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Type III secretion system genes of *Edwardsiella tarda* associated with intracellular replication and virulence in zebrafish

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ABSTRACT: The Type III secretion system (T3SS) is essential for intracellular replication of *Edwardsiella tarda* in phagocytes of fish and mammals. Two possible effector candidate genes (*eseE* and *eseG*) and 7 hypothetical genes (*esaB, escC*, *orf13*, *orf19*, *orf26*, *orf29*, and *orf30*) located in the T3SS gene cluster were inactivated by an allelic exchange method, and we found that *E. tarda* strains carrying insertion mutations in *escC*, *orf13*, *orf19*, *orf29*, and *orf30* were unable to replicate within J774 macrophages and HEp-2 epithelial cells. Furthermore, the virulence of these mutants to zebrafish was severely attenuated as well. These data suggest that the *escC*, *orf13*, *orf19*, *orf29*, and *orf30* genes are required for intracellular replication and virulence of *E. tarda*.

KEY WORDS: *E. tarda* T3SS genes · Intracellular replication · Virulence · *Danio rerio*

INTRODUCTION

Edwardsiella tarda, a member of the family *Enterobacteriaceae*, infects a wide variety of invertebrates and vertebrates such as fish, amphibians, reptiles, birds, and mammals, including humans. *E. tarda* infections (edwardsiellosis) in fish have caused severe economic losses in the aquaculture industry for cultured freshwater and marine fish species, including Japanese eel *Anguilla japonica* (Hoshina 1962, Wakabayashi & Egusa 1973), channel catfish *Ictalurus punctatus* (Meyer & Bullock 1973), red sea bream *Pagrus major* (Yasunaga et al. 1982), and Japanese flounder *Paralichthys olivaceus* (Nakatsugawa 1983), as reviewed by Evans et al. (2011). A variety of virulence factors of *E. tarda* have been reported, including the ability to invade and replicate in epithelial cells (Marques et al. 1984, Janda et al. 1991, Ling et al. 2000), to resist phagocyte-mediated killing (Srinivasa Rao et al. 2001), and to produce catalases, hemolysins, and dermato- or lethal toxins (Ullah & Arai 1983, Janda & Abbott 1993, Suprapto et al. 1996, Srinivasa Rao et al. 2003a). However, it is difficult to fully explain the infection mechanism of *E. tatda* only by these known virulence factors.

Recently, a Type III secretion system (T3SS), a 'weapon' possessed by many pathogenic bacteria, was also found in *E. tarda* (Tan et al. 2002, Srinivasa Rao et al. 2003b, 2004). T3SS injects effector proteins into the cytoplasm of host cells to modify host cell physiology or to subvert host immunity for promoting bacterial infection (Hueck 1998). The T3SS gene cluster is largely composed of genes encoding effectors, regulators, apparatuses, chaperones, and translocons. *E. tarda* T3SS was reported to consist of 35 open reading frames (ORFs), which are homologous to a T3SS encoded by *Salmonella* pathogenicity island 2 (SPI2), which is required for intracellular proliferation of *S. enterica* serovar Typhimurium (Tan et al. 2005). T3SS of *E. tarda* was also essential for survival and replication of the

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bacterium in J774 murine macrophages (Okuda et al. 2006). To our knowledge, no studies have reported on the identification of proteins in *E. tarda* T3SS which are essential for survival and replication of the bacterium in murine macrophages, although Xie et al. (2010) recently reported that EseG is an effector protein of *E. tarda* which triggers microtubule destabilization in HeLa cells. However, the contribution of microtubule destabilization activity of EseG to antiphagocytosis of *E. tarda* is still unclear.

In this study, to find further proteins in *E. tarda* T3SS which are associated with survival and replication in J774 murine macrophages and virulence to zebrafish *Danio rerio*, we inactivated the 2 genes (*eseE* and *eseG*) which are homologous to the known effector candidate and effector genes (*sseE* and *sseG*) of *S. enterica* serovar Typhimurium and 7 hypothetical genes (*esaB*, *escC*, *orf13*, *orf19*, *orf26*, *orf29*, and *orf30*) in the *E. tarda* T3SS gene cluster by using an allelic exchange method. We demonstrate that 5 genes (*escC*, *orf13*, *orf19*, *orf29*, and *orf30*) are required both for survival and replication within J774 murine macrophages and HEp-2 human epithelial cells and for virulence to zebrafish.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Edwardsiella tarda* wild-type strain FK1051 was isolated from a diseased Japanese flounder. mET1229 is a T3SS-deficient mutant of FK1051 (Okuda et al. 2006). FK1051 and the mutants were cultured at 30°C on Trypto-Soya Agar (TSA, Nissui) or in Trypto-Soy Broth (TSB, Eiken). *Escherichia coli* were routinely grown on Luria-Bertani (LB) agar or in LB broth at 37°C. Stock cultures were maintained at −80°C as a suspension in supplemented broth containing 25% (v/v) glycerol.

Construction of *E. tarda* insertion mutants

Information on the ORFs of *E. tarda* T3SS genes tested in this study is provided in Table 2. Insertion mutations of 9 genes (*esaB*, *eseE*, *eseG*, *escC*, *orf13*, *orf19*, *orf26*, *orf29*, and *orf30*) encoded in the T3SS gene cluster of *E. tarda* FK1051 were performed as

Table 1. Bacterial strains and plasmids used in this study. Col^r: colistin resistance; Cm^r: chloramphenicol resistance; Km^r: kanamycin

Strain/mutant or plasmid	Characteristics	Source or reference
***Edwardsiella tarda* strains**		
FK1051	Wild type, virulent, Col^r	Diseased Japanese flounder[b]
mET1229	*esaV* insertion mutant from FK1051, Col^r Km^r	Okuda et al. (2006)
orf19	*orf19* insertion mutant from FK1051, Col^r Cm^r	This study
orf13	*orf13* insertion mutant from FK1051, Col^r Cm^r	This study
esaB	*esaB* insertion mutant from FK1051, Col^r Cm^r	This study
eseG	*eseG* insertion mutant from FK1051, Col^r Cm^r	This study
eseE	*eseE* insertion mutant from FK1051, Col^r Cm^r	This study
escC	*escC* insertion mutant from FK1051, Col^r Cm^r	This study
orf26	*orf26* insertion mutant from FK1051, Col^r Cm^r	This study
orf29	*orf29* insertion mutant from FK1051, Col^r Cm^r	This study
orf30	*orf30* insertion mutant from FK1051, Col^r Cm^r	This study
***E. coli* strains**		
SY327λ*pir*	Strain containing the *pir* factor	Okuda et al. (2001)
SM10λ*pir*	*thi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA* RP4-2-*Tc*::*Mu* λ*pir*	Okuda et al. (2001)
Plasmids		
pRE112	Suicide vector, *sacB* Cm^r	Okuda et al. (2001)
pRE112-*orf19*	pRE112 with internal fragment of *orf19* (229 bp)	This study
pRE112-*orf13*	pRE112 with internal fragment of *orf13* (164 bp)	This study
pRE112-*esaB*	pRE112 with internal fragment of *esaB* (272 bp)	This study
pRE112-*eseG*	pRE112 with internal fragment of *eseG* (255 bp)	This study
pRE112-*eseE*	pRE112 with internal fragment of *eseE* (126 bp)	This study
pRE112-*escC*	pRE112 with internal fragment of *escC* (214 bp)	This study
pRE112-*orf26*	pRE112 with internal fragment of *orf26* (254 bp)	This study
pRE112-*orf29*	pRE112 with internal fragment of *orf29* (510 bp)	This study
pRE112-*orf30*	pRE112 with internal fragment of *orf30* (638 bp)	This study

Table 2. Information on open reading frames (ORFs) of *Edwardsiella tarda* Type III secretion system (T3SS) genes tested in this study. ORF names follow Tan et al. (2005), except EscC, which follows Zheng et al. (2007). *E. tarda* EIB202 genomic sequence data were retrieved from GenBank (accession number CP001135.1), and a BLAST search was carried out for the analyses of similar proteins, conserved domains, and functions

ORF	Gene name	Locus tag	Accession number	Similar protein (% identity)		Conserved domain	Function
				Salmonella enterica serovar Typhimurium	*Chromobacterium violaceum*		
ORF19	*orf19*	ETAE_0856	ACY83701.1	STM1410 (28)	CV2589 (36)	Type III secretion apparatus associated with locus of enterocyte effacement (LEE)	Hypothetical protein
ORF13	*orf13*	ETAE_0862	ACY83707.1		CV2595 (38)	T3SS protein, YseE family	Hypothetical protein
EsaB	*esaB*	ETAE_0865	ACY83710.1		CV2598 (30)		Hypothetical protein
EseG	*eseG*	ETAE_0866	ACY83711.1	SseF (34)		Pathogenicity island 2 effector protein SseF and SseG domains	Hypothetical protein
EseE	*eseE*	ETAE_0868	ACY83713.1	SseE (25)	SseE (33)	Pathogenicity island 3 effector candidate protein SseE domain	T3SS effector candidate protein E
EscC	*escC*	ETAE_0873	ACY83718.1			PRK11820 hypothetical protein domain	T3SS chaperone
EsaO	*esaO*	ETAE_0876	ACY83721.1				T3SS ATPase
ORF26	*orf26*	ETAE_0884	ACY83729.1		Peptidoglycan N-acetylmuramoyl-hydrolase (51)	Lytic transglycosylase (LT) and goose egg white lysozyme (GEWL) domains	Putative transglycosylase signal peptide protein
ORF29	*orf29*	ETAE_0887	ACY83732.1				Hypothetical protein
ORF30	*orf30*	ETAE_0888	ACY83733.1				Putative T3SS effector protein

described previously with a slight modification using the suicide vector plasmid pRE112 (Okuda et al. 2001, 2009, Tan et al. 2005). Internal fragments of each gene were amplified by PCR using the primers listed in Table 3. The primers were designed where the amplicon has a *Sac*I site and stop codons at the 5′-end and a *Sac*I site at the 3′-end. The PCR amplification was performed using *Taq* polymerase (Ex Taq; Takara) and the following protocol: 30 s denaturation at 95°C, 1 min annealing at 50°C, and an extension step at 72°C for 1.5 min (30 cycles); followed by a final extension step at 72°C for 5 min. The amplified fragments were digested using *Sac*I and cloned into *Sac*I-cleaved pRE112. The resulting plasmids were first constructed using an *E. coli* SY327λ*pir* background, and *E. coli* SM10λ*pir* was transformed with them. These plasmids were mobilized into *E. tarda* FK1051 using conjugation. Transconjugants were selected on TSA supplemented with chloramphenicol (30 µg ml^{-1}) and colistin (10 µg ml^{-1}) at 25°C. Insertional inactivation then occurred, resulting in the integration of the cloned suicide vector into each gene in the wild-type genome as described previously (Tan et al. 2005). After confirming the integration of the cloned suicide vector into each gene by PCR, single strains retaining each mutant gene were selected as the *E. tarda* insertion mutants, respectively.

Tissue culture

J774, a murine macrophage cell line derived from BALB/c mice, and HEp-2 cells from human laryngeal carcinoma were used in this study. Both cell lines were maintained in RPMI1640 (Sigma) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2.

Intracellular replication assay

A 24-well tissue culture plate (Iwaki) was inoculated with 7.5×10^4 cells $well^{-1}$ and incubated overnight at 37°C under 5% CO_2. The cells were infected with either FK1051, mET1229, or 1 of 9 insertion mutants at a multiplicity of infection (MOI) of 1 for 30 min, before pre-warmed tissue culture medium containing gentamicin was added at a final concentration

Table 3. Primers used for construction of insertion mutants. Restriction site sequences are underlined

Gene	Primer name	Sequence (5′ to 3′)
orf19	5-SacI-stop-orf19	GAGAGAGCTCTGACTGACTGAACTGCTGGCAGTCGATCTA
	3-SacI-orf19	AGAGGAGCTCTTTCTCGGTACGGCTTGAG
orf13	5-SacI-stop-orf13	GAGAGAGCTCTGACTGACTGACTAACGCATCTTGAAGATTC
	3-SacI-orf13	AGAGGAGCTCAGACACGACTGACGCTGA
esaB	5-SacI-stop-esaB	GAGAGAGCTCTGACTGACTGATACCCTAATGACATGAACAG
	3-SacI-esaB	AGAGGAGCTCCAGAACTGCTCCTGTTCAAC
eseG	5-SacI-stop-eseG	GAGAGAGCTCTGACTGACTGATAAGGAGCCTATGATGAT
	3-SacI-eseG	AGAGGAGCTCACTTTGGCGACAAAGCTGC
eseE	5-SacI-stop-eseE	GAGAGAGCTCTGACTGACTGATGGAGAGCGAGTTTCTCAT
	3-SacI-eseE	AGAGGAGCTCTAACGGCCTGTCCATCGTCATT
escC	5-SacI-stop-escC	GAGAGAGCTCTGACTGACTGATTGCAACTGATGCAAGACCTG
	3-SacI-escC	AGAGGAGCTCTTTGCAGGCGGCTGATATTG
orf26	5-SacI-stop-orf26	GAGAGAGCTCTGACTGACTGATGAATCCTCCTATCGTTATG
	3-SacI-orf26	AGAGGAGCTCTTTCAGGATAGGGAGGAATC
orf29	5-SacI-stop-orf29	GAGAGAGCTCTGACTGACTGATATGACGATACGACACAGGA
	3-SacI-rf29	AGAGGAGCTCGAACAGGAGATTGAACGTAT
orf30	5-SacI-stop-orf30	GAGAGAGCTCTGACTGACTGATCAATCAGCAGGTGTATCAG
	3-SacI-orf30	AGAGGAGCTCAAACAGCGTATCCTGTATCA

of 200 µg ml^{-1}. After 30 min of incubation, the medium was removed, the cell culture plates were washed twice with phosphate-buffered saline (PBS), and new tissue culture medium without gentamicin was added. Both J774 and HEp-2 cells were incubated for 0, 12, or 24 h at 37°C under 5% CO_2. At the end of each time period, PBS containing 1% Triton X-100 was added to each well in order to lyse the cells. The lysed solution diluted with PBS was plated on TSA, and the number of viable bacteria was counted.

Fish and experimental infection

Healthy zebrafish were kept in an aquarium with a recirculation filtration system at a water temperature of 25°C. Fish were 2.59 ± 0.29 (SD) cm long and weighed 0.229 ± 0.061 g. Before infection, fish were acclimated for several days.

Overnight cultures of *E. tarda* FK1051, mET1229, and 9 insertion mutants were inoculated into TSB and incubated at 30°C until the OD_{600} reached 0.7. The cultures were then diluted 1000-fold with PBS and used for injection into zebrafish.

Zebrafish were injected intraperitoneally (i.p.) with 5 µl (10^3 CFU) of either *E. tarda* FK1051, mET1229, 1 of the 9 insertion mutants, or PBS for control fish. We injected 20 fish per strain. After injection, fish were moved to a 12 l aquarium with a recirculation filtration system at a water temperature of 25°C, and the course of mortality was recorded daily for 10 d. Bacterial isolation from dead fish was carried out on TSA supplemented with colistin at 25°C. *E. tarda* was identified by an agglutination reaction with a rabbit anti-*E. tarda* serum.

RESULTS

Intracellular replication of *Edwardsiella tarda* mutants

J774 cells. All genes examined, except for *orf1B*, were inactivated successfully by the allelic exchange method with the suicide vector pRE112 (Fig. 1). In order to clarify whether the 7 hypothetical genes, except *esaO*, are required for survival and replication of *E. tarda* in macrophages, we compared the level of intracellular replication of the 9 insertion mutants including the *eseE* and *eseG* genes after infection of J774 macrophages (MOI = 1). The wild-type strain FK1051 efficiently replicated in the cells but its T3SS mutant mET1229 showed greatly reduced replication, as described previously (Okuda et al. 2006). On the other hand, intracellular survival or replication of the 9 insertion mutants in J774 cells at 0, 12, and 24 h after infection varied depending on each inactivated gene (Fig. 2A). Although the *esaB*, *eseG*, *eseE*, and *orf26* mutants replicated in the cells at comparable levels (12 h post infection) to the wild-type strain or at slightly decreased levels (24 h post

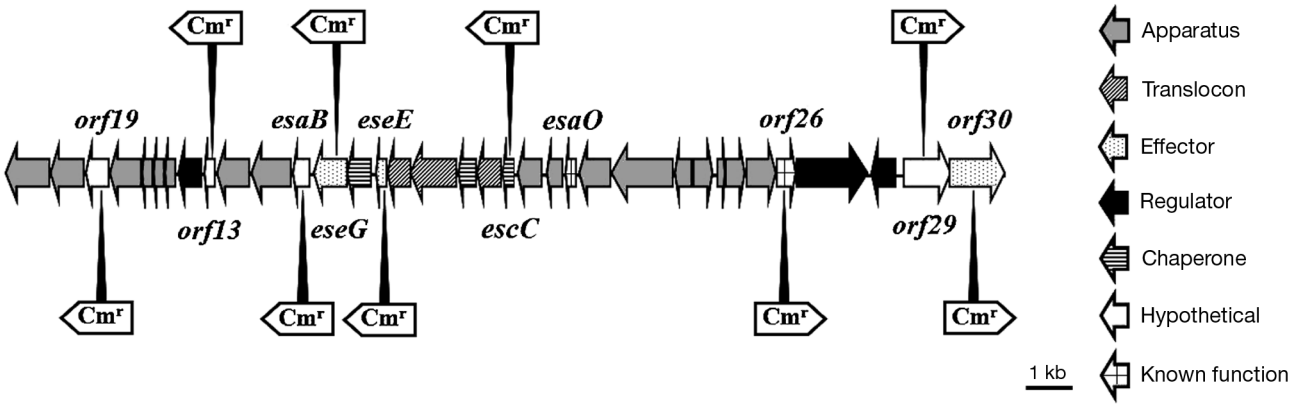

Fig. 1. *Edwardsiella tarda* Type III secretion system (T3SS) gene cluster and location of insertional mutations. *E. tarda* T3SS is composed of 35 genes encoding apparatuses, translocons, effectors, regulators, chaperones, and known-function and hypothetical proteins (Tan et al. 2005, Yang et al. 2012). The chloramphicol resistance (Cm^r) gene was inserted in the possible effector and hypothetical genes by an allelic exchange method using the suicide vector pRE112

infection), intracellular replication of the *escC*, *orf13*, *orf19*, *orf29*, and *orf30* mutants was greatly reduced compared to the wild-type strain (Fig. 2A).

HEp-2 cells. The *esaB*, *eseG*, *eseE*, and *orf26* mutants replicated in HEp-2 cells to the same or slightly decreased levels compared to the wild-type strain at 12 and 24 h post infection, while the *escC*, *orf13*, *orf19*, *orf29*, and *orf30* mutants were completely killed in HEp-2 cells at 24 h post infection (Fig. 2B).

Virulence to zebrafish

In case of the wild-type strain FK1051, fish died at 1 to 5 d post injection and the cumulative mortality for 10 d reached 85%, while that of fish infected with mET1229 was only 10% (Fig. 3A). The cumulative mortality of zebrafish injected with the *escC*, *orf13*, *orf19*, *orf29*, and *orf30* mutants, which showed greatly attenuated replication ability in J774 and HEp-2 cells (Fig. 2), was 20, 25, 35, 10, and 15%, respectively (Fig. 3B). On the other hand, the cumulative mortality of fish infected with the *esaB*, *eseE*, *eseG*, and *orf26* mutants was 85, 90, 80, and 65%, respectively (Fig. 3C).

DISCUSSION

Anti-phagocytosis is an important virulence factor for *Edwardsiella tarda* to establish infection in its host (Srinivasa Rao et al. 2001, Okuda et al. 2006). After phagocytosis, phagocytes produce reactive oxygen species (ROS) having scavenger activity such as hydrogen peroxide (H_2O_2) and hydroxy radicals (OH^-) from superoxide (O_2^-) to kill bacteria in the phagosome. Although *E. tarda* does produce superoxide dismutase (SodB) and catalase (KatB) to neutralize the bactericidal action by ROS (Yamada & Wakabayashi 1999, Srinivasa Rao et al. 2003a), it is difficult to explain how *E. tarda* escapes from phagocytotic killing and replicates in the phagosome only by secretion of SodB and KatB. Okuda et al. (2006) reported that *E. tarda* T3SS was essential for replication of the bacterium within the phagosome of murine macrophages. The present study suggests that 2 possible effector candidate genes (*eseE* and *eseG*) and 7 hypothetical genes (*esaB*, *escC*, *orf13*, *orf19*, *orf26*, *orf29*, and *orf30*) found in the *E. tarda* T3SS gene cluster are effector genes (Fig. 2). The 2 candidate genes (*eseE* and *eseG*) have homology to known effector candidate and effector genes (*sseE* and *sseG*) encoded by SPI2 of *Salmonella enterica*, both of which are associated with proliferation in RAW264.7 murine macrophages (Hensel et al. 1998). The SseG protein contributes to the formation of *Salmonella* induced-filament (Sif) and the positioning of *Salmonella*-containing vacuoles in a juxtanuclear and Golgi network (Kuhle & Hensel 2002, Salcedo & Holden 2003). The function of the SseE protein is unknown at present. Based on previous findings for SseE and SseG, EseG and EseE proteins of *E. tarda* are probably effector proteins providing functions other than intracellular replication. Xie et al. (2010) reported that EseG is an effector protein of *E. tarda* which triggers microtubule destabilization, although the contribution of microtubule destabilization activity of EseG to *E. tarda* pathogenesis is still unclear since knockout of the *eseG* gene exhibited only slight attenuation of virulence to blue gourami *Trichogaster trichopterus*.

The *esaB* and *orf26* gene mutants replicated within J774 macrophages. The *esaB* gene has weak

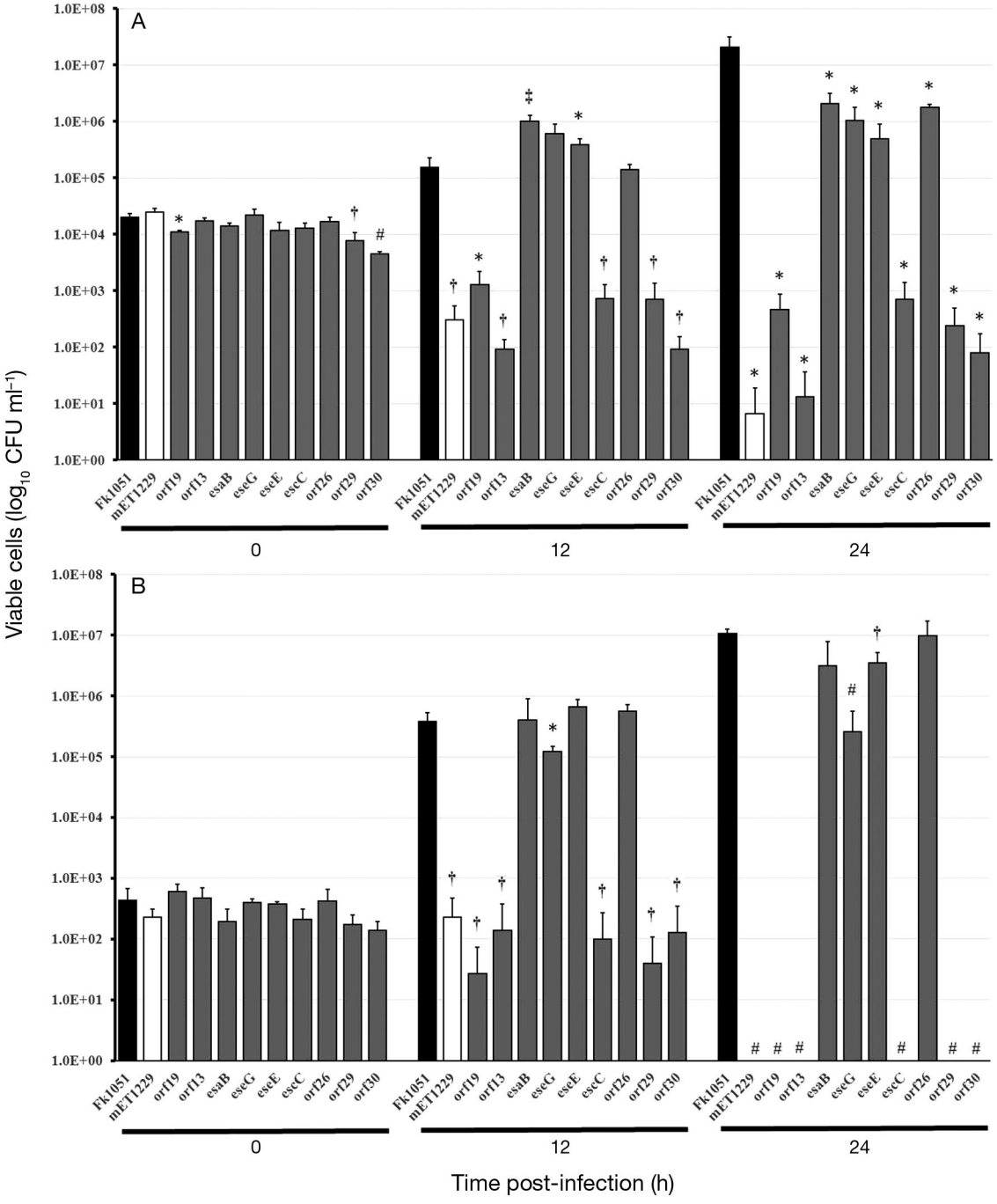

Fig. 2. Intracellular survival and replication of *Edwardsiella tarda* in (A) J774 macrophages and (B) HEp-2 cells. J774 macrophages and HEp-2 cells were infected with the *E. tarda* wild-type strain FK1051 (black bars), mET1229 (a Type III secretion system mutant of FK1051; white bars) or 1 of 9 insertion mutants of *E. tarda* (grey bars) at a multiplicity of infection of 1. Macrophages and HEp-2 cells were lysed and the number of viable bacteria in the cells was counted at 0, 12, and 24 h post infection. Each assay was performed in triplicate, and the results are means ± SD. Statistical analysis was performed using a 2-tailed *t*-test. Significant differences compared to FK1051 are shown by symbols: $^{*}p < 0.05$, $^{\ddagger}p < 0.02$, $^{\ddagger}p < 0.01$, $^{\#}p < 0.002$

homology both to the *spiC* effector gene and the *ssaB* T3SS apparatus gene of *S. enterica*. As the *Salmonella spiC* mutant was reported to show reduced replication ability in macrophages (Uchiya et al. 1999), the *esaB* gene is more likely to code an apparatus protein.

In contrast, 5 strains with insertion mutations in the *escC*, *orf13*, *orf19*, *orf29*, and *orf30* genes failed to

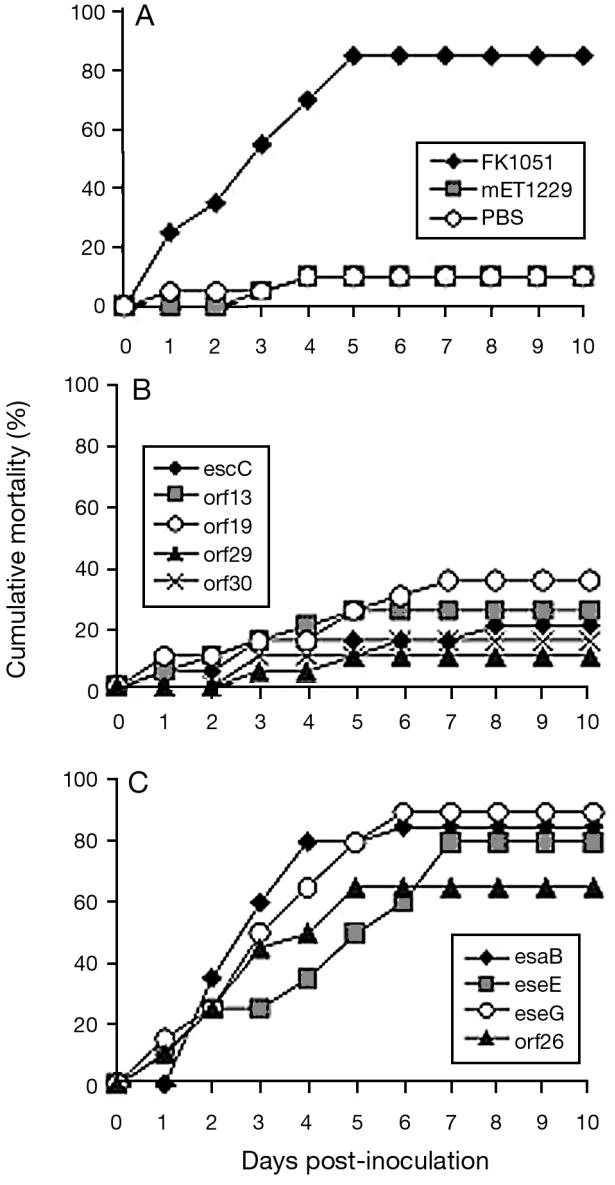

Fig. 3. Cumulative mortality (%) of zebrafish *Danio rerio* infected with *Edwardsiella tarda* strains. *E. tarda* (10^3 CFU) was intraperitoneally injected into 20 zebrafish, and their viability was recorded for 10 d. Phosphate-buffered saline (PBS) was injected into control fish. Results are shown for fish injected with (A) *E. tarda* wild-type strain FK1051, mET1229 (a Type III secretion system mutant of FK1051), and PBS (controls); (B) insertion mutants of the *escC*, *orf13*, *orf19*, *orf29*, and *orf30* genes; and (C) insertion mutants of the *esaB*, *eseE*, *eseG*, and *orf26* genes

proliferate in J774 macrophages. The *escC*, *orf13*, and *orf19* genes have homology to CV2581, CV2596, and CV2589 of *Chromobacterium violaceum*, respectively, but their function is not well known. The *orf29* and *orf30* genes were reported to be regulated by both the EsrA-EsrB 2-component system and EsrC (Zheng et al. 2005). The present genes (*escC*, *orf13*, *orf19*, *orf29*, and *orf30*) may be effector genes required for survival and replication within macrophages, although further study using the *cyaA* gene fusion system (Sory et al. 1995) is needed to confirm translocation of the present genes into macrophages.

E. tarda can invade and replicate in epithelial cells such as HeLa and HEp-2 cells (Marques et al. 1984, Janda et al. 1991). Further, T3SS is required for proliferation of *E. tarda* in HEp-2 cells (Okuda et al. 2008). These observations suggest that T3SS is essential for survival and replication of *E. tarda* not only in phagocytes but also in epithelial cells. In the present study, the 5 strains with insertion mutations in the *escC*, *orf13*, *orf19*, *orf29*, and *orf30* genes were completely killed in HEp-2 cells at 24 h after infection (Fig. 2), which suggests that these genes may play a key role in the replication of *E. tarda* within epithelial cells as well as in murine macrophages. *Salmonella enterica* harbors 2 SPIs, each encoding a T3SS for virulence proteins. SPI1 is required for invasion into epithelial cells, while intracellular accumulation of *Salmonella* in macrophages is dependent on SPI2 function (Hansen-Wester & Hensel 2001). In contrast, a whole-genome comparison of multiple *E. tarda* strains revealed that *E. tarda* genotypes were broadly clustered into 2 major subpopulations: pathogenic *E. tarda* genotype EdwGI, which represents a genotype of fish pathogens and possesses virulence factor genes such as T3SS and the Type VI secretion system (T6SS); and the nonpathogenic or environmental *E. tarda* genotype EdwGII (Yang et al. 2012). Further, *E. tarda* isolated from red sea bream had T3SS (Et-LEE) and T6SS (Et-T6SS2) genes, which suggests that the genotype EdwGI could be divided into 2 sub-genotypes, viz. a typical T3SS/T6SS-bearing sub-genotype and an atypical Et-LEE/T6SS2-bearing one (Nakamura et al. 2013). In present study, we used *E. tarda* FK1051 strain as a wild-type strain. The FK1051 strain was isolated from a diseased Japanese flounder, suggesting that FK1051 belongs to a typical T3SS/T6SS-bearing EdwGI genotype strain.

We assumed that *E. tarda* injects effector candidates such as the *escC*, *orf13*, *orf19*, *orf29,* and *orf30* both for invasion into epithelial cells and for proliferation in macrophages by using the T3SS.

T3SS is important for *E. tarda* virulence against hosts such as blue gourami (Tan et al. 2005). In the present study, virulence of insertion mutants to the host was evaluated by exploiting zebrafish. Zebrafish have been used as model vertebrate organisms in various fields including microbiology (Neely et al.

2002, Prouty et al. 2003), because this species is easy to cultivate and maintain and its whole genome has been sequenced. Pressley et al. (2005) reported that adult zebrafish were susceptible to challenge by i.p. injection of *E. tarda*. We used the adult zebrafish model to evaluate the virulence of *E. tarda* mutants. The virulence of *E. tarda* FK1051 after i.p. injection to zebrafish was very strong, whereas 5 insertion mutants of the *orf19*, *orf13*, *escC*, *orf29*, and *orf30* genes, which could not survive and replicate in J774 macrophages and HEp-2 cells, showed attenuated virulence to zebrafish as compared with the wild-type strain (Fig. 3). Taken together, our results strongly suggest that intracellular growth of *E. tarda* is a key factor for *E. tarda* to show virulence against its host and that T3SS is important for pathogenicity of this bacterium, and also that 5 genes (*escC*, *orf13*, *orf19*, *orf29*, and *orf30*) are essential for virulence of *E. tarda* in zebrafish. However, we cannot deny the possibility that the observation of reduced survival and replication of *E. tarda* within host cells and attenuated virulence to zebrafish may be due to polar effects of insertion mutagenesis on genes downstream of the target gene. Complementation of the mutants with the functional genes will help to clarify this point.

In conclusion, the *orf19*, *orf13*, *escC*, *orf29,* and *orf30* genes were identified as the T3SS genes that are responsible for survival and replication of *E. tarda* within host cells. Our next aim is to clarify the unknown mechanism(s) of intracellular replication of *E. tarda* by exploiting these hypothetical genes.

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