperones (Hueck, 1998). The translocon proteins of the apparatus form a needle structure 2.8 nm in diameter to inject effector proteins directly into eukaryotic cells (Marlovits *et al.*, 2004), where the normal cellular functions are subverted for the benefit of the bacteria. In *Ed. tarda*, a T3SS was identified by using a combination of genomics and proteomics approaches (Tan *et al.*, 2002; Srinivasa Rao *et al.*, 2003a; Tan *et al.*, 2005). The T3SS of *Ed. tarda* PPD130/91 contains 35 open reading frames, which include *Ed. tarda* secretion system apparatus (*esa*), chaperones (*esc*), effectors (*ese*), regulators (*esr*) and some unknown genes (*orf*) (Tan *et al.*, 2005). By using two-dimensional electrophoresis (2-DE), EseB, EseC and EseD were identified as the major components of the extracellular proteins (ECPs) and their secretion was shown to be T3SS-dependent. 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). Sequence analysis showed that EseB, EseC and EseD are homologous to EspA, EspD and EspB, respectively, of enteropathogenic *Escherichia coli* (EPEC) (Tan *et al.*, 2005). EspA forms a sheath-like structure, and EspB and EspD form a translocon pore in EPEC. EspA, EspB and EspD together constitute a molecular syringe and channel effector proteins into the host cells (Ide *et al.*, 2001). In addition, the homologues of EseB, EseC and EseD in *Salmonella* (SseB, SseC and SseD, respectively) have also been shown to function as translocon components, and to be essential for translocation of the effectors (Nikolaus *et al.*, 2001). The sequence similarities of EseB, EseC and EseD to their homologues in EPEC and *Salmonella* suggest that these three Ese proteins may have similar functions, forming a translocon complex to facilitate the translocation of effectors.

Many of the T3SS secreted proteins are dependent on specific chaperones for secretion as well as stabilization in the bacterial cytosol prior to secretion. The binding of a chaperone with the secreted protein can prevent premature interaction of a secreted protein with other secreted protein(s) and/or with parts of the secretion and translocation machinery (Wattiau *et al.*, 1996; Bennett & Hughes, 2000). In addition, chaperones were shown to be required for the regulation of genes in T3SS (Darwin & Miller, 2000, 2001) and conferred secretion pathway specificity to their cognate secreted proteins (Lee & Galan, 2004). In *Ed. tarda*, EscA and EscB have been proposed to be the chaperones for T3SS proteins (Tan *et al.*, 2005). However, the chaperones for the secreted proteins EseB, EseC and EseD have not been characterized. In the present study, we first determined that EseB, EseC and EseD formed a complex after their secretion, which is consistent with their possible roles as translocon components. We also found that Orf2 is a chaperone of EseB and EseD, and thus it was renamed EscC.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *Ed. tarda* strains were

grown in tryptic soy broth (TSB) or Dulbecco's modified Eagle medium (DMEM) at 25 °C without shaking, while *E. coli* strains were cultured in Luria broth (LB) at 37 °C. Cultivation of bacteria in DMEM was carried out in a 5% (v/v) CO₂ atmosphere. The appropriate antibiotics were added when necessary at the following concentrations: ampicillin (Amp, 100 µg ml⁻¹); kanamycin (Km, 50 µg ml⁻¹); colistin (Col, 12.5 µg ml⁻¹); chloramphenicol (Cm, 30 µg ml⁻¹).

Construction of deletion mutants and plasmids. Overlap extension PCR (Ho *et al.*, 1989) was used to generate in-frame deletion of *orf2* (*escC*) and *eseE* on the *Ed. tarda* wild-type PPD130/91 chromosome as described previously (Zheng *et al.*, 2005). For the construction of Δ *escC*, two PCR fragments were generated from PPD130/91 genomic DNA with the primer pairs *escC*-for (5'-AGGTACCGTTAGTCCTCGGCTGGTGCT-3') plus *escC*-int-rev (5'-ATGGATGGCGTGTGGCT-3') and *escC*-int-for (5'-GCCAAC-ACGCCATCCATTTCATCTAACTCCATCCCTC-3') plus *escC*-rev (5'-TGGTACCGGCGTAGTCCTCTGCGTCG-3'). The resulting products were a 767 bp fragment, containing the upstream region of *escC*, and a 791 bp fragment, containing the downstream region of *escC*, respectively. A 17 bp overlap in the sequences (underlined) permitted amplification of a 1558 bp product during a second PCR with primers *escC*-for and *escC*-rev, both of which introduced a *KpnI* restriction site (bold). The resulting PCR product, containing a deletion from nucleotides 22 to 339 of *escC*, was ligated into suicide vector pRE112 (Cm^r) (Edwards *et al.*, 1998) and transformed into SM10 λ pir. The single crossover mutants were obtained by conjugal transfer of the resulting plasmid into *Ed. tarda* PPD130/91. Deletion mutants were screened on 10% sucrose TSA plates. The construction of the *eseE* deletion mutant followed the same protocol. The primers used for Δ *eseE* were *eseE*-for (5'-TGGTACCGCAGTTCATGCTGGAGGA-3') plus *eseE*-int-rev (5'-GTCATCCATCGTGCTCC-3') and *eseE*-int-for (5'-GAGGCACGATGGATGACCTGTGGCTGCGCTATTGCTGC-3') plus *eseE*-rev (5'-AGGTACCGGCTCCTTAGGGTTGATT-3').

For the construction of LacZ transcriptional or translational strains, the *eseB* internal fragment was amplified with the forward primer *eseB*-int-for (5'-GAATTCCATGACGCTATTCACCGAT-3') and the reverse primer *eseB*-int-rev (5'-TTCTAGACGGATATTCTGGGCG-ATGG-3') from PPD130/91 genomic DNA. The PCR product was digested with *XbaI* and *EcoRI* and ligated into the same sites within pVIK112 or pVIK111. The resulting plasmids were then transferred from S17- λ pir into *Ed. tarda* by conjugation and the LacZ transcriptional or translational strains were selected for kanamycin and colistin resistance.

To generate GST hybrid proteins, *eseB*, *eseC* and *eseD* were amplified from PPD130/91 chromosomal DNA and ligated into the pGEX-4T-1 vector. The plasmid expressing the His-tagged EseB protein was constructed by ligating the *eseB* gene into the pET32a vector.

For the construction of pSAescC-FLAG, the complete *escC* gene was amplified from PPD130/91 genomic DNA. The PCR product was digested with *Sall* and *EcoRI* and ligated into the same sites within the pSA10 plasmid (Schlosser-Silverman *et al.*, 2000).

To generate co-expression plasmids pETB-D and pETD-D, *eseB* and *eseD*, respectively, were amplified from the *Ed. tarda* PPD130/91 chromosome and cloned into *BamHI* and *HindIII* (*eseB*) or *BamHI* and *EcoRI* (*eseD*) sites in pETDuet-1 (Novagen). The resulting plasmids were then digested with *NdeI* and *XhoI*, and *escC* PCR product amplified from the *Ed. tarda* wild-type chromosome and digested with the same enzymes was introduced into the constructs.

Production of recombinant EseB, EseC and EseD and generation of polyclonal antibodies. The recombinant proteins GST-EseB, GST-EseC and GST-EseD were expressed overnight at 25 °C

Table 1. Strains and plasmids used for this study

Strain or plasmid	Description	Reference or source
<i>Ed. tarda</i>		
PPD130/91	Wild-type, Km ^s , Col ^r , Amp ^s , LD ₅₀ =10 ^{5.0}	Ling <i>et al.</i> (2000)
Δ <i>eseE</i>	PPD130/91, in-frame deletion of <i>eseE</i>	This study
Δ <i>escC</i>	PPD130/91, in-frame deletion of <i>escC</i>	This study
Δ <i>escC</i> + <i>escC</i>	Δ <i>escC</i> with pSAescC-FLAG	This study
Δ <i>esrC</i>	PPD130/91, in-frame deletion of <i>esrC</i>	Zheng <i>et al.</i> (2005)
Δ <i>eseB</i>	PPD 130/91, in-frame deletion of <i>eseB</i>	This study
<i>eseB</i>	<i>eseB</i> : : pFS- <i>eseB</i> (Km ^r)	Tan <i>et al.</i> (2005)
<i>eseD</i>	<i>eseD</i> : : pFS- <i>eseD</i> (Km ^r)	Tan <i>et al.</i> (2005)
<i>eseB</i> : : pVIK212	PPD130/91; <i>eseB</i> – <i>lacZ</i> transcriptional fusion	This study
Δ <i>escC</i> <i>eseB</i> : : pVIK212	Δ <i>escC</i> ; <i>eseB</i> – <i>lacZ</i> transcriptional fusion	This study
Δ <i>esrC</i> <i>eseB</i> : : pVIK212	Δ <i>esrC</i> ; <i>eseB</i> – <i>lacZ</i> transcriptional fusion	This study
<i>eseB</i> : : pVIK211	PPD130/91; in-frame <i>eseB</i> – <i>lacZ</i> translational fusion	This study
Δ <i>escC</i> <i>eseB</i> : : pVIK211	Δ <i>escC</i> ; in-frame <i>eseB</i> – <i>lacZ</i> translational fusion	This study
Δ <i>esrC</i> <i>eseB</i> : : pVIK211	Δ <i>esrC</i> ; in-frame <i>eseB</i> – <i>lacZ</i> translational fusion	This study
<i>E. coli</i>		
JM109	Km ^s , Col ^s , Cm ^s	Promega
MC1061 (λ <i>pir</i>)	<i>thi thr-1 leu-6 proA2 his-4 argE2 lacY1 galK2 ara-14 xyl-5 supE44 pir</i>	Rubirés <i>et al.</i> (1997)
SM10 (λ <i>pir</i>)	<i>thi thr leu tonA lacY supE recA</i> -RP4 : 2-Tc : Mu Km ^r <i>pir</i>	Rubirés <i>et al.</i> (1997)
S17-1 (λ <i>pir</i>)	Tp ^r Sm ^r <i>recA thi pro hsdR</i> [−] M ⁺ RP4 : 2-Tc : Mu : Km Tn7 λ <i>pir</i>	Simon <i>et al.</i> (1983)
BL21(DE3)/pLysS	F [−] <i>ompT hsdS_B</i> (r _{β} [−] m _{β} [−]) <i>dcm gal</i> (DE3) <i>tonA</i> pLysS (Cm ^R)	Stratagene
Plasmids		
pGEM-T Easy	Amp ^r	Promega
pRE112	pGP704 suicide plasmid, <i>pir</i> dependent, Cm ^r , <i>oriT oriV sacB</i>	Edwards <i>et al.</i> (1998)
pSA10	pKK177-3 derivative, <i>lacI</i> , Amp ^r	Schlosser-Silverman <i>et al.</i> (2000)
pSAescC-FLAG	pSA10 with wild-type <i>escC</i> plus FLAG tag	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)
pVIK211	pVIK111, with <i>eseB</i> internal fragment	This study
pVIK212	pVIK112, with <i>eseB</i> internal fragment	This study
pETduet-1	Amp ^r	Novagen
pETB-D	pETDuet-1 with full-length <i>eseB</i> fused to 6His at the N-terminal and <i>escC</i>	This study
pETD-D	pETDuet-1 with full-length <i>eseD</i> fused to 6His at the N-terminal and <i>escC</i>	This study
pETescC	pETDuet-1 with <i>escC</i>	This study
pEThis- <i>eseB</i>	pET32a with full-length <i>eseB</i> fused to 6His at the N-terminal	This study

and purified by affinity chromatography with Sepharose 4B-glutathione resin under conditions recommended by the manufacturer (Amersham Biosciences). The purified GST-EseB, GST-EseC and GST-EseD proteins were used to immunize New Zealand White rabbits. For co-immunoprecipitation, the anti-EseB antibody in the immune serum was purified by affinity adsorption onto a nitrocellulose membrane blotted with the His-tagged EseB fusion protein. Briefly, His-tagged EseB protein was expressed from the pET32a expression vector and purified with Ni-NTA resin. The purified His-tagged EseB was then blotted onto the nitrocellulose membrane and blocked with 5% milk. The membrane was then incubated with the anti-EseB serum. The anti-EseB antibody adsorbed on the membrane was eluted with 100 mM glycine (pH 2.0). The eluate was centrifuged briefly and neutralized with 0.1 vol. 1.5 M Tris (pH 8.0). The Tris buffer was replaced with PBS by spinning through an Amicon column (Millipore).

SDS-PAGE, 2-DE and Western analysis of proteins. Protein samples were prepared as described previously (Srinivasa Rao *et al.*,

2004). Proteins were separated on 12% SDS-polyacrylamide gels. For Western analysis, proteins were transferred to PVDF membrane with a semi-dry system and examined by using the SuperSignal WestPico Chemiluminescent substrate under conditions recommended by the manufacturer (Pierce). EseB, EseC and EseD were detected by the addition of diluted anti-EseB (1 : 10 000), anti-EseC (1 : 10 000) and anti-EseD (1 : 5000) polyclonal antisera, respectively, followed by a 1 : 5000 dilution of mouse anti-rabbit IgG HRP (Santa Cruz Biotechnology). For detection of EscC-FLAG, the anti-FLAG M2 monoclonal antibody (Sigma) was used at 1 : 2500. Protein isolation and 2-DE were performed as previously described (Srinivasa Rao *et al.*, 2004). Proteins were identified by MALDI-TOF MS as described elsewhere (Zheng *et al.*, 2005). Anti-DnaK monoclonal antibody (Stressgen) was used at 1 : 2000, and anti-EvpA1 (J. Zheng & K. Y. Leung, unpublished data) was used at 1 : 5000.

Co-immunoprecipitation of EseB, EseC and EseD. ECPs from *Ed. tarda* PPD130/91 were prepared and quantified as described previously to obtain high concentrations (Tan *et al.*, 2002). The Seize

Primary Immunoprecipitation kit (Pierce) was used for the co-immunoprecipitation experiment. Generally, 200 μ l of slurry and 100 μ g of purified anti-EseB antibody were mixed and incubated at room temperature for 4 h with gentle shaking. After washing the mixture according to the product manual, a 50 μ g freshly prepared ECP sample was loaded. The mixtures were incubated overnight at 4 °C with shaking. The beads were eluted with elution buffer and the eluted sample was subjected to SDS-PAGE, followed by Western analysis with the anti-EseB, anti-EseC or anti-EseD antibody.

Fractionation of bacterial cells. Bacteria were fractionated as described by Neyt & Cornelis (1999). *Ed. tarda* cultured in DMEM was harvested by 10 min centrifugation at 5000 g at 4 °C. The ECPs were prepared by precipitation of the supernatant with TCA as described previously (Srinivasa Rao *et al.*, 2004). The bacterial pellet was resuspended in cold 10 mM Tris/HCl (pH 8.0), 5 mM MgCl₂, then sonicated. The intact cells were pelleted by centrifugation at 10 000 g for 5 min. The supernatant was then centrifuged for 30 min at 100 000 g to separate the membrane fraction (insoluble fraction, pellet) from the cytosolic fraction (soluble fraction). The membrane fraction (pellet) was washed once with cold 10 mM Tris/HCl (pH 8.0), 5 mM MgCl₂ and dissolved in an appropriate volume of ReadyPrep reagent 3 (Bio-Rad) for SDS-PAGE.

β -Galactosidase assays. *Ed. tarda* strains were grown in DMEM for 24 h at 25 °C with 5% (v/v) CO₂. β -Galactosidase activities were determined with cells permeabilized with SDS and chloroform as described by Miller (1972).

Co-purification assay. The ability of a Ni²⁺-NTA column (Qiagen) to bind 6His-EseB-EseC and 6His-EseD-EseC complexes was determined according to the product manual with slight modifications. Briefly, 6His-EseB and EseC, and 6His-EseD and EseC were expressed from plasmids pETB-D and pETD-D, respectively, by induction with 0.5 mM IPTG. The bacterial pellets were resuspended in buffer containing 300 mM NaCl, 50 mM Tris (pH 8.0) and 10 mM β -mercaptoethanol supplemented with 20 mM imidazole, and lysed by sonication. The lysates were cleared by centrifugation and incubated at 4 °C for 1 h with 50 μ l Ni²⁺-NTA resin (Qiagen) with gentle mixing. The suspensions were then transferred into a column, followed by five washes with buffer containing 300 mM NaCl, 50 mM Tris (pH 8.0) and 10 mM β -mercaptoethanol supplemented with 30 mM imidazole. The bound proteins from the column were eluted with 50 μ l buffer containing 300 mM NaCl, 50 mM Tris (pH 8.0) and 10 mM β -mercaptoethanol supplemented with 200 mM imidazole.

RESULTS

EseB, EseC and EseD form a protein complex after secretion

EseB, EseC and EseD are secreted via a T3SS of *Ed. tarda*, and are homologous to the translocon components of other bacteria (Tan *et al.*, 2005), suggesting that they may have similar functions in *Ed. tarda*. In several Gram-negative pathogens, translocon components of T3SS have been shown to form homo- and hetero-oligomers after their secretion (Hartland *et al.*, 2000; Büttner & Bonas, 2002). Bioinformatics analysis with COILS (http://www.ch.embnet.org/software/COILS_form.html) revealed the coiled-coil regions in EseB, EseC and EseD, which are implicated in the mediation of homo- or hetero-oligomeric protein-protein interactions (Lupas, 1996; Pallen *et al.*, 1997). To test whether these three proteins are interacting with one another, co-immunoprecipitations were performed. The ECPs from *Ed. tarda* PPD130/91 were harvested and the anti-EseB antibody was used to co-immunoprecipitate the protein complex. The Δ *eseB* mutant was used as a negative control. The flow-throughs and the immunoprecipitates were then subjected to Western blot analysis. As shown in Fig. 1, EseB, EseC and EseD, and EseC and EseD were detected in the flow-throughs of the wild-type ECPs and the Δ *eseB* ECPs, respectively (lanes 1 and 2). However, the three Ese proteins (EseB, EseC and EseD) were only detected in the immunoprecipitate of the wild-type ECPs and not in the immunoprecipitate of the Δ *eseB* ECPs (lanes 3 and 4). These results indicated that EseC and EseD specifically interact with EseB, and these three Ese proteins form a protein complex. Furthermore, a control using anti-GFP rabbit antibody did not co-immunoprecipitate any EseB, EseC or EseD proteins, suggesting that the immunoprecipitation was specific (data not shown). Therefore, our bioinformatics analysis and co-immunoprecipitation results suggested that EseB, EseC and EseD form a protein complex after secretion, which is consistent with their putative functions as translocon components.

Fig. 1. Co-immunoprecipitation of EseB, EseC and EseD with the anti-EseB antibody. The flow-throughs and immunoprecipitates were separated by SDS-PAGE and examined independently with the anti-EseB (a), anti-EseC (b) and anti-EseD (c) sera, respectively. For each Western blot: lane 1, flow-through from the immunoprecipitation of the *Ed. tarda* wild-type ECPs; lane 2, flow-through from the immunoprecipitation of the Δ *eseB* ECPs; lane 3, immunoprecipitate of the wild-type ECPs; lane 4, immunoprecipitate of the Δ *eseB* ECPs; lane 5, PBS control.

The secretion of EseB and EseD is dependent on Orf2

The secretion and the stabilization of the T3SS effectors or translocon components generally require chaperones. T3SS chaperone genes are often located near genes encoding their target proteins. In an effort to characterize the chaperone for the putative translocon proteins EseB and EseD, we examined several candidate genes that were encoded in the vicinity of and inside the T3SS gene cluster of *Ed. tarda* PPD130/91. These include *EseA*, *EseB* and *EseE* (Fig. 2a). Three deletion mutants were constructed and autoaggregation of overnight *Ed. tarda* cultures in DMEM was used as a quick assay to confirm the secretion of EseB. Our previous results suggested that extracellular EseB is required for the autoaggregation phenotype (Tan *et al.*, 2005). Autoaggregation was found for mutants Δ *escA* and Δ *escB* (Z. L. Mo & K. Y. Leung, unpublished data) as well as Δ *eseE*, which suggests that EseB is secreted in these mutants. We also analysed Orf2, which is located immediately upstream of *eseB* (Fig. 2a). A homology search of Orf2 against the NCBI database did not retrieve any characterized homologues. However, the close proximity of *orf2* and *eseB* suggests that Orf2 may be the chaperone for EseB. To investigate the role of *orf2*, an in-frame deletion mutant Δ *orf2* was constructed. The resulting mutant lacked an internal fragment of 318 bp, and

contained the first 21 bp and the last 6 bp of the *orf2* gene. The Δ *orf2* mutant did not show an autoaggregation phenotype after 24 h of culture in DMEM. The results of 2-DE revealed that EseB and EseD but not EseC were absent in the ECPs of the Δ *orf2* mutant (Fig. 2b, d). Complementation of the Δ *orf2* mutant *in trans* with pSA*orf2*-FLAG restored the secretion of EseB and EseD in the supernatant and the autoaggregation phenotype. These results suggested that Orf2 is essential for the secretion of EseB and EseD. In contrast, EseC but not EseB and EseD was absent from the ECPs in the deletion mutant Δ *eseE* (Fig. 2c), indicating that EseE is essential for the secretion of EseC.

Orf2 has features of a chaperone and is not secreted into the culture

The typical features of T3SS chaperones include low molecular mass and a structure rich in α -helices (Bennett & Hughes, 2000; Cornelis & Van Gijsegem, 2000), and these characteristics were observed in Orf2. The predicted molecular mass of Orf2 is 13 kDa, which is within the 10–15 kDa range of most known T3SS chaperones. It has an α -helical structure, and a stretch of N-terminal sequence with strong characteristics of coiled-coils motifs. Thus, we renamed *orf2* as *escC* (*Ed. tarda* secretion system chaperone C). However, the predicted pI of EscC is 7.3, which is near

Fig. 2. (a). Schematic representation of *eseBCD* region of *Ed. tarda*. Arrows represent each of the open reading frames. Secreted proteins or putative effectors (*ese*) of the T3SS are indicated by hatched arrows. Genes encoding apparatus (*esa*), chaperones (*esc*) and hypothetical proteins (*orf*) are represented by checked, black and open arrows, respectively. *orf2* was renamed *escC*. (b–d). Proteome analysis of *Ed. tarda* PPD130/91 (b), Δ *eseE* (c) and Δ *escC* (d) mutants. ECPs in DMEM were separated on Immobiline DryStrips (pH 3–10) combined with 2-DE analysis (12.5% polyacrylamide). ECPs were adjusted according to the relative secreted protein amount of each strain: wild-type (10 μ g), Δ *eseE* (5 μ g), Δ *orf2* (5 μ g). Gels were silver stained.

neutral and differs from the predicted acidic pI of most identified type III cytosolic chaperones as well as the basic pI of chaperone SseA from *Salmonella* SPI-2 T3SS (Zurawski & Stein, 2003).

To localize EscC in *Ed. tarda*, the Δ escC mutant was complemented with pSAescC-FLAG and the secreted proteins were collected. After sonication, the soluble and insoluble fractions of *Ed. tarda* were separated by centrifugation. The three fractions were then analysed by Western blotting with an anti-FLAG antibody. As shown in Fig. 3, EscC-FLAG was found in both the soluble (bacterial cytoplasm) and the insoluble fractions (bacterial membranes) but not in the ECPs. In contrast, no EscC-FLAG was detected in the Δ escC background (Fig. 3). DnaK, a cytosolic protein, was found only in the soluble fractions and not in the ECPs or in the insoluble fractions (Fig. 3), indicating that the membrane fraction was not contaminated by the bacterial cytoplasm. Taken together, these results suggest that EscC is not a secreted protein but a protein located in the cytoplasm or on the bacterial membrane. However, we could not eliminate the possibility that EscC formed small insoluble aggregates and thus was detected in the insoluble fractions.

EscC is required for the accumulation of both EseB and EseD in the cytoplasm

To investigate if EscC is required for the accumulation of EseB and EseD in the cytoplasm of *Ed. tarda*, the total proteins of the wild-type, the Δ escC mutant and its complemented strain were separated by SDS-PAGE and immunoblotted with either anti-EseB or anti-EseD antibody. Samples were loaded to represent the same number of bacteria for each strain. As shown in Fig. 4(a), the amounts of EseB and EseD were reduced in the Δ escC background. After pSAescC-FLAG was introduced into the Δ escC mutant, EseB and EseD were restored to nearly the same levels as in the wild-type (Fig. 4a). However, the

Fig. 3. Localization of EscC in *Ed. tarda*: Western analysis with anti-FLAG M2 monoclonal antibody of the secreted proteins, insoluble fraction and soluble fraction of Δ escC and its complemented strain. Anti-DnaK serum was used to confirm effective fractionation.

Fig. 4. EscC affects the accumulation of EseB and EseD. (a) Western analysis of total bacterial proteins from the same number of *Ed. tarda* PPD130/91 wild-type and its mutants. The Western blots were probed with anti-EseB or anti-EseD polyclonal antibody, and the first lane in each blot is of the total bacterial proteins from an *eseB* or *eseD* mutant, respectively, as a negative control. Anti-EvpA1 was used to demonstrate that the same amounts of bacterial proteins were loaded. (b) Proteome analysis of the total bacterial proteins from *Ed. tarda* PPD130/91 wild-type (A) and the Δ escC mutant (B) grown in DMEM culture at 25 °C. The total bacterial proteins were separated on an Immobiline DryStrip (pH 3–10) combined with 2-DE analysis (12.5% polyacrylamide). Gels were silver stained. Identical amounts of protein were loaded, and corresponding sections of representative gels are shown. The identities of EseB and EseD were confirmed with MALDI-TOF MS.

amount of EvpA1, a non-T3SS secreted protein used as a control, was nearly the same in the different strains (Fig. 4a). Taken together, these results suggest that EscC is required for the accumulation of EseB and EseD. We also used 2-DE to analyse the total bacterial proteins of the wild-type and the Δ escC mutant, and the results further confirmed the reduction of EseB and EseD in Δ escC (Fig. 4b). Thus, EscC is required for the accumulation and stabilization of EseB and EseD in *Ed. tarda*.

EscC is not required for the transcription of *eseB*

Chaperones generally function in the stabilization of their target proteins. However, some chaperones also have regulatory functions, such as SicA of *Salmonella enterica* serovar Typhimurium (Darwin & Miller, 2000, 2001). The effect of $\Delta escC$ on EseB and EseD accumulation could also be explained if $\Delta escC$ affected their transcription. To address whether or not the decrease of EseB and EseD accumulation was due to decreased transcription, we examined the effect of a deletion mutation in *escC* on EseB at the transcription and translation levels. pVIK112 is a transcriptional fusion suicide plasmid carrying *lacZY* and has been used to study the regulation of genes at the transcriptional level (Kalogeraki & Winans, 1997). We cloned a 462 bp fragment of *eseB* and generated the plasmid pVIK212, in which *eseB* was in transcriptional fusion with *lacZY*. The plasmid was integrated by conjugation into the chromosomes of *Ed. tarda* wild-type PPD130/91, $\Delta escC$ and $\Delta esrC$ mutants, creating the strains *eseB* : pVIK212, $\Delta escC$ *eseB* : pVIK212 and $\Delta esrC$ *eseB* : pVIK212, respectively. The β -galactosidase activity was then examined. Strong β -galactosidase activity was detected in *eseB* : pVIK212 (172 ± 9.8 Miller units; $n=3$), and nearly the same β -galactosidase activity was found in $\Delta escC$ *eseB* : pVIK212 (185 ± 11 Miller units; $n=3$). However, the β -galactosidase activity decreased significantly ($P<0.01$) in $\Delta esrC$ *eseB* : pVIK212 (24 ± 1.0 Miller units; $n=3$). These results further confirm the role of *esrC* as a transcriptional regulator (Zheng *et al.*, 2005) and suggest that the deletion of *escC* does not affect the transcription of *eseB*. Thus, EscC is not a transcriptional regulator like EsrC. In order to further address the role of *escC* in the stabilization of EseB and EseD, pVIK111, which is a translational fusion suicide plasmid carrying *lacZY* (Kalogeraki & Winans, 1997), was used to construct the translational fusion of *eseB* with *lacZY*. The internal fragment *eseB* was cloned into pVIK111 and generated the plasmid pVIK211. The resulting plasmid was integrated by conjugation into the chromosome of *Ed. tarda* wild-type PPD130/91, $\Delta escC$ and $\Delta esrC$ mutants, creating the strains *eseB* : pVIK211, $\Delta escC$ *eseB* : pVIK211 and $\Delta esrC$ *eseB* : pVIK211. The β -galactosidase activity in these strains was examined. As expected, mutation of the transcriptional regulator *esrC* decreased the level of EseB–LacZ fusion proteins in the bacteria and the β -galactosidase activity in $\Delta esrC$ *eseB* : pVIK211 (437 ± 10 Miller units; $n=3$) was reduced more than sixfold compared to that in *eseB* : pVIK211 (3075 ± 124 Miller units; $n=3$). A decreased level of β -galactosidase activity in $\Delta escC$ *eseB* : pVIK211 (2183 ± 300 Miller units; $n=3$) was also observed, demonstrating that mutation of *escC* affected the EseB–LacZ level in the bacteria. These results suggest that deletion of *escC* affected the stability of EseB in the cytoplasm, as $\Delta escC$ did not affect the transcription of *eseB*. Taken together, these results suggest that EscC is not required for the transcription of *eseB* but is important for the accumulation of EseB in the cytoplasm. However, we

still can not rule out the possibility that EscC could have a role in the translation of EseB.

EscC interacts with both EseB and EseD

To determine the interaction between EscC and EseB, we co-expressed EseB and EscC in plasmid pETDuet-1. The resulting plasmid produced EseB with a 6His tag at the N-terminus (6His–EseB) and EscC in *E. coli*. Lysates of *E. coli* expressing 6His–EseB and EscC were incubated with a Ni^{2+} -NTA resin column. The binding substrates were eluted and examined on a Coomassie blue stained SDS-PAGE gel. As shown in Fig. 5(a), co-expression of 6His–EseB with EscC in *E. coli* led to two proteins being retained on the column and they could be eluted together by using a high concentration (200 mM) of imidazole. The molecular masses of these two proteins on the SDS-PAGE corresponded to those of 6His–EseB and EscC, respectively, and the identity of the protein corresponding to EscC was further confirmed by MALDI-TOF MS. However, when lysate of *E. coli* expressing 6His–EseB alone was applied to the column, the band corresponding to EscC was not observed from the eluted sample (Fig. 5a). In another control experiment, EscC was not retained on a Ni^{2+} -NTA column when lysates of *E. coli* expressing EscC only were used (Fig. 5a). Thus, our results suggest that EscC specifically interacts with EseB.

The interaction of EseD and EscC was also examined by using a similar assay. As shown in Fig. 5(b), EscC co-purified with 6His–EseD when lysates of *E. coli* expressing 6His–EseD together with EscC were incubated in a Ni^{2+} -NTA resin column (Fig. 5b). As a control, EscC alone could

Fig. 5. Association between 6His–EseB or 6His–EseD and EscC. SDS-PAGE analysis (Coomassie blue staining) of the proteins bound to Ni^{2+} -NTA resin incubated with cleared extracts from *E. coli* BL21(DE3)/pLysS expressing (a) 6His–EseB plus EscC, 6His–EseB and EscC, respectively, or (b) 6His–EseD plus EscC, 6His–EseD and EscC, respectively.

not bind to Ni-NTA resin and flowed through the column (Fig. 5b), suggesting that EseD specifically interacts with EscC. The co-purification of EseB or EseD with EscC strongly supports the direct association of EseB or EseD with EscC, which is consistent with the chaperone function of EseBD.

EscC is required for virulence

The contribution of EscC to the pathogenesis of *Ed. tarda* was investigated by examining the LD₅₀ values in blue gourami fish. The deletion of *escC* increased the LD₅₀ value by about 1 log ($10^{5.9 \pm 0.3}$; $n=3$) as compared to the wild-type ($10^{5.0 \pm 0.2}$, $n=3$). This increase is comparable to that of *eseB* or *eseD* mutants (LD₅₀ of *eseB*= $10^{6.0}$, LD₅₀ of *eseD*= $10^{6.1}$) (Tan *et al.*, 2005), but is less than that of the regulator mutant Δ *esrC* (LD₅₀= $10^{7.6}$) (Zheng *et al.*, 2005). These results suggest that EscC is required for virulence and contributes to the pathogenesis of *Ed. tarda*.

DISCUSSION

Like many other Gram-negative pathogens, *Ed. tarda* appears to rely on a T3SS for its pathogenesis (Tan *et al.*, 2005). EseB, EseC and EseD are the major ECPs in *Ed. tarda* and their secretion is T3SS-dependent. The amino acid sequences of these three Ese proteins are homologous to those of the translocon components in other bacteria (Tan *et al.*, 2005). EseB was predicted to be a major component of the *Ed. tarda* T3SS translocon, based on its similarity to EspA, and to form a translocon complex together with EseC and EseD on the bacterial cell surface (Tan *et al.*, 2005). In this study, we showed that EseB, EseC and EseD formed a complex after secretion, which is consistent with their roles as translocon components. The interaction of EseB, EseC and EseD is reminiscent of that of EspA and EspB from EPEC. EspA interacted with EspB and this interaction was necessary for EPEC type III effector translocation. However, an interaction between EspA (EseB) and EspD (EseC) was not shown in EPEC as co-immunoprecipitation of EspA filaments with EspD failed to yield positive results (Hartland *et al.*, 2000). In contrast to EPEC, EseB (EspA) interacted not only with EseD (EspB) but also with EseC (EspD). The reason for this difference is not clear, but it may be due to the organisms' respective infection mechanisms. The interaction of EseB, EseC and EseD is probably essential for the translocation of *Ed. tarda* T3SS effector(s) although no effector proteins have been reported in *Ed. tarda* thus far. Future study will investigate the actual roles of EseB, EseC and EseD by evaluating their abilities to form pores in biological membranes.

The secretion of T3SS translocon components is generally chaperone-dependent. Up to now, various chaperones involved in the translocon components have been reported in EPEC, *Salmonella* and *Shigella* species. However, their roles in the secretion of EseB and EseD in *Ed. tarda* are still

unclear. We report here the identification and characterization of EscC, a specific chaperone for EseB and EseD. EscC shares most of the characteristics of typical T3SS chaperones, such as α -helical propensity, small size, and a carboxy-terminal amphipathic α -helical segment (Wattiau *et al.*, 1996; Bennett & Hughes, 2000). However, the predicted pI of EscC is near neutral, which is different from most characterized acidic or basic type III chaperones (Wattiau *et al.*, 1996; Bennett & Hughes, 2000; Page & Parsot, 2002). Chaperones with near neutral pI are not unprecedented. CesD in EPEC has a predicted pI of 7.4 (Wainwright & Kaper, 1998). Furthermore, the association of neutral pI chaperones with their cognate proteins remains uncharacterized. Crystallographic study of the EscC complex is needed to provide insights into the mechanism. Another atypical feature of EscC is its location both on the membrane and in the cytoplasm. Most of the characterized type III chaperones are found in the cytosol, and only a few chaperones have membrane associations. For example, CesD from EPEC and SseA from *Salmonella* were localized to both the cytoplasmic membrane and cytoplasm (Wainwright & Kaper, 1998; Zurawski & Stein, 2003). The mechanism of membrane localization of chaperones is unclear. Cycles of chaperone membrane insertion and deinsertion may be a possible explanation for the dual location. The cytoplasmic chaperone CesT has been demonstrated to recruit Tir and co-localize to the EPEC inner membrane (Elliott *et al.*, 1999; Thomas *et al.*, 2005). The ATPase of T3SS plays critical roles in chaperone release from and unfolding of the cognate secreted protein in an ATP-dependent manner (Akeda & Galan, 2005). Thus, membrane insertion may be the initial step in the reorganization of the secreted protein-chaperone complex with the T3SS ATPase.

T3SS chaperones have been divided into three classes: class I, chaperones which associate with one or several effectors, typified by the *Yersinia* SycE chaperone, which serves a single substrate (YopE); class II, chaperones which associate with translocators, typified by the *Yersinia* SycD protein, which is required for the proper secretion of two proteins (YopB and YopD); and class III, chaperones of the flagellar export system, which are believed to be the ancestors of the T3SS (Neyt & Cornelis, 1999; Page & Parsot, 2002; Parsot *et al.*, 2003). Chaperones from the flagellar system participate in flagellar assembly and recognize the C-terminal domain of their substrates, and these C-terminal domains are thought to form amphipathic helices that mediate interactions between the subunits of the flagellum (Fraser *et al.*, 1999; Auvray *et al.*, 2001). Despite the evidence for EscC as a chaperone, this protein also possesses features different from most other chaperones. For example the EscC substrate of EseB is neither an effector nor a translocon pore, and EscC could also be detected in the bacterial membrane. The characteristics of EscC are reminiscent of SseA of *Salmonella* SPI-2. SseA interacts with the C-terminal coiled-coil domain of SseB and is classified as a class III flagellar chaperone

(Zurawski & Stein, 2004). Similarly, coiled-coil domains in the C-terminal regions of EseB and EseD were predicted with COILS. EscC may interact with EseB and EseD at their coiled-coil domains. Thus, EscC seems functionally more similar to SseA. However, EscC does not share sequence similarity with SseA. The predicted coiled-coil domain of EscC is in the N-terminal region while that of SseA is in the C-terminal region. Another chaperone functionally similar to EscC is CesAB of EPEC, which is required for the secretion and stability of EspA (the homologue of EseB) and EspB (the homologue of EseD) (Creasey *et al.*, 2003). CesAB has been shown to interact with EspA with the N-terminal coiled-coil domain (Yip *et al.*, 2005). Although there is a lack of sequence similarity between EscC and CesAB, they may have similar three-dimensional structures, and the coiled-coil domain of EscC may be important for its binding with EseB or EseD. Further studies to delineate how EscC interacts with EseB and EseD will allow better comparisons with well defined chaperone families.

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