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Role of type III secretion in *Edwardsiella tarda* virulence

Y. P. Tan,[1] J. Zheng,[1] S. L. Tung,[1] I. Rosenshine[2] and K. Y. Leung[1]

[1]Department of Biological Sciences, Faculty of Science, National University of Singapore, Science Drive 4, Singapore 117543

[2]Department of Molecular Genetics and Biotechnology, Faculty of Medicine, The Hebrew University, PO Box 12272, Jerusalem 91120, Israel

Correspondence
K. Y. Leung
dbslky@nus.edu.sg

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Edwardsiella tarda is a Gram-negative enteric bacterium affecting both animals and humans. Recently, a type III secretion system (TTSS) was found in *Ed. tarda*. Such systems are generally used by bacterial pathogens to deliver virulence factors into host cells to subvert normal cell functions. Genome-walking was performed from the *eseB* and *esrB* genes (homologues of *Salmonella sseB* and *ssrB*, respectively) identified in previous studies, to determine the sequences of the TTSS. Thirty-five ORFs were identified which encode the TTSS apparatus, chaperones, effectors and regulators. Mutants affected in genes representing each category were generated and found to have decreased survival and growth in fish phagocytes. LD_{50} values of the mutants were increased by at least 10-fold in comparison to those of the wild-type strain. The adherence and invasion rates of the *esrA* and *esrB* mutants were enhanced while those of the other mutants remained similar to the wild-type. The *eseC* and *eseD* mutants showed slight autoaggregation in Dulbecco's Modified Eagle Medium, whereas the rest of the mutants failed to autoaggregate. Regulation of the TTSS was found to involve the two-component regulatory system *esrA–esrB*. This study showed that the TTSS is important for *Ed. tarda* pathogenesis. An understanding of this system will provide greater insight into the virulence mechanisms of this bacterial pathogen.

INTRODUCTION

Edwardsiella tarda is a wide-host-range Gram-negative bacterium, infecting lower and higher vertebrates such as 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, 1993a). Its virulence factors are largely unknown. One of the established factors is its ability to invade non-phagocytic cells (Janda *et al.*, 1991; Ling *et al.*, 2000). When viewed by electron microscopy, the bacterium was observed to induce extensive plasma membrane projections or ruffles in HEp-2 cells (Phillips *et al.*, 1998). It is also capable of surviving in phagocytes (Srinivasa Rao *et al.*, 2001, 2003b), and produces haemolysins (Janda & Abbott, 1993b; Chen *et al.*, 1996; Hirono *et al.*, 1997) and catalases (Srinivasa Rao *et al.*, 2003b).

More recently, two genes, *eseB* and *esrB*, homologous to the *sseB* and *ssrB* respectively of the type III secretion system (TTSS) encoded by *Salmonella* pathogenicity island 2 (SPI2) (Hensel *et al.*, 1998), were found in *Ed. tarda* (Tan *et al.*, 2002; Srinivasa Rao *et al.*, 2003a). At the same time, three TTSS proteins, EseB, EseC and EseD, homologous to SseB, SseC and SseD respectively of SPI2, were identified by MS in the extracellular proteome of *Ed. tarda* (Srinivasa Rao *et al.*, 2004). These findings led to speculation of the presence of a SPI2-like pathogenicity island (PAI) in *Ed. tarda*.

TTSSs are used by many bacterial pathogens for the delivery of virulence factors into host cells (Hueck, 1998). They encode various subsets of proteins involved in different aspects of pathogenesis. Some of them are secreted effector molecules, whereas others are involved in regulating TTSS expression, form the translocation apparatus (Knutton *et al.*, 1998; Roine *et al.*, 1997), or cause pore formation in the host cell membrane (Tardy *et al.*, 1999; Wachter *et al.*, 1999) to facilitate translocation of effector proteins into host cells.

In addition to SPI2, a SPI1-encoded TTSS is also present in *Salmonella* (Ochman *et al.*, 1996). SPI1 is involved in the interaction of the bacterium with the cells of the gastrointestinal mucosa and facilitates invasion of epithelial cells (Galan & Curtiss, 1989; Galan, 1996), whereas SPI2 is primarily associated with bacterial survival and proliferation within phagocytes (Hensel, 2000).

Abbreviations: 2-D, two-dimensional; DMEM, Dulbecco's Modified Eagle's Medium; ECP, extracellular protein; EPC, epithelioma papillosum of carp; EPEC, enteropathogenic *Escherichia coli*; PAI, pathogenicity island; SPI2, *Salmonella* pathogenicity island 2; TSA, tryptic soy agar; TTSS, type III secretion system.

The GenBank accession number for the complete TTSS including the flanking sequences on the left and right sides of the cluster is AY643478.

In this paper, we report the identification of a TTSS in *Ed. tarda*. Based on sequence homology, many of the *Ed. tarda* TTSS genes resemble counterparts in the SPI2-encoded TTSS. We also show that the *Ed. tarda* TTSS is important for virulence.

METHODS

Bacterial strains, plasmids, media and culture conditions. The bacterial strains and plasmids used are described in Table 1. *Ed. tarda* cultures were routinely grown in tryptic soy broth (BD Biosciences) or on tryptic soy agar (TSA, BD Biosciences) and *Escherichia coli* strains were cultured on Luria–Bertani (LB, BD Biosciences) agar or in LB broth. *Ed. tarda* and *E. coli* cultures were incubated at 25 and 37 °C respectively unless otherwise stated. Stock cultures were maintained in a suspension of broth with 25 % (v/v) glycerol and stored at −80 °C. When required, the appropriate antibiotics were supplemented at the following concentrations: ampicillin (100 μg ml^{-1}), colistin (12·5 μg ml^{-1}), gentamicin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}).

For the study of the autoaggregation phenotype of *Ed. tarda* wild-type and its mutants, bacterial cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) without phenol red as described previously (Srinivasa Rao *et al.*, 2004). The bacteria were streaked on TSA and incubated for 48 h, after which a single colony was inoculated into 5 ml DMEM and cultured at 25 °C in a 5 % CO_2 incubator for 24 h. The strains or mutants which aggregated to form cell clumps covering the entire round bottom of the tube (clear medium at the top) were scored '+', while those which remained suspended in the culture medium (turbid) with few cells settling at the bottom of the tube were scored '−' for autoaggregation. A '±' score denotes bacteria that formed small clumps at the bottom of the tube. The experiment was repeated three times in three independent trials.

Table 1. Bacterial strains, mutants and plasmids

Strain/mutant or plasmid	Genotype description and/or relevant property	Source/reference(s)
Ed. tarda		
PPD130/91	Col^r	*Serpae tetra*, AVA*, Singapore
eseB	Amp^r Col^r Kan^r	This study
eseC	Amp^r Col^r Kan^r	This study
eseD	Amp^r Col^r Kan^r	This study
esaC	Amp^r Col^r Kan^r	This study
escA	Amp^r Col^r Kan^r	This study
esrA	Amp^r Col^r Kan^r	This study
esrB	Amp^r Col^r Kan^r	This study
Δ*esrA*	Deleted 2610 bp from total 2718 bp of *esrA*	This study
Δ*esrB*	Deleted 516 bp from total 645 bp of *esrB*	This study
E. coli		
DH5α	*supE44* Δ*lac*U169 (*ϕ*80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, Amp^s	Hanahan (1983)
MC1061(*λpir*)	(*λpir*), *thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara14 xyl5 supE44*, *λpir*	Rubires *et al.* (1997)
SM10(*λpir*)	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r *pir*	Miller & Melakanos (1988)
Plasmids		
PGEM-T Easy	Cloning vector, Amp^r	Promega
pFS100	pGP704 suicide vector, *λpir* dependent, Km^r Amp^r, low copy number	Rubires *et al.* (1997)
pFS-*esaC*	pFS100 with internal fragment of *esaC* (832 bp)	This study
pFS-*escA*	pFS100 with internal fragment of *escA* (158 bp)	This study
pFS-*eseB*	pFS100 with internal fragment of *eseB* (178 bp)	This study
pFS-*eseC*	pFS100 with internal fragment of *eseC* (929 bp)	This study
pFS-*eseD*	pFS100 with internal fragment of *eseD* (440 bp)	This study
pFS-*esrA*	pFS100 with internal fragment of *esrA* (458 bp)	This study
pFS-*esrB*	pFS100 with internal fragment of *esrB* (452 bp)	This study
pKK177-3	P_{tac} promoter, derivative of pKK223-3, Amp^r	Brosius & Holy (1984)
pKK-*eseB*	pKK177-3 with the full *eseB* gene	This study
pACYC184	Cm^r Tc^r	Fermentas Life Sciences
pACYC-*esrAB*	pACYC184 with full *esrA* and *esrB* genes, Cm^r Tet^r	This study
pRE112	*oriT oriV sacB*, Cm^r	Edwards *et al.* (1998)
pRE112 + *esrA*	pRE112 with 2610 bp deleted from the *esrA* gene, Cm^r	This study
pRE112 + *esrB*	pRE112 with 516 bp deleted from the *esrB* gene, Cm^r	This study

*AVA, Agri-Veterinary Authority of Singapore.

Genome-walking, cloning and sequencing. In order to obtain the sequences of the TTSS cluster, genome-walking and/or inverse PCR (Sambrook & Russell, 2000) were performed concurrently from both the *eseB* and *esrB* genes obtained from our earlier work (Tan *et al.*, 2002; Srinivasa Rao *et al.*, 2003a). Genome-walking libraries (*Eco*RV, *Dra*I, *Hin*cII, *Pvu*II, *Sca*I, *Stu*I) were constructed and PCR amplification was carried out according to the Universal GenomeWalker kit (BD Biosciences) user manual. DNA fragments amplified were cloned into the pGEM-T Easy vector system (Promega) and transformed into *E. coli* TOP10F′ or *E. coli* DH5α.

The Applied Biosystems (ABI) PRISM BigDye Terminator v3.0 or 3.1 Cycle Sequencing Kit was used for the sequencing PCR. The ABI 3100 Genetic Analyser was then used for automated DNA sequencing. Sequence assembly and editing were performed with the DNASIS sequence analysis software (Hitachi Software). BLASTP and/or PSI-BLAST sequence homology analyses were done using the National Center for Biotechnology BLAST network service.

Construction of *Ed. tarda* mutants. Insertion mutation was carried out in various TTSS genes (*esaC*, *escA*, *eseB*, *eseC*, *eseD*, *esrA* and *esrB*) in *Ed. tarda* PPD130/91. Briefly, internal fragments of each gene were obtained by PCR using the primers given in Table 2 and cloned into the pGEM-T Easy vector (Promega). These partial gene fragments were released from the vector by *Eco*RI digestion and religated into the suicide plasmid pFS100 (Rubires *et al.*, 1997). The construct obtained was transformed into *E. coli* MC1061. pFS100 carrying the internal fragment of each TTSS gene was isolated from *E. coli* MC1061 and transformed into *E. coli* strain SM10λ*pir*. Subsequently plate-mating was performed to allow mobilization of the suicide vector with the internal gene fragment from *E. coli* SM10λ*pir* to *Ed. tarda* PPD130/91. Insertional inactivation then occurred, resulting in the integration of the cloned suicide vector into the respective TTSS gene of interest in the *Ed. tarda* genome.

In addition to the insertion mutants, non-polar deletion mutation was also performed for the *esrA* and *esrB* genes following the procedure of Edwards *et al.* (1998). The target gene, including at least 500 bp flanking sequences, was amplified using primers (Table 2) containing the *Kpn*I restriction site and cloned into the pGEM-T Easy vector. Inverse PCR was then performed using these constructs as templates. The resulting PCR product was purified and self-ligated to obtain the gene of interest with non-polar deletion, which was then excised with *Kpn*I and cloned into the suicide vector pRE112 (Edwards *et al.*, 1998). The construct obtained was transformed into *E. coli* SM10λ*pir* and mobilized into wild-type *Ed. tarda* PPD130/91 by conjugal transfer. Double crossover mutation was achieved by plating the bacterial cells on TSA plates with 10 % sucrose. The deletion mutants were confirmed by PCR and sequencing.

Phagocyte isolation and intracellular replication assay. Phagocytes were isolated from the head kidney of healthy naïve blue gourami obtained from a commercial fish farm following the method of Secombs (1990) and as described by Srinivasa Rao *et al.* (2001) with slight modifications. Approximately $4–5 \times 10^6$ phagocytic cells suspended in L-15 medium (Sigma) supplemented with 5 % fetal bovine serum (Invitrogen) were seeded into each well of a 48-well tissue culture plate (Costar) and incubated for 4 h at 25 °C in a 5 % CO_2 atmosphere. Afterwards, the cells were washed once with Hanks' balanced salt solution (HBSS, Sigma) and infected with *Ed. tarda* at an m.o.i. of 1 : 1 for 30 min (four wells per strain/mutant). The cells were then washed twice with HBSS and extracellular bacteria were killed by gentamicin (100 μg ml^{-1}) treatment for

Table 2. Primers used for PCR amplification for the construction of insertional and deletion mutants

Gene	Primer or primer pair	Primer sequence (5′ to 3′)
	Primers used to amplify TTSS gene internal fragments	
eseB	eseB-IF	CTACGACAACAACCTGGATCGTCGCAT
	eseB-IR	GTCGATCTGATTAGCCACCTGCTG
eseC	eseC-IF	GGATCACGATCCTAAGCG
	eseC-IR	CTTGGCCGACATTTCGAC
eseD	eseD-IF	GGACGGCCATCGTTACGTTTCAC
	eseD-IR	TTTCCAGCGTTTCATCCG
esaC	esaC-IF	TATGCCTGCCAGGTCCGC
	esaC-IR	CCTCGATATTGAGGATCAGCAAT
escA	escA-IF	GATCTCGCTACGCTGCATGGT
	escA-IR	GCCAGCAGCAGGTAAAAGCGT
esrA	esrA-IF	GGAGTAAGCCTTATCGCAG
	esrA-IR	CGTCAGGCGCTGCATAATG
esrB	esrB-IF	GATCATGCCTTGCTAGCC
	esrB-IR	TCGGCGACCAGCTTGAGA
	Primers used in the construction of the non-polar deletion mutants	
esrA	int-rev	CATAGGGGATTCCTTTATGTGC
	DelrA-int-for	CTGACGCTGGTGCATGAGG
	DelrA-for	AGAGCTCAGAAGAAGAGGAGGAGGA
	DelrA-rev	AGAGCTCCCGTAATCTCTTGGACAG
esrB	DelrB-int-for	CAAGAGATTACGGATGCCA
	DelrB-int-rev	GCGGAGCTGAGCAACTGGGC
	DelrB-for	AGGTACCGGCGACAATCCCGATCTG
	DelrB-rev	AGGTACCGAAAGGCTCTCCGTTGAC

1·5 h. At the end of this time period, the cells were washed twice with HBSS and those in two of the wells were lysed with 1 % Triton X-100 (Sigma) and plated to obtain the bacterial count in c.f.u. Five hundred microlitres fresh L-15 medium was added into each of the remaining wells and the cells were incubated for a further 4·5 h before being lysed with 1 % Triton X-100 (Sigma) and plated.

The ratio of the c.f.u. count at the end of the 6·5 h period and that at 2 h after gentamicin treatment was obtained for each mutant and the wild-type strain. The ratio of the two time points for each mutant was expressed as a quotient of that of the wild-type strain. A value less than one indicated reduced replication in phagocytes. The experiment was repeated three times for each mutant and the wild-type strain was kept for comparison in every trial.

Adherence and internalization assays. These assays were performed following the procedure by Wang *et al.* (1998) with slight modifications. Epithelioma papillosum of carp, *Cyprinus carpio* (EPC), cells (Wolf & Mann, 1980) were grown for 72 h to 100 % confluence in 24-well tissue culture plates (Falcon). The cells were then washed once with HBSS and infected with *Ed. tarda* and its mutants in minimal essential medium (MEM, Invitrogen) at an m.o.i. of 1 : 1 (four wells per bacterial strain/mutant). The plate was centrifuged at 800 ***g*** for 5 min at 4 °C and incubated at 25 °C in a 5 % CO_2 incubator for 30 min. To measure the number of bacteria adhering to the EPC monolayer, the cells were washed six times with HBSS, lysed with 1 % Triton X-100 (Sigma) and the bacterial number quantified by plate counting (Elsinghorst, 1994). For measuring the number of internalized bacteria, the monolayer was washed three times with HBSS and incubated for 2 h in MEM with gentamicin (100 μg ml^{-1}) (Sigma) to kill any remaining extracellular bacteria. The EPC cells were washed three times again with HBSS to remove gentamicin; 1 ml 1 % Triton X-100 was added to lyse the cells and the number of viable intracellular bacteria was quantified by plate counting. The adherence and invasion rates were calculated from the mean of two wells in triplicate experiments.

LD_{50} determination. The LD_{50} of *Ed. tarda* PPD130/91 wild-type and mutants was determined using healthy naïve blue gourami, *Trichogaster trichopterus* (Pallas), obtained from a commercial fish farm as described previously (Ling *et al.*, 2000). Fish mortality was observed for 7 days and the LD_{50} values were calculated according to the method of Reed & Muench (1938).

Preparation of extracellular proteins (ECPs) and two-dimensional (2-D) PAGE analysis. ECPs from *Ed. tarda* PPD130/91 and its mutants were prepared as described before (Leung & Stevenson, 1988; Tan *et al.*, 2002) and the proteins were quantified using the Bio-Rad protein assay.

2-D PAGE was used to resolve the proteins. Eighteen-centimetre Immobiline Drystrips pH 3–10 (Amersham Biosciences) were used and passive rehydration of the strips was carried out. IEF of the proteins was done using the Ettan IPGphor Isoelectric Focusing System (Amersham) under the following conditions: 50 V for 1 h, 500 V for 2 h, 4000 V for 1 h 30 min, 8000 V for 40 000 V h or 500 V for 2 h, 4000 V for 1 h 30 min, 8 000 V for 40 000 V h. The cup loading method was employed to load the protein samples prior to IEF. Silver staining was performed following the procedure of Blum *et al.* (1987).

Complementation of mutants. The *eseB* gene and the *esrA–esrB* regulatory genes, including more than 1 kb of flanking sequences upstream of each gene, were amplified by PCR and cloned into pKK177-3 (Brosius & Holy, 1984), adjacent to the P_{tac} promoter, and pACYC184 (Fermentas Life Sciences) respectively. Each of the constructs was first transformed into *E. coli* DH5α and plasmid DNA was isolated. The *eseB* and *esrA–esrB* genes carried on the vector were then transformed into the respective *eseB* and *esrA* and *esrB* mutants. Expression of the *eseB* gene in these constructs is inducible by 0·1 mM IPTG.

The complemented mutants harbouring a wild-type copy of the *eseB* gene carried on the pKK177-3 vector were then inoculated into DMEM with and without IPTG, cultured for 24 h and compared with the wild-type and the *eseB* mutant. The ability of the *esrA* and *esrB* mutant complemented with the wild-type *esrA–esrB* genes provided *in trans* on the pACYC184 plasmid to autoaggregate in DMEM were also checked and their adherence and invasion rates were measured.

Statistical analysis. The data obtained for adherence and internalization and phagocyte intracellular replication were expressed as mean ± SEM. Data analysis was performed using the paired *t*-test (MINITAB Release 14.0 Statistical Software, Minitab) for comparison between the wild-type and each mutant. $P<0·05$ was considered statistically significant.

RESULTS AND DISCUSSION

Identification of a TTSS in *Ed. tarda*

Using the comparative proteomics (Tan *et al.*, 2002) and transposon tagging approaches (Srinivasa Rao *et al.*, 2003a), we found two genes, *eseB* and *esrB*, in *Ed. tarda* that are homologous to members of the SPI2-encoded TTSS. Furthermore, the EseB protein and two other major proteins, homologous to SPI2 SseC and SseD, were found in the *Ed. tarda* secretions (Srinivasa Rao *et al.*, 2004). These findings suggested the presence of a SPI2-like PAI. Subsequent PCR and genome-walking confirmed our hypothesis and 35 ORFs encoding the TTSS from the virulent *Ed. tarda* PPD130/91 were identified.

The TTSS encoding region is 30 756 bp and its organization is shown in Fig. 1(a). The cluster has a mean G+C content of 64·3 mol%, which is approximately 6–9 percentage points higher than that of the *Ed. tarda* chromosome (55–58 mol%) (Farmer & McWhorter, 1984). This observation is in contrast to SPI2, in which the G+C content is generally lower than the rest of the *Salmonella* genome (Hensel *et al.*, 1997, 1999). Such variation in the G+C content is a distinctive feature of PAIs observed in many bacteria (Hacker *et al.*, 1997). It suggests that the group of genes could have been acquired from an external source. The presence of this TTSS (as surveyed using *eseB* and *esrB*) in most virulent but not avirulent strains (Tan *et al.*, 2002; Srinivasa Rao *et al.*, 2003a) also suggests the possibility of horizontal gene transfer.

The predicted ORFs of the TTSS cluster are listed in Table 3 and their putative functions are given as deduced from those of the homologues in *Salmonella enterica* serovar Typhimurium, enteropathogenic *E. coli* (EPEC) and *Yersinia enterocolitica*. As in the case of SPI2, the genes of the *Ed. tarda* TTSS cluster are likewise grouped into four categories, namely *Ed. tarda* secretion system apparatus (*esa*), chaperones (*esc*), effectors (*ese*) and regulators (*esr*). The designation of each gene is based on its sequence homologue.

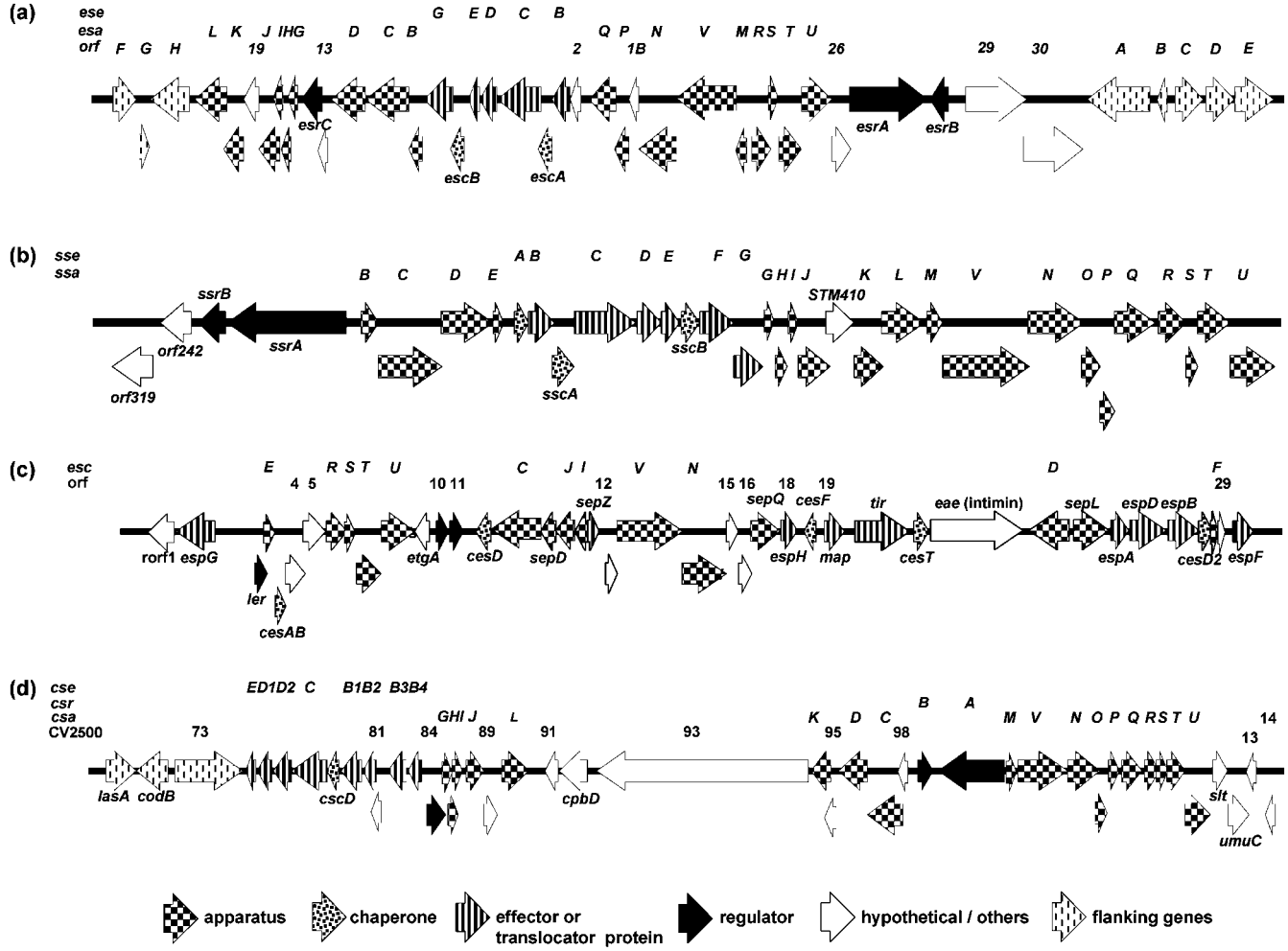

Fig. 1. Genetic organization of TTSSs in (a) *Ed. tarda* PPD130/91, (b) *S. enterica* serovar Typhimurium (SPI2), (c) EPEC (LEE) and (d) *C. violaceum* ATCC 12472 (CPI2). The secretion systems essentially consist of four categories of genes, namely apparatus, chaperones, effectors and regulators, but the arrangement of the genes varies. The direction of transcription of each gene is indicated by the arrow. In *Ed. tarda*, the cluster is flanked by *orfA–E* and *orfF–H* on both sides. OrfA is homologous to the catalase–peroxidase. OrfB is unknown and OrfC–E are homologous to members of the *tdc* operon, *tdcA*, *tdcB* and *tdcC*. OrfF is homolgous to a hypothetical protein in *E. coli* and the sulfite oxidase in *Burkholderia fungorum*. OrfH is homologous to the putative *Photobacterium profundum* guanine aminohydrolase and *E. coli* guanine deaminase. OrfG has no homologue.

Previously, the ORFs were presented in two separate DNA regions (Zheng *et al.*, 2005). Five additional ORFs have since been identified which join the two regions together and they have been designated ORF1A–ORF1E. The DNA region 1 was reversed in this case so as to join DNA region 2 to obtain a complete, contiguous TTSS cluster. For ease of reference, we have kept to the ORF numbering used earlier (Table 3).

The *Ed. tarda* TTSS and its counterparts SPI2, LEE and CPI2

The *Ed. tarda* TTSS identified in the present study showed both similarities and differences in organization to the TTSSs encoded by SPI2 (Hensel, 2000), LEE (locus of enterocyte effacement; Elliott *et al.*, 1998) and CPI2 (*Chromobacterium* pathogenicity island 2; Brazilian National Genome Project Consortium, 2003; Betts *et al.*, 2004) encoded in *S. enterica* serovar Typhimurium, EPEC and *Chromobacterium violaceum* respectively (Fig. 1). It also has homologues in the *Y. enterocolitica* TTSS (Table 3). The functions of several SPI2 (Ruiz-Albert *et al.*, 2003; Zurawski & Stein, 2003) and LEE (Elliott *et al.*, 2001; Creasey *et al.*, 2003; Jepson *et al.*, 2003; Tu *et al.*, 2003; Deng *et al.*, 2004; Kanack *et al.*, 2004; Nougayrede & Donnenberg, 2004; O'Connell *et al.*, 2004; Pallen *et al.*, 2005) proteins (Fig. 1b, c) were updated based on more recent studies. In contrast to the other four bacteria, *C. violaceum* is generally free-living, but occasional fatal human infections have been reported

Table 3. ORFs of *Ed. tarda* TTSS and the sequence homologues in *S. enterica* serovar Typhimurium, EPEC, *C. violaceum* and *Y. enterocolitica*

Ed. tarda ORF*	Gene name	Similar protein (% identity, % similarity)				Putative function†
		S. enterica serovar Typhimurium	EPEC	*C. violaceum*	*Y. enterocolitica*	
rORF21	*esaL*	SsaL (23·9, 38·3)	SepL (19·9, 33·9)	CsaL (27·3, 41·5)	YscL (8·8, 17·7)	Apparatus
rORF20	*esaK*	SsaK (11·7, 19·0)		CsaK (17·8, 26·8)	YscK (14·8, 24·2)	Apparatus
rORF19				CV2589 (31·6, 44·4)		Unknown
rORF18	*esaJ*	SsaJ (38·6, 52·8)	EscJ (30·9, 43·9)	CsaJ (50·2, 62·4)	YscJ (32·9, 49·4)	Apparatus
rORF17	*esaI*	SsaI (23·8, 44·0)	EscI (14·0, 23·8)	CsaI (24·8, 31·9)	YscI (17·2, 26·7)	Apparatus
rORF16	*esaH*	SsaH (24·7, 36·0)				Apparatus
rORF15	*esaG*	SsaG (39·2, 54·1)	EscF (35·1, 55·4)	CsaG (50·0, 64·9)	YscF (15·9, 39·8)	Apparatus
rORF14	*esrC*			CV2584 (27·5, 40·8)	VirF (20·5, 34·4)	Regulator
rORF13				CV2595 (36·0, 45·3)		Unknown
rORF12	*esaD*	SsaD (24·3, 38·0)	EscD (22·3, 39·7)	CsaD (33·0, 47·8)	YscD (20·5, 32·3)	Apparatus
rORF11	*esaC*	SpiA (44·9, 60·1)	EscC (39·8, 57·7)	CsaC (55·4, 67·9)	YscC (26·8, 39·7)	Apparatus
rORF10	*esaB*	SsaB (16·3, 29·4)		CV2598 (19·6, 29·4)		Apparatus/effector?
rORF9	*eseG*	SseG (13·4, 24·7)				Effector
rORF8	*escB*	SscB (24·4, 37·8)			SycB (20·0, 33·5)	Chaperone
rORF7	*eseE*	SseE (10·2, 20·4)		CseE (29·6, 48·6)		Effector
rORF6	*eseD*	SseD (23·9, 39·1)	EspB (9·3, 19·9)	CseD1 (22·2, 37·4)‡	YopD (9·2, 17·6)	Translocon component
rORF5	*eseC*	SseC (24·4, 38·5)	EspD (11·2, 22·5)	CseC (33·1, 48·7)	YopB (14·4, 26·0)	Translocon component
rORF4	*escA*	SscA (26·3, 43·8)	CesD (35·0, 50·3)	CscD (44·4, 58·0)	SycD (20·7, 39·1)	Chaperone
rORF3	*eseB*	SseB (29·5, 46·4)	EspA (21·4, 35·2)	CseB2 (32·2, 52·2)‡		Translocon component
rORF2				CV2581 (25·2, 39·8)		Unknown
rORF1	*esaQ*	SsaQ (11·9, 21·6)	SepQ (12·1, 22·9)	CsaQ (21·9, 32·2)	YscQ (16·3, 26·6)	Apparatus
rORF1A	*esaP*			CsaP (23·6, 29·2)		Apparatus
rORF1B						Unknown
rORF1C	*esaN*	SsaN (51·6, 61·8)	EscN (46·9, 64·4)	CsaN (57·8, 71·2)	YscN (49·6, 63·7)	Apparatus
rORF1D	*esaV*	SsaV (50·6, 65·1)	EscV (45·6, 63·4)	CsaV (63·5, 78·6)	YscV (43·1, 61·0)	Apparatus
rORF1E	*esaM*	SsaM (7·0, 12·4)		CsaM (28·3, 37·0)		Apparatus
ORF22	*esaR*	SsaR (57·9, 73·1)	EscR (48·4, 66·1)	CsaR (64·5, 75·1)	YscR (52·3, 67·4)	Apparatus
ORF23	*esaS*	SsaS (52·2, 66·7)	EscS (44·4, 67·8)	CsaS (66·7, 76·7)	YscS (51·1, 64·4)	Apparatus
ORF24	*esaT*	SsaT (41·0, 60·5)	EscT (32·3, 54·0)	CsaT (52·7, 65·9)	YscT (29·9, 48·5)	Apparatus
ORF25	*esaU*	SsaU (35·7, 50·1)	EscU (34·4, 50·9)	CsaU (50·4, 64·9)	YscU (30·1, 43·4)	Apparatus
ORF26			EtgA (10·4, 20·3)	Slt (35·2, 44·7)		Soluble lytic murein transglycosylase
ORF27	*esrA*	SsrA (29·9, 42·0)		CsrA (33·5, 46·3)		Regulator
rORF28	*esrB*	SsrB (42·4, 56·7)		CsrB (59·5, 72·3)		Regulator
ORF29						Unknown
ORF30						Unknown

*The ORFs were numbered (Fig. 1, from left to right) following our previous numbering system (Zheng *et al.*, 2005); r indicates that the direction of transcription is reverse.

†Putative functions are deduced based on the sequence homologues.

‡Multiple SseB/EspA and SseD/EspB homologues are found in CPI2. The *Ed. tarda* EseB and EseD were compared only with the CPI2 homologues with the highest homology.

(Ti *et al.*, 1993). Recently, TTSS genes which are likely to be involved in pathogenesis were found in the genome of this bacterium (Brazilian National Genome Project Consortium, 2003; Alves de Brito *et al.*, 2004; Betts *et al.*, 2004).

The gene members of these TTSSs are essentially categorized into the four groups mentioned above and those involved in similar or related functions (apparatus, effectors and the corresponding chaperone, regulators) are located in close proximity (Fig. 1). In the four bacteria compared, the apparatus genes essentially exist in two regions in the cluster, and those encoding the apparatus proteins R, S, T and U are present and arranged in tandem in the exact same order and transcribed in the same direction. As for the other apparatus

genes, their positions, though similar, are not identical in the four bacteria.

The gene arrangement of the *Ed. tarda* system (Fig. 1a) most closely resembles that in *S. enterica* serovar Typhimurium (Fig. 1b). The putative effector genes (*eseB*, *eseC*, *eseD*, *eseE*) and the interspersed putative chaperone genes (*escA* and *escB*) are arranged in the same order in the two bacteria. However, although both of them possess a two-component regulatory system, their locations differ. The *Ed. tarda* system is situated further away from the group of effector and chaperone genes unlike in SPI2, in which it is positioned immediately next to the *ssaB* gene and only four ORFs away from *sseA*. Moreover, the *Ed. tarda* regulatory gene members are transcribed in opposite directions, in contrast to the *Salmonella ssrA–ssrB*, which form one transcriptional unit (Shea *et al.*, 1996). Therefore, with respect to the location and direction of transcription of the *Ed. tarda* regulatory system, there is greater similarity to the *C. violaceum* system (Fig. 1d).

Besides the putative *esrA–esrB* two-component system, an additional transcriptional regulator, which we named *esrC*, is also present in the *Ed. tarda* TTSS cluster (Zheng *et al.*, 2005). This protein has homology to proteins of the AraC/XylS family of positive transcriptional regulators (Gallegos *et al.*, 1997) such as VirF in *Yersinia* species (Wattiau & Cornelis, 1994). CV2584 of *C. violaceum* is also a transcriptional regulator homologous to VirF (Betts *et al.*, 2004).

Orf10 shows weak homology to SpiC of *S. enterica* subsp. enterica Paratypi, SsaB of *S. enterica* serovar Typhimurium and a hypothetical protein (CV2598) of *C. violaceum* (Table 3), but it is unknown at this stage if it is an apparatus or effector protein. Orf19 has homology to a hypothetical protein (CV2589) in *C. violaceum* (NCBI accession no. AAQ60259) and *Y. pseudotuberculosis* (NCBI accession no. YP_068866), and a putative PAI protein of *S. enterica* subsp. enterica serovar Typhi (NCBI accession no. NP_805081) and Paratypi (NCBI accession no. YP_150694).

There are six novel genes (*orf1B*, *orf2*, *orf13*, *orf19*, *orf29* and *orf30*) with unknown function. Although *orf19* has homology to putative PAI genes, the function of the latter is not known. *orf29* and *orf30* located next to *esrB* were found to belong to the TTSS cluster (Zheng *et al.*, 2005).

TTSS boundary

Several ORFs (*orfA–E* and *orfF–H*) flanking the *Ed. tarda* TTSS were obtained (Fig. 1a). At one end, *orfA* encodes a catalase–peroxidase-like protein, *orfB* is unknown and *orfC–orfE* are homologous to the *tdc* operon genes. The catalase–peroxidase enzyme mainly functions in protecting bacteria under oxidative stress against the deleterious effects of reactive oxygen species (Schellhorn & Hassan, 1988; Schellhorn, 1995), although it has also been implicated in virulence in some bacteria (Pym *et al.*, 2002; Ng *et al.*, 2004). The *E. coli tdc* operon is involved in amino acid transport and metabolism during anaerobic growth to provide a source of metabolic energy (Datta *et al.*, 1987; Goss *et al.*, 1988; Sumantran *et al.*, 1990; Ganduri *et al.*, 1993). At the other end, *orfF* has homology to a hypothetical protein in *E. coli* (Dobrindt *et al.*, 2002) and sulfite oxidase enzymes in *Burkholderia fungorum* (NCBI accession no. ZP 00280377). *orfG* has no sequence homologue and *orfH* is homologous to the putative guanine aminohydrolase (NCBI accession no. YP 130136) and guanine deaminase in *Photobacterium profundum* and *E. coli* (Maynes *et al.*, 2000) respectively. The guanine aminohydrolase or deaminase is involved in the conversion of guanine to xanthine and ammonia during the first step of utilization of guanine as a nitrogen source (Maynes *et al.*, 2000). The homologues in *Ed. tarda* may have similar functions. These two groups of genes appear to play roles in metabolism and housekeeping for the general well-being of the bacteria. The mean G+C content of the two flanking regions (54·8 mol% for *orfA–orfE* and 53·5 mol% for *orfF–orfH*) is 54·2 mol%, which is very close to the *Ed. tarda* genome range (55–58 %). Therefore, they could be the flanking genes lying outside the cluster defining the TTSS boundary.

Construction and characterization of mutants

Since TTSSs are known to play important roles in virulence in many pathogenic bacteria (Hueck, 1998), there is a need to establish if this secretion system is also crucial for *Ed. tarda* pathogenesis. In order to address this question and to assign a more defined role to this newly discovered system in *Ed. tarda*, various TTSS genes, including those homologous to the regulators (*esrA* and *esrB*), apparatus (*esaC*), chaperone (*escA*) and effectors (e*seB*, *eseC* and *eseD*), were disrupted by insertional inactivation. For the regulator genes, non-polar deletion was also carried out. The mutants' ability to survive and replicate within phagocytes, to adhere to and internalize within non-phagocytic EPC cells, and to kill fish (LD_{50} determination) were examined and compared to the properties of the wild-type strain. These three phenotypes are the ones primarily associated with the SPI2 analogue (Hensel, 2000). The ability of the mutants to autoaggregate in DMEM was also checked. This phenotype was previously found to correlate with *Ed. tarda* virulence (Srinivasa Rao *et al.*, 2004); hence it may be used as a rapid preliminary screening tool to identify potential attenuated mutants.

Reduced survival and growth of TTSS mutants in fish phagocytes

The results of the mutant characterization are as shown in Table 4. Both the insertion and deletion *esrA* and *esrB* mutants showed essentially similar phenotypes. All the mutants tested had decreased ability to survive and replicate within phagocytes when compared to the wild-type strain, thus showing that the TTSS may be important for *Ed. tarda* to resist phagocytic killing and to replicate within the immune cells. This observation was similar to that in *Salmonella*. Disruption of SPI2 genes such as *spiR* (*ssrA*),

Table 4. Characterization of wild-type *Ed. tarda* PPD130/91 and its TTSS mutants

For adherence and internalization and macrophage replication, values are presented as mean ± SEM of triplicate trials. Values with different superscript letters are significantly different ($P<0{\cdot}05$).

Strain/mutant	Adherence*	Internalization†	Macrophage replication‡	LD_{50}	Aggregation in DMEM§
Wild-type	$6{\cdot}6\pm1{\cdot}1^{a}$	$2{\cdot}6\pm1{\cdot}0^{a}$	$1\pm0{\cdot}00^{a}$	$10^{4{\cdot}8}$	+
eseB	$7{\cdot}5\pm1{\cdot}4^{a}$	$0{\cdot}8\pm0{\cdot}03^{a}$	$0{\cdot}41\pm0{\cdot}10^{b}$	$10^{6{\cdot}0}$	−
eseC	$7{\cdot}3\pm1{\cdot}6^{a}$	$1{\cdot}4\pm0{\cdot}5^{a}$	$0{\cdot}22\pm0{\cdot}04^{b}$	$10^{6{\cdot}1}$	±
eseD	$5{\cdot}3\pm0{\cdot}3^{a}$	$1{\cdot}1\pm0{\cdot}2^{a}$	$0{\cdot}42\pm0{\cdot}15^{a}$	$10^{6{\cdot}1}$	±
esaC	$5{\cdot}3\pm2{\cdot}1^{a}$	$1{\cdot}0\pm0{\cdot}2^{a}$	$0{\cdot}23\pm0{\cdot}03^{b}$	$10^{6{\cdot}1}$	−
escA	$4{\cdot}6\pm2{\cdot}1^{a}$	$3{\cdot}4\pm1{\cdot}9^{a}$	$0{\cdot}68\pm0{\cdot}04^{b}$	$10^{5{\cdot}7}$	−
esrA	$45{\cdot}3\pm7{\cdot}3^{b}$	$12{\cdot}7\pm0{\cdot}6^{b}$	$0{\cdot}47\pm0{\cdot}07^{b}$	$10^{7{\cdot}6}$	−
esrB	$38{\cdot}3\pm5{\cdot}1^{b}$	$11{\cdot}5\pm3{\cdot}3^{a}$	$0{\cdot}47\pm0{\cdot}06^{b}$	$10^{8{\cdot}2}$	−
esrA + pACYC-*esrAB*	$12{\cdot}2\pm3{\cdot}7^{a}$	$2{\cdot}1\pm0{\cdot}5^{a}$	ND	ND	+‖
esrB + pACYC-*esrAB*	$10{\cdot}4\pm0{\cdot}7^{a}$	$1{\cdot}9\pm0{\cdot}3^{a}$	ND	ND	+‖
Δ*esrA*	$32{\cdot}6\pm4{\cdot}3^{b}$	$8{\cdot}5\pm0{\cdot}2^{b}$	ND	$10^{7{\cdot}6}$	−
Δ*esrB*	$36{\cdot}7\pm6{\cdot}1^{b}$	$10{\cdot}2\pm0{\cdot}7^{b}$	ND	$10^{8{\cdot}0}$	−

ND, Not determined.

*Expressed as percentage of bacteria still adherent after washing without gentamicin treatment ($n=3$).

†Expressed as percentage of input bacteria surviving after gentamicin treatment for 2 h ($n=3$).

‡The ratio of bacterial c.f.u. count at 6·5 h and 2 h was obtained and the ratio of each mutant was expressed as a quotient of the wild-type ratio. A value of 1 was obtained for the wild-type and a value <1 indicated reduced replication.

*†‡Paired *t*-test was performed for comparison between the wild-type and each mutant. Values with different superscript letters are significantly different ($P<0{\cdot}05$) from each other.

§'+', '±' and '−' denote aggregation, slight aggregation and no aggregation respectively.

‖Observation of the autoaggregation phenotype in the complemented *esrA* and *esrB* mutants was delayed and autoaggregation was seen to increase gradually with longer incubation time.

spiA (*ssaC*) (Ochman *et al.*, 1996), *sseB* and *sseC* (Hensel *et al.*, 1998) affected intracellular survival and replication of the mutants in murine macrophages.

esrA and *esrB* mutants show increased adherence to and internalization within EPC cells but greatly reduced LD_{50}

Earlier work showed that *Ed. tarda* is able to adhere to and invade epithelial cells (Janda *et al.*, 1991; Ling *et al.*, 2000). In this study, the *esrA* and *esrB* insertion and non-polar deletion mutants were found to have increased adherence and internalization, whereas the rest of the mutants showed adherence and internalization similar to the wild-type strain. The high internalization is probably a consequence of increased adherence because this is a prerequisite for bacterial entry (Neves *et al.*, 1994; Edelman *et al.*, 2003). Our observations are in contrast to the reduced adherence seen in *S. enterica* serovar Typhimurium SPI2 mutants (Deiwick *et al.*, 1998). In *Salmonella* species, SPI2 mutations (*ssaT* and *ssaV*) affect the expression of SPI1 genes and hence decrease the ability of the bacteria to invade cultured epithelial cells. In *Ed. tarda*, it is not known if a SPI1-like cluster involved in internalization is present and, so far, only the haemolysin-associated genes, homologous to *shlA* and *hpmA*, have been shown to be related to bacterial entry into epithelial cells (Strauss *et al.*, 1997). However, the putative *esrA–esrB* regulatory system does appear to affect the genes associated with adherence and invasion in a manner opposite to that observed in *Salmonella* species (Deiwick *et al.*, 1998). EsrA–EsrB may thus control a system with some anti-internalization activity, which maintains only a certain level of bacterial entry into epithelial cells. Therefore, internalization increases when the putative two-component system is disrupted. Alternatively, the effect of the regulatory system on adherence and invasion may be indirect, mediated through the action of other sets of genes. Complementation of the *esrA* and *esrB* insertion mutants with a wild-type copy of the genes restored adherence and internalization to levels similar to the wild-type strain (Table 4).

Mutations in *Salmonella spiA* (*ssaC*) and effector genes like *spiC* do not affect bacterial invasion (Ochman *et al.*, 1996; Uchiya *et al.*, 1999) as seen in *Ed. tarda esaC* (*spiA* homologue) and effector (*eseB*, *eseC* and *eseD*) mutants in the present study.

When the virulence of these mutants was compared, all of them were found to have attenuated virulence. Virulence of mutants with disruption in *esrA* and *esrB* was highly attenuated, with LD_{50} values raised at least two orders of magnitude above that of the wild-type, whereas the rest of the mutants had LD_{50} values increased by about 10-fold. In

the case of *Salmonella*, mutants harbouring SPI2 insertions were attenuated in mice (Hensel *et al.*, 1998; Shea *et al.*, 1996). Inactivation of regulator (*ssrA*) (Ochman *et al.*, 1996), effector (*sseA*, *sseB*, *sseC* and *spiC*) or apparatus (*spiA*) genes led to strong attenuation of virulence (Hensel *et al.*, 1998; Uchiya *et al.*, 1999). This differs from the case of *Ed. tarda* infection, in which the regulator genes appear to play a more significant role in virulence, as their disruption led to more dramatic attenuation. However, mutations in other *Salmonella* effector genes caused only slight (*sseF* and *sseG*) or no detectable attenuation of virulence (*sseE*) (Hensel *et al.*, 1998). Therefore, the extent of involvement of each gene in pathogenesis may vary in different bacteria.

EseB and autoaggregation in DMEM

All the TTSS mutants were attenuated and their ability to autoaggregate was reduced or abolished (Table 4). From the results obtained, failure of the *eseB* mutant to autoaggregate may indicate that secretion of EseB is required for autoaggregation. The EseB protein, homologous to EspA of EPEC, may be a component of an extracellular filamentous organelle or appendage that mediates autoaggregation. Therefore, when this gene is disrupted, the mutant fails to autoaggregate. Further evidence that EseB is likely to be involved in mediating the autoaggregation phenotype was provided by the restoration of this ability in the complemented *eseB* mutant upon IPTG induction of the *eseB* gene (data not shown). This phenotype was not observed in the complemented but uninduced mutant. In EPEC, *espD* mutants secrete low levels of EspA and produce shorter filaments (Knutton *et al.*, 1998) and in enterohaemorrhagic *E. coli*, synthesis of the EspD protein is essential for the formation of EspA-containing filaments (Kresse *et al.*, 1999). Mutations in *eseC* and *eseD* (homologous to *espD* and *espB* respectively) in *Ed. tarda* may also have affected the proper formation of putative EseB filaments which, in turn, reduced autoaggregation (Table 4). The EPEC CesD chaperone is essential for the proper secretion of EspB and EspD (Wainwright & Kaper, 1998). EscA, a homologue of CesD, may also be a putative chaperone essential for the proper secretion of EseB, EseC and/or EseD proteins. Disruption of EscA may therefore affect the proper formation of the putative EseB appendages, thus making autoaggregation impossible.

These results are clearly indicative of the participation of the putative *Ed. tarda* TTSS in phagocyte survival and virulence. Although this system was shown to affect the adherence and invasion of *Ed. tarda*, its precise involvement awaits further investigation in our ongoing experiments. For the insertion mutants, even though there could be potential polar effects on TTSS genes downstream, the characterization of mutants with disruption in genes from all the categories (apparatus, chaperone, effector and regulator), and the restoration of phenotypes in complemented mutants, should reflect the contribution of the TTSS to *Ed. tarda* pathogenesis, particularly in the three virulence-associated phenotypes examined.

The *Ed. tarda* TTSS is regulated by a putative *esrA–esrB* two-component system

Bacteria often sense environmental stimuli via two-component sensor-response regulatory systems (Garcia Vescovi *et al.*, 1996; Pratt & Silhavy, 1995). In *Salmonella* species, the expression of SPI2 genes is dependent on the SsrA–SsrB system which is encoded within the cluster itself (Cirillo *et al.*, 1998; Deiwick *et al.*, 1999; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996). Since *Ed. tarda* also possess a very similar EsrA–EsrB two-component system, this could likewise be involved in the regulation of the putative TTSS. When the ECP profiles of the wild-type and *esrA* and *esrB* mutants were analysed using 2-D PAGE (Fig. 2a, b; only the *esrB* mutant ECP profile is presented), the EseB, EseC and EseD proteins were missing or much reduced. This shows that the two-component system is likely to be controlling TTSS expression and/or protein secretion. For the *eseD* mutant (Fig. 2c), the EseB protein, but not EseC and EseD, was seen in the ECP, indicating that production and/or secretion of the latter two proteins may have been co-regulated. As described and discussed above, the *esrA* and *esrB* mutants were highly attenuated. This may be attributed to the loss of TTSS involvement in pathogenesis as a result of the disruption of its regulatory system. Without these important virulence factors, the bacteria failed to establish an infection in the fish host.

Conclusion

The identification of a putative TTSS in *Ed. tarda* certainly offers greater insight into the pathogenesis of this bacterium. The results of the characterization studies demonstrate the importance of the secretion system in phagocyte survival and bacterial replication and virulence. It also appears to affect *Ed. tarda* adherence to and internalization into epithelial cells. The restoration of these phenotypes in complemented mutants confirms the involvement of the TTSS in these functions. The *Ed. tarda* system is similar to that in *Salmonella* species in terms of the gene categories, the associated functions and regulation by a two-component system. However, differences do exist between the two systems, such as the position of the genes, their arrangement and organization, and regulation of the TTSS. Therefore, the two bacteria could have acquired the virulence gene cluster from different sources during evolution.

In *Ed. tarda*, the *esrA–esrB* two-component system is critical for regulating not only the expression of the secretion system genes, but also genes outside the cluster, such as the *evp* genes (Srinivasa Rao *et al.*, 2004; Zheng *et al.*, 2005). Therefore, it could also be regulating the adherence and invasion genes, either directly or indirectly, as in the case of *ssrA–ssrB* in SPI2, which controls gene expression both within the cluster and elsewhere in the chromosome (Beuzon *et al.*, 2000; Brumell *et al.*, 2003; Cirillo *et al.*, 1998).

The apparatus, chaperone and effector genes, on the other hand, may play more defined roles in the system, unlike the

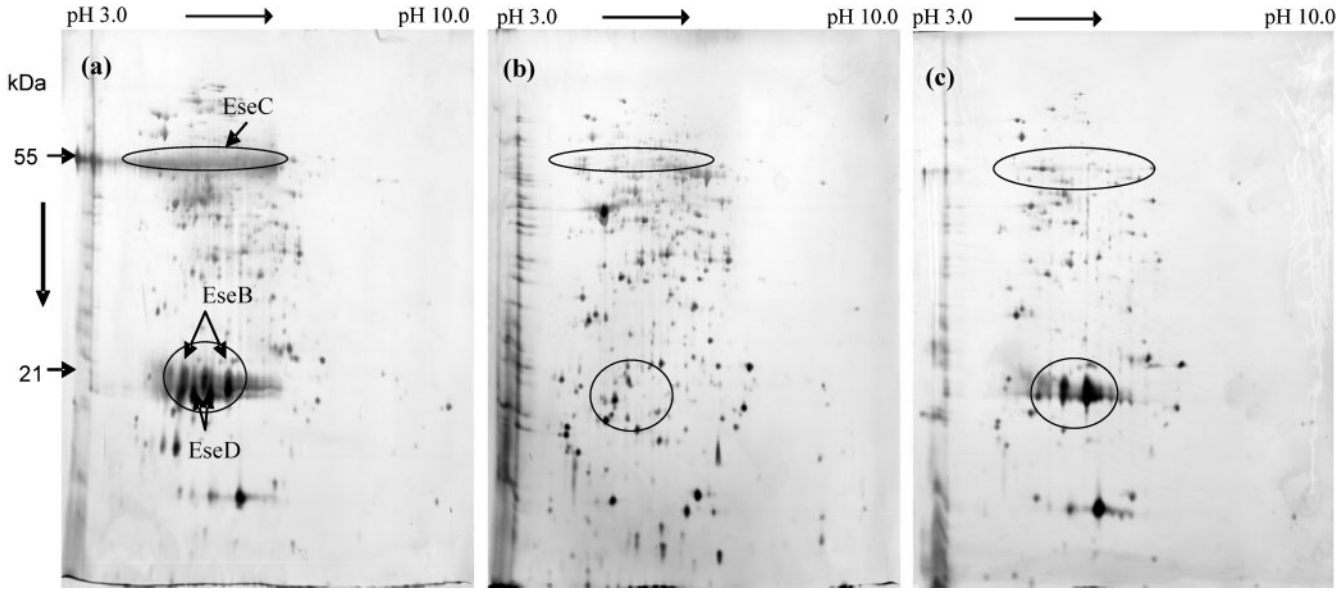

Fig. 2. 2-D ECP profile of (a) wild-type, (b) *esrB* and (c) *eseD* insertional mutants. The TTSS-encoded proteins (circled) are lost or much diminished in the *esrB* mutant. The *esrA* mutant has the same ECP profile as the *esrB* mutant (not shown). In the *eseD* mutant, only the EseB protein is present in the ECP.

regulators, so their inactivation brings about more specific changes. This is clearly reflected in the high attenuation of the mutants with regulator disruption because additional virulence factors (e.g. Evp proteins) may be lost (Srinivasa Rao *et al.*, 2004) besides those encoded by the TTSS. This contrasts with the more subtle decrease in virulence of the other mutants resulting from the loss of specific TTSS virulence factors. However, further studies are essential to understand the mechanism of the TTSS and to assign a more definitive role to each member of the cluster. These studies are currently ongoing in our laboratory.

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