RESEARCH LETTER

# The type III secretion system is involved in the invasion and intracellular survival of *Escherichia coli* K1 in human brain microvascular endothelial cells

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*Escherichia coli* K1; meningitis; type III secretion system; blood–brain barrier.

## Abstract

Type III secretion systems (T3SSs) have been documented in many Gram-negative bacteria, including enterohemorrhagic *Escherichia coli*. We have previously shown the existence of a putative T3SS in meningitis-causing *E. coli* K1 strains, referred to as *E. coli* type III secretion 2 (ETT2). The sequence of ETT2 in meningitis-causing *E. coli* K1 strain EC10 (O7:K1) revealed that ETT2 comprises the *epr*, *epa* and *eiv* genes, but bears mutations, deletions and insertions. We constructed the EC10 mutants deleted of ETT2 or *eivA* gene, and their contributions to bacterial pathogenesis were evaluated in human brain microvascular endothelial cells (HBMECs). The deletion mutant of ETT2 exhibited defects in invasion and intracellular survival compared with the parental *E. coli* K1 strain EC10. The mutant deleted of *eivA* within ETT2 was also significantly defective in invasion and intracellular survival in HBMECs, and the defects of the *eiv* mutant were restored to the levels of the parent strain EC10 by transcomplementation. These findings suggest that ETT2 plays a role in the pathogenesis of *E. coli* K1 infection, including meningitis.

## Introduction

Type III secretion systems (T3SSs) are found exclusively among Gram-negative bacteria and have been shown to be involved in the transport of structurally diverse bacterial virulence proteins across the bacterial membranes and host cell barriers, into the host cell interior (Lee, 1997; Cornelis, 2002). Intracellular pathogens, including *Salmonella*, *Shigella* and *Chlamydia*, use T3SSs for invasion of and/or multiplication within host cells (Menard *et al.*, 1996; Galan, 2001; Fields *et al.*, 2003) or use the T3SS to resist uptake by phagocytic cells (Cornelis & Van Gijsegem, 2000). *Escherichia coli* strains causing diarrheal disease use the T3SS to deliver effector proteins that result in the formation of attaching/effacing lesions (Celli *et al.*, 2000). Sequence analyses have revealed that most bacteria contain only one virulence-associated T3SS, but some pathogens have two T3SSs (Haller *et al.*, 2000; Foultier *et al.*, 2002). The genome sequence of enterohemorrhagic *E. coli* O157 strain, a major food-borne infectious pathogen that causes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome, revealed the presence of a second type III secretion or *E. coli* type III secretion 2 (ETT2) (Hayashi *et al.*, 2001; Perna *et al.*, 2001). The ETT2 gene cluster is shown to be present in whole or in part in the majority of *E. coli* strains but is unable to encode a functional secretion system in most strains (Ren *et al.*, 2004; Zhang *et al.*, 2004).

Neonatal bacterial meningitis is a devastating disease. Despite the availability of effective bactericidal antibiotics over the last 50 years, neonatal bacterial meningitis remains an important cause of high mortality and morbidity (Kim, 2003). In a previous paper, we have demonstrated by comparative genome hybridization that *E. coli* K1 strain EC10 isolated from the cerebrospinal fluid (CSF) of a neonate with meningitis harbors the *epr*, *epa* and *eiv* genes encoding ETT2 apparatus proteins (Yao *et al.*, 2006). The schematic representation of the ETT2 genes in the *E. coli* strain EC10 is shown in Fig. 1. However, the role of ETT2 in the pathogenicity of *E. coli* K1 infection is unclear.

Bacterial pathogens breach the blood–brain barrier (BBB) and enter the central nervous system (CNS) through paracellular, transcellular mechanism(s) or using the Trojan







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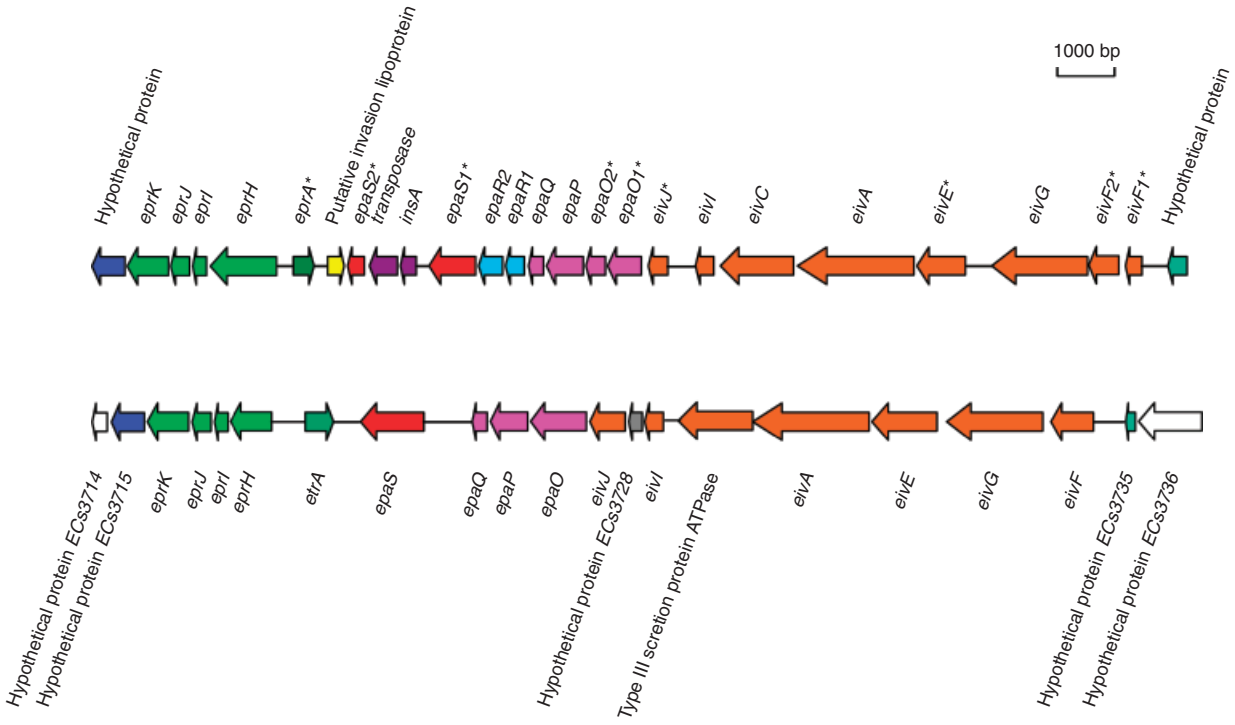


**Fig. 1.** The ORFs of sequenced T3SS locus from *Escherichia coli* K1 strain EC10 (right) were compared with those of *E. coli* strain Sakai (left). All the truncated ORFs in the strain EC10 were marked with an asterisk.

horse mechanism (Kim, 2008). We have developed the *in vitro* BBB model by isolation and cultivation of human brain microvascular endothelial cells (HBMECs) (Stins *et al.*, 1997). The HBMEC monolayers have been shown to exhibit morphological and functional properties of tight junction formation as well as polar monolayer (Stins *et al.*, 2001; Kim, 2003). The HBMECs have been shown to be useful for elucidating the microbial–host interactions that are involved in traversal of the BBB by meningitis-causing microorganisms, including *E. coli* (Kim, 2003). For example, we have shown that meningitis-causing *E. coli* K1 strains are able to invade HBMEC monolayers, as shown by the localization of internalized *E. coli* K1 within membrane-bound vacuoles (Kim, 2003; Kim *et al.*, 2003), and traverse the HBMEC monolayers without intracellular multiplication (Nemani *et al.*, 1999; Kim, 2003). However, *E. coli* K1 traversal of the HBMEC monolayers as live bacteria requires the K1 capsule (Hoffman *et al.*, 1999; Kim *et al.*, 2003).

In the present study, we investigated whether ETT2 plays a role in *E. coli* K1 interaction with HBMECs, using mutants deleted of the whole ETT2 locus (designated as TTKO) or *eivA* gene, a homologue of *invA* gene, which encodes a protein involved in the *Salmonella enterica* invasion of the eukaryotic cells. We showed that the ETT2 is involved in *E. coli* K1 invasion and survival in HBMECs, indicating that the ETT2 we identified from the meningitis-causing *E. coli* K1 may play a role in the microbial–host interactions relevant to *E. coli* K1 infection, including meningitis.

## Materials and methods

### *Escherichia coli* strains and growth conditions

*Escherichia coli* K1 strain EC10 (O7:K1) was isolated from the CSF of a neonate with meningitis (Yao *et al.*, 2006). A laboratory *E. coli* K-12 strain HB101 was used as a negative control. All bacteria were grown in Luria–Bertani (LB) broth with appropriate antibiotics.

### Confirmation of T3SS locus in *E. coli* K1 strain EC10

Based on the ETT2 sequence of *E. coli* O157 strain Sakai, primers for amplifying each ORF and intergenic region were designed by PRIMER 3 (http://frodo.wi.mit.edu/primer3). PCR products were sequenced at Johns Hopkins Medical Institution DNA Sequencing Facility. The DNA sequence of the *E. coli* strain EC10 ETT2 locus was submitted to GenBank (accession number EU179217).

## Construction of mutants deleted of ETT2 gene cluster or *eivA* gene

The mutants deleted of the ETT2 or *eivA* of *E. coli* EC10 were constructed using the protocol described by Datsenko & Wanner (2000). Briefly, strain *E. coli* EC10 was transformed with plasmid pKD46, which encodes the arabinose-inducible lambda red recombinase that promotes gene recombination between linear DNA and the host chromosome based on extremely short stretches of homology (50 nucleotides). PCR primers TTKO-F and TTKO-R (for entire ETT2 deletion), or eivA-KOF and eivA-KOR (for *eivA* deletion) (Table 1), contain 50 nucleotides of 5′-flanking sequences exactly matching with the 5′ and 3′ ends of the targeted deletion regions. The resultant PCR product was gel purified and then electrotransformed into the pKD46-containing strain EC10, which had been incubated with 10 mM of arabinose during the procedure of making competent cells. After cultivation in Super Optimal Catabolite medium (Invitrogen, Carlsbad, CA) for 1 h at 37 °C, the bacteria were plated onto LB agar containing antibiotics to select for recombinant transformants. The successful deletions were confirmed by PCR and sequencing with primers (TTKO CHK-F and TTKO CHK-R for ETT2 deletion mutant, and eivA-CHK-F and eivA-CHK-R for *eivA* single gene deletion mutant; Table 1). Of note, *eivA* is located in the middle of the *eiv* operon. To avoid the polar effect of the gene replacement, the antibiotic-resistance cassette was flipped out through transiently expressed FLP recombinase from temperature-sensitive plasmid pCP20, which resulted in a 'scar' that contained essential ribosomal binding sequence for translation of downstream genes.

## Transcomplementation of the *eivA* deletion mutant

As *eivA* gene is located in the middle of the *eiv* operon, it is unclear whether an independent promoter exists for *eivA* gene. Therefore, we assembled a construct whose expression of *eivA* gene is under the control of the native *eiv* operon promoter. Briefly, 673 bp of the upstream region of *eivF*, the first gene in the *eiv* operon, was amplified with the eivA-IF-RF and eivA-IF-RR primer pair using high-fidelity Pfu Ultra II DNA polymerase (Stratagene) (Table 1). The entire *eivA* gene including start and stop codons was amplified with the eivA-IF-LF and eivA-IF-LR primer pair (Table 1). Seamless assembly of *eiv* promoter, *eivA* gene and pACYC184 vector backbone (prelinearized with overnight BamHI and SalI double-digestion) was achieved using the In-Fusion PCR cloning kit (Clontech), where three modules were mixed and used to dissolve In-Fusion cloning beads. After electroporation, transformants were confirmed by checking PCR and plasmid sequencing using tetA-CHK-F and tetA-CHK-R primers (Table 1). The complementation vector was designated as pACYC-eivAc. For complementation, EC10-ΔeivA or TTKO mutants were transformed with either pACYC-eivAc or vector control pACYC184.

## Culture of HBMECs and *E. coli* invasion and intracellular survival assays

HBMECs were isolated, cultured and used for assays as described previously (Stins *et al.*, 1997). Invasion assays used approximately $10^6$ bacteria added to a well containing a confluent monolayer of HBMECs at a multiplicity of infection of 10 and a 2-h incubation period (Prasadarao *et al.*, 1996). The number of invaded bacteria was determined after the extracellular bacteria were killed by incubation of the monolayer with experimental medium containing gentamicin ($100\,\mu g\,mL^{-1}$) for 1 h and lysis of HBMECs in sterile distilled water. The released intracellular bacteria were enumerated by culturing on sheep-blood agar plates. All the experiments were repeated three times in triplicate. Results were expressed as percent invasion, determined as $100 \times$ [(number of internalized bacteria recovered)/

**Table 1.** Oligonucleotides used in the experiments

| Primers | Sequences |
|---|---|
| TTKO-F | CGAGTGCATCTGCAGGGAACATCTGCGATATTTGAATATCA-GGCTTACCCGTGTAGGCTGGAGCTGCTTC |
| TTKO-R | TTGTTTGCTGATGCCTTGCCCTTTGCATCAGAAGTTGTGCTA-TTCAGTAACATATGAATATCCTCCTTA |
| TTKO CHK-F | GATTGCTCTAACCGCTGCTC |
| TTKO CHK-R | CCGAAGCCAAATTGAGGTAA |
| eivA-KOF | AATGGTGATGATTATCGCCATGCTCATAATTCCATTGCCCA-CCTACTTAATCGTGTAGGCTGGAGCTGCTTC |
| eivA-KOR | GATAAAACGACGAATATCCACAGATCCCAGTAAAATAATG-TCTTTAATAGGAATATGGGAATTAGCCATGGTCC |
| eivA-CHK-F | ATGTTTAACAAAGTTTTGATAGGGTTA |
| eivA-CHK-R | AATAGTTTTCAGAATATTAACGGGAAC |
| eivA-IF-RF | CCCGTCCTGTGGATCTCCGCTAAATTGGTCGAAAG |
| eivA-IF-RR | GTTTCATCTCCTTATTCCATATTCTC |
| eivA-IF-LF | ATGGAATAAGGAGATGAAACATGTTTAACAAAGTTTTGAT-AGGGTTA |
| eivA-IF-LR | AAGGGCATCGGTCGAATGCTCCATATCGCAAGACA |
| tetA-CHK-F | CTGCTCGCTTCGCTACTTG |
| tetA-CHK-R | CGTCATCTACCTGCCTGGAC |

(number of bacteria inoculated)] or percent relative invasion compared with percent invasion of the parent strain.

To determine whether *E. coli* strains survive within HBMECs, invasion assays were performed as described above, except that the time between the 100 μg mL$^{-1}$ gentamicin treatment and lysis of HBMECs was lengthened to 24 h. The extended incubation was done with medium containing a lower concentration of gentamicin (20 μg mL$^{-1}$) to prevent extracellular multiplication of any bacteria released into the medium (Badger *et al.*, 1999). All the experiments were repeated three times in triplicate. Results were expressed as percent survival, determined by (number of intracellular bacteria recovered compared with the number of bacteria inoculated) × 100 or percent relative intracellular survival compared with percent survival of the parent strain.

## Results

### Sequence analysis of the ETT2 in *E. coli* K1 strain EC10

Our sequencing analysis revealed that the locus coding the ETT2 from *E. coli* K1 strain EC10 and strain Sakai shared a highly conserved gene content and order. The nucleotide sequence of the ETT2 of *E. coli* K1 is over 91% identical to that of *E. coli* strain Sakai. Twenty-six ORFs were identified and annotated using GLIMMER and BLAST in ETT2 locus from *E. coli* K1 strain EC10 (Fig. 1). Of these 26 putative proteins, EprKJI, EpaQP and EivICAG in *E. coli* strain EC10 were almost identical to their counterparts in *E. coli* strain Sakai. Compared with *E. coli* strain Sakai, EprH in strain EC10 has 149 extra amino acid residuals at the N terminal, and EivEFJ, EpaOS and EtrA were truncated due to the stop codons. In addition, there are two insertions in this locus compared with *E. coli* strain Sakai. Insertion 1 is downstream of *epaS2*, and insertion 2 in the middle of this gene. Insertion 1 encodes a small putative invasion lipoprotein, which may be involved in ETT2 pathogenicity. Insertion 2 bears IS1 transposase and an insertion element protein and divides the *epaS* into two ORFs: *epaS1* and *epaS2*, which indicates that *epaS* may not be functional in the *E. coli* K1 strain EC10. In EHEC O157:H7, EtrA and EivF exert profound negative effects on the transcriptional level of genes within the locus of enterocyte effacement (LEE) and the mutation in the two proteins greatly increased secretion of LEE proteins and the ability of adhesion to human intestinal cells (Zhang *et al.*, 2004). Interestingly, both of these two genes were truncated in *E. coli* K1 strain EC10.

### *Escherichia coli* invasion and intracellular survival in HBMECs

*Escherichia coli* K1 strain EC10 was isolated from the CSF of a neonate with meningitis, but the microbial–host interactions involved in EC10 penetration into the CNS remain unclear. We have shown that *E. coli* K1 invasion of HBMECs is a prerequisite for its penetration into the CNS (Kim, 2003). Therefore, we examined the ability of the deletion mutant of ETT2 (TTKO) to invade into HBMECs compared with the parent strain EC10. As shown in Fig. 2, the mutant was significantly defective ($P < 0.05$) in invasion of HBMECs compared with the parent strain. We also showed that the ability of the TTKO to survive intracellularly within HBMECs was significantly decreased compared with the parent strain.

We next examined whether ETT2 was indeed involved in HBMEC invasion and intracellular survival by single gene deletion and complementation. InvA, which is essential for *Salmonella* entering epithelial cells (Galan & Curtiss, 1991), is a member of a family of proteins involved in the secretion of virulence factors by many plant and mammalian pathogens. The EivA identified in *E. coli* strain EC10 is homologous to InvA (65% identity). We constructed a nonpolar deletion mutant of *eivA* within ETT2 and complemented the mutant with *eivA* supplied *in trans* on pACYC184. As shown in Fig. 3, the Δ*eiv*A mutant was defective in invasion and

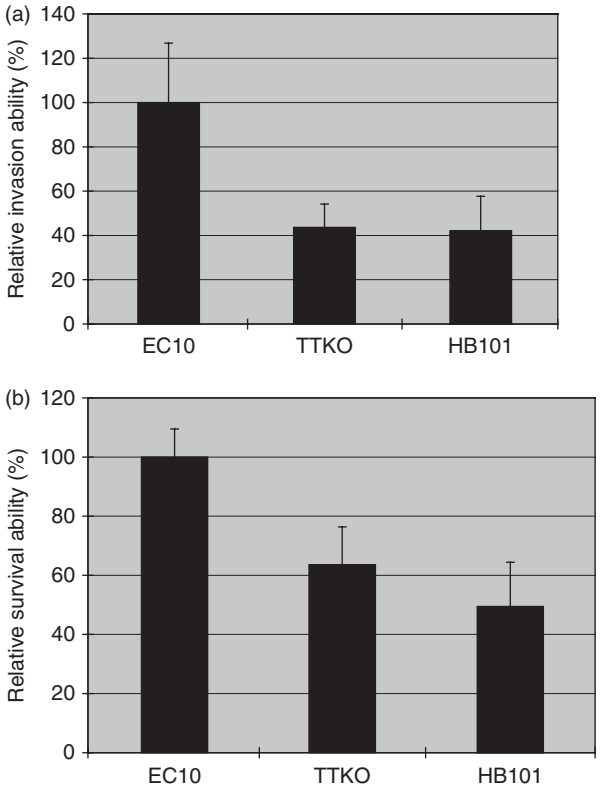


**Fig. 2.** The T3SS of *Escherichia coli* strain EC10 is involved in the invasion and intracellular survival in HBMECs. (a) The invasion frequency in HBMECs. (b) The intracellular survival in HBMECs. The bars represent the means of three independent experiments, and the error bars indicate the SDs.

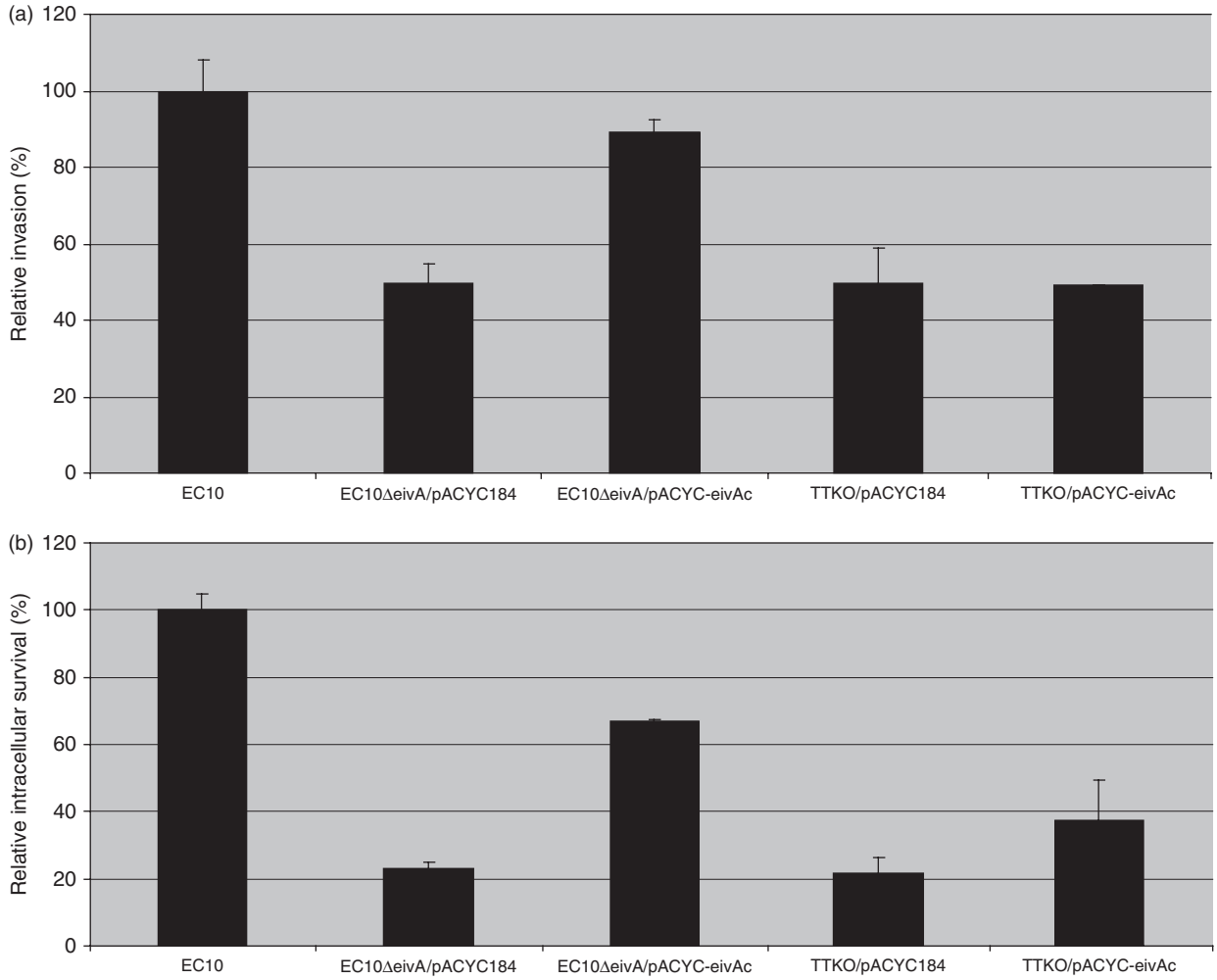


**Fig. 3.** Invasion and intracellular survival of the *eivA* mutant of strain EC10 and the complemented strain in HBMECs. (a) The invasion frequency in HBMECs. (b) The intracellular survival in HBMECs. The bars represent the means of three independent experiments, and the error bars indicate the SDs.

intracellular survival of HBMECs, similar to the defects of the TTKO. Transcomplementation with *eivA* restored the invasion and intracellular survival ability of the Δ*eiv*A mutant to the levels of the parent strain, while it failed to restore the invasion and intracellular survival of TTKO. These findings indicate that successful restoration of invasion and intracellular survival by transcomplementation with *eivA* is specific to the Δ*eiv*A mutant.

## Discussion

*Escherichia coli* strains possessing the K1 capsular polysaccharide are predominant (approximately 80%) CSF isolates from neonatal meningitis and most of these K1 isolates are associated with a limited number of O types (e.g. O18, followed by O7, O1 and O16) (Sarff *et al.*, 1975). We have previously shown using comparative genome hybridization that *E. coli* K1 strains isolated from CSF can be categorized into two groups based on their putative virulence factors, lipoproteins, proteases, outer membrane proteins and secretion systems (Yao *et al.*, 2006). We showed that group 1 strains comprise phylogenetic groups B2 and contain the known invasin proteins such as Ibe proteins and CNF1, whereas groups 2 strains contain phylogenetic groups A and D and lack the known invasin proteins. *Escherichia coli* strain EC10 (O7:K1) belongs to the group 2 strains, but represents a serotype commonly associated with neonatal meningitis. We demonstrated for the first time the existence of ETT2 in group 2 strains, but the role of ETT2 in the pathogenesis of *E. coli* infection, including meningitis, remains unclear.

A previous study has shown the presence of an ETT2 from septicemic *E. coli*, where it contributed to virulence in a 1-day-old chick model, although it has several premature stop codons in *eprI* and *eprJ* encoding the needle structure, and a 5-kb deletion encoding several ETT2 apparatus

proteins, compared with the ETT2 of *E. coli* strain Sakai (Ideses *et al*., 2005). In the present study, we showed that the approximately 18-kb sequenced fragment from *E. coli* K1 strain EC10 bears the genes encoding ETT2 apparatus proteins (e.g. *epr*, *epa* and *eiv* genes). *Escherichia coli* K1 strain EC10 showed significant defects in invasion and intracellular survival in HBMECs when the ETT2 coding locus or its *eivA* gene was deleted. The defects of the *eivA* deletion mutant were successfully restored to the levels of the parent strain by transcomplementation with *eivA*. These findings suggest that the ETT2 in *E. coli* K1 strain EC10 is involved in the interaction with HBMECs, but the mechanisms involved in invasion and intracellular survival associated with the ETT2 remain unclear, requiring further investigation.

A large amount of effectors secreted via the bacterial T3SS have been identified and characterized (Collmer *et al*., 2000; Ehrbar *et al*., 2002; Matsumoto & Young, 2009; Shrivastava & Miller, 2009). In *Salmonella*, $>20$ effectors have been discovered, some of them involved in bacterial intracellular survival (Waterman & Holden, 2003). For instance, SifA permits survival and replication of *Salmonella typhimurium* in murine macrophages (Brumell *et al*., 2001), and mutation of *spiC* resulted in a strong defect in intracellular trafficking in macrophages (Uchiya *et al*., 1999). Our previous studies have shown that the OmpA and the K1 capsules affect *E. coli* K1 interaction with HBMECs, such as invasion and intracellular survival, respectively (Kim *et al*., 2003; Shin *et al*., 2005). The mutant deleted of the ETT2 exhibited the same levels of OmpA and the K1 capsule compared with the parent strain EC10. Thus, the defect in intracellular survival with the ETT2 mutant was not related to the loss of the K1 capsule, implying a novel property of the ETT2 associated with *E. coli* EC10. We postulate that the ETT2 delivers bacterial proteins into HBMECs to enhance bacterial interaction with host cells, such as invasion and intracellular survival. Our previous comparative genome hybridization study failed to reveal the presence of known effectors and translocators in *E. coli* K1 strain EC10, such as Esp and Tir (McDaniel & Kaper, 1997). The ETT2 of *E. coli* K1 strain EC10 may, therefore, utilize unidentified effector(s) to interfere with host defense or use an unknown mechanism to invade and survive in HBMECs.

In summary, we determined the sequence of ETT2 in meningitis-causing *E. coli* K1 strain EC10 and constructed the EC10 mutants deleted of ETT2 and *eiv*A. The deletion mutants of ETT2 and *eiv*A exhibited the defects in the invasion of HBMECs, the key step shown to be involved in the development of *E. coli* meningitis, as well as intracellular survival in HBMECs compared with the parental *E. coli* K1 strain. Further investigation of how the ETT2 from *E. coli* K1 strain EC10 affects invasion of HBMECs will help in our elucidation of the pathogenesis of *E. coli* meningitis.

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