YgfZ contributes to secretion of cytotoxic necrotizing factor 1 into outer-membrane vesicles in *Escherichia coli*

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Cytotoxic necrotizing factor 1 (CNF1), a Rho GTPase-activating bacterial toxin, has been shown to contribute to invasion by meningitis-causing *Escherichia coli* K1 of human brain microvascular endothelial cells (HBMEC), which constitute the blood–brain barrier. However, CNF1 is a cytosolic protein and it remains unclear how its secretion occurs, contributing to *E. coli* invasion of HBMEC. To investigate the genetic requirement for CNF1 secretion in *E. coli* K1 strain RS218, we performed mini-Tn5 *in vitro* mutagenesis and constructed a transposon mutant library of strain NBC, in which β -lactamase was fused to the C-terminus of CNF1 in the chromosome of strain RS218. We identified a transposon mutant (NBC-1E6) that exhibited reduced β -lactamase activity in its culture supernatant and had the transposon inserted into the *ygfZ* gene. When *ygfZ* was deleted from the genome of strain RS218 ($\Delta ygfZ$), the translocation of CNF1 into HBMEC was impaired. Subcellular localization analysis of CNF1 demonstrated that YgfZ, a periplasmic protein, contributes to secretion of CNF1 into outer-membrane vesicles (OMVs). The $\Delta ygfZ$ mutant was significantly defective in invasion of HBMEC compared to the parent *E. coli* K1 strain. The defects of the $\Delta ygfZ$ mutant in CNF1 secretion into OMVs and translocation into HBMEC as well as invasion of HBMEC were abrogated by complementation with *ygfZ*. Taken together, our findings demonstrate that YgfZ contributes to CNF1 secretion into OMVs in meningitis-causing *E. coli* K1.

Received 18 August 2011
Revised 1 November 2011
Accepted 12 December 2011

INTRODUCTION

CNF1, the paradigm of the Rho GTPase-activating bacterial toxins (Boquet, 2001; Knust & Schmidt, 2010; Lemonnier *et al.*, 2007), is frequently associated with uropathogenic and meningitis-causing *Escherichia coli* strains (Khan *et al.*, 2002; Landraud, *et al.*, 2000). CNF1 activates Rho GTPases by deamidation of glutamine 63, converting it into glutamic acid, thus inhibiting GTP-hydrolysing activity, resulting in constitutive activation of Rho GTPase. CNF1 has been shown to contribute to polymerization of F-actin, increased formation of stress fibres and phagocytosis (Caprioli *et al.*, 1983; Falzano *et al.*, 1993; Flatau *et al.*, 1997; Schmidt *et al.*, 1997; Vouret-Craviari *et al.*, 1999). CNF1-like toxins have also been found in other bacteria, including the dermonecrotic toxin of *Bordetella* spp. and the CNF γ from *Yersinia pseudotuberculosis* (Knust & Schmidt, 2010; Kume *et al.*, 1986; Lockman *et al.*, 2002).

We have shown that CNF1 contributes to invasion by *E. coli* K1 of human brain microvascular endothelial cells

(HBMEC) and penetration into the brain via the interaction with its receptor, 37 laminin receptor precursor (37LRP)/67 laminin receptor (67LR) (Chung *et al.*, 2003; Khan *et al.*, 2002; Kim *et al.*, 2005). CNF1 is a cytoplasmic protein and its secretion is, therefore, a strategy utilized by meningitis-causing *E. coli* K1 to invade the blood–brain barrier (Khan *et al.*, 2002; Kim, 2003, 2008). However, it remains incompletely understood how CNF1 secretion occurs. No typical signal peptide is found in the CNF1 sequence. Recent studies have shown that CNF1 is transported to the culture supernatant in a complex with outer-membrane vesicles (OMVs) in uropathogenic *E. coli* strains J96 and CP9 (Davis *et al.*, 2006; Koukam *et al.*, 2006).

OMVs from a number of bacterial species have been shown to contain virulence factors, some exhibiting immunomodulatory effects, and others exhibiting adherence to and intoxication of host cells (Mashburn-Warren & Whiteley, 2006). Several studies suggest that the secretion of cargo proteins into OMVs is a regulated process. For example, in enterotoxigenic *E. coli*, delivery of toxins via OMVs has been shown to be associated with host cells in a time- and receptor-dependent manner (Kesty *et al.*, 2004), suggesting that OMVs serve as transporters mediating entry of

Abbreviations: AP, alkaline phosphatase; Bla, β -lactamase; HBMEC, human brain microvascular endothelial cells; CNF1, cytotoxic necrotizing factor 1; OMV, outer-membrane vesicle.

bacterial virulence factors into host cells. In *Pseudomonas aeruginosa*, interaction of quorum signals with outer-membrane lipids was shown to be required for OMV formation (Mashburn-Warren *et al.*, 2008). However, it is unclear whether delivery of toxins and other bacterial molecules into target cells from those membrane blebs can be regarded as true secretion, because there was no evidence of the involvement of ‘secretion’ factors independent of membrane perturbation (Economou *et al.*, 2006).

In order to study the genetic requirements for secretion of CNF1 in meningitis-causing *E. coli* K1, we designed a Tn5 mutational screening strategy by applying TEM β -lactamase as the reporter to monitor CNF1 secretion. We demonstrated that YgfZ, a periplasmic protein, contributes to secretion of CNF1 into OMVs.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are shown in Table 1. *E. coli* K1 strain

RS218 (O18:K1:H7) is a cerebrospinal fluid isolate from a neonate with meningitis (Khan *et al.*, 2002). Strain NBC was used for Tn5 mutagenesis, in which the β -lactamase reporter gene was translationally fused to the C-terminus of the *cnf1* gene in the chromosome of strain RS218, as previously described (Yu & Kim, 2010). *E. coli* K-12 strain DH5 α was used as the host for plasmids, and EC100D *pir*116+ (Epicentre Biotechnologies) as the host for R6k γ origin plasmid. *E. coli* strains were routinely grown at 37 °C in Luria Broth (LB). Where appropriate, the medium was supplemented with ampicillin (100 μ g ml⁻¹), spectinomycin (100 μ g ml⁻¹), tetracycline (10 μ g ml⁻¹) or chloramphenicol (20 μ g ml⁻¹).

Transposome formation and transposition mutagenesis. These were performed as described previously (Geddes *et al.*, 2005; Yu & Kim, 2010). Briefly, the transposome complex was formed by incubating Tn5 transposome SR and hyperactive Tn5 transposase (Epicentre Biotechnologies) for 1 h at 37 °C. Transposomes were electroporated into competent NBC cells. Transposon insertion mutants were selected with spectinomycin.

β -Lactamase (Bla) activity assay. Bla activity was determined as described previously (Yu & Kim, 2010). Briefly, nitrocefin at a final concentration of 0.1 mM was incubated with bacterial culture supernatant (obtained by centrifugation at 4000 g for 10 min) for

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>E. coli</i> strains		
RS218	O18:K1:H7 strain isolated from the cerebrospinal fluid of a neonate with <i>E. coli</i> meningitis	Khan <i>et al.</i> (2002)
EC100D	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80d <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ^- <i>rpsL</i> <i>nupG</i> <i>pir</i> ⁺ (DHFR)	Epicentre Biotechnologies
DH5 α	F' ϕ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r _k ⁻ m _k ⁺) <i>phoA</i> <i>supE44</i> λ^- <i>thi-1</i>	Lab stock
NBC	β -Lactamase reporter gene translationally fused to the C-terminus of the <i>cnf1</i> gene in the chromosome of strain RS218	Yu & Kim (2010)
NBC-1E6	NBC with Tn5 inserted in the <i>ygfZ</i> gene	This study
YK6	RS218 (O18:K1:H7) <i>ygfZ</i> deletion mutant	This study
HNS	RS218 (O18:K1:H7) <i>hns</i> deletion mutant	Lab stock
YK7	RS218 (O18:K1:H7) <i>ygfZ</i> deletion mutant complemented with <i>ygfZ</i> under the control of its native promoter	This study
YK14	NBC-1E6 complemented with <i>ygfZ</i> under the control of its native promoter	This study
YK15	RS218 (O18:K1:H7) <i>ygfZ</i> and <i>hns</i> double deletion mutant	This study
YK16	RS218 (O18:K1:H7) <i>ygfZ</i> and <i>hns</i> double deletion mutant complemented with <i>ygfZ</i> under the control of its native promoter	This study
Plasmids		
pFLAG-CTC	5348 bp <i>E. coli</i> expression vector used for cytoplasmic expression of a properly inserted ORF as a C-terminal FLAG fusion protein	Sigma
pFLAG-CTS-BAP	<i>E. coli</i> plasmid used for efficient and controlled periplasmic expression of C-terminal FLAG-BAP fusion protein	Sigma
pCTC-YgfZ	Expression vector of YgfZ fused with FLAG at the C-terminus	This study
pCXN	CNF1 coding region cloned into the <i>KpnI</i> site of pCX340, tetracycline resistant	Yu & Kim (2010)
pKD3	Containing chloramphenicol-resistance gene and R6k γ replication origin	Datsenko & Wanner (2000)
pKD47	Derivative of pKD46 (Datsenko & Wanner, 2000), with the only modification that <i>blaM</i> was replaced by spectinomycin-resistance gene	This study
pGRG36	Tn7 insertion vector, ampicillin resistance, temperature sensitive	McKenzie & Craig (2006)
pSR	Tn5 vector, spectinomycin resistant, R6k γ replication origin	Yu & Kim (2010)
pG-ygfZ	DNA fragment containing <i>ygfZ</i> gene obtained by PCR amplification from genomic DNA, and ligated into <i>AvrII</i> and <i>PacI</i> sites of pGRG36	This study

20 h. Nitrocefin, as the chromogenic substrate of Bla, undergoes a distinctive colour change from yellow (λ_{max} 390 nm at pH 7.0) to red (λ_{max} 486 nm at pH 7.0) as the amide bond in the β -lactam ring is hydrolysed by Bla. Bla activity was read as positive if the colour change to red occurred. Spectrophotometric assays for Bla using nitrocefin were also carried out by measuring changes in A_{486} .

Genomic DNA isolation and sequencing. Genomic DNA was isolated from Tn5 mutants. Quantity of chromosomal DNA was measured with the Quant-iT dsDNA BR assay kit (Invitrogen); 12 μ l genomic DNA (0.5 μ g μ l⁻¹) and 12 μ l sequencing primer SR-Seq (8 μ M) were sent to the DNA Synthesis and Sequencing Facility, Johns Hopkins University School of Medicine, for sequencing.

ygfZ gene deletion and complementation. To delete the *ygfZ* gene, a chloramphenicol-resistance cassette was amplified from pKD3 using primers 1E6-KOF and 1E6-KOR (Table 2). The PCR product was inserted into the chromosome by Lambda Red-mediated allele replacement (Datsenko & Wanner, 2000). The correct insertion was verified by PCR with primers 1E6CKF and 1E6CKR (Table 2).

For gene complementation, Tn7 site-specific insertion of the *ygfZ* gene into the second benign site in the chromosome of the mutant was carried out as described previously (McKenzie & Craig, 2006; Yu & Kim, 2010). Briefly, the *ygfZ* gene together with its native promoter was amplified from the genomic DNA of strain RS218 by primers 1E6 s and 1E6-a (Table 2), and then ligated into *AvrII* and *PacI* sites of pGRG36. The resulting plasmid was designated pG-*ygfZ*. pG-*ygfZ* was then electroporated into NBC-1E6 (Tn5::*ygfZ*), the Δ *ygfZ* mutant, or Δ *ygfZ*/ Δ *hns*, and transformants were selected on LB plates containing ampicillin at 32 °C. The insertion of Tn7 in the attachment site was verified by PCR with primers Tn7-ckf and Tn7-ckr (Table 2) (Fig. 1c).

Assessment of CNF1 translocation into HBMEC. The CNF1 translocation assay was done as described previously (Mills *et al.*, 2008; Yu & Kim, 2010). Briefly, HBMEC grown in monolayers were preloaded with CCF4/AM dye and incubated with bacteria harbouring pCXN. The expression of the CNF1-Bla fusion from pCXN was induced with 1 mM IPTG. Upon entry into HBMEC, the non-fluorescent esterified CCF4/AM substrate is converted to fluorescent green CCF4 by cellular esterases. Translocation of CNF1-Bla into HBMEC induces catalytic cleavage of the CCF4 β -lactam ring, which produces an easily detectable colour change in CCF4 fluorescence from green to blue emission (Charpentier & Oswald, 2004; Yu & Kim, 2010; Zlokarnik *et al.*, 1998). After 45 min of infection, the

translocation of CNF1-Bla hybrid into HBMEC was observed with a Nikon fluorescence microscope.

Plasmid construction. To subclone *ygfZ* into the FLAG-tagged expression vector pFLAG-CTC (Sigma), the *ygfZ* coding region was amplified from RS218 genomic DNA with primers 1E6-s2 and 1E6-a2 (Table 2), and the PCR product was ligated into the *NdeI* and *Sall* sites of pFLAG-CTC, resulting in plasmid pCTC-*YgfZ*.

Cell fractionation. Cytoplasmic, periplasmic and outer-membrane fractions were obtained by the osmotic shock method as described previously (Wai *et al.*, 2003; Yu & Kim, 2010). Periplasmic suspension was filtered through a 0.22 μ m filter to remove any residual bacterial cells, and then precipitated by the sodium deoxycholate/trichloroacetic acid method (Yu & Kim, 2010). OMVs were isolated by ultracentrifugation (100 000 g for 2 h; SW41Ti rotor, Beckman) as described previously (Kouokam *et al.*, 2006).

Western blot assays. Cell fractions were separated by SDS-PAGE, and then protein samples were transferred to nitrocellulose membrane. The blots were blocked with 5% skim milk in TBS (25 mM Tris, pH 7.4, 150 mM NaCl) for 60 min at 22 °C. The membrane was then incubated for 2 h at 22 °C with primary antibody. Primary antibodies used in this study were anti-CNF1 monoclonal antibody (DD1) (Meysick *et al.*, 2001), alkaline phosphatase (AP or PhoA) monoclonal antibody (Millipore), β -Gal polyclonal antibody (Millipore), NuoCD polyclonal antibody (Castro-Guerrero *et al.*, 2010), anti-FLAG BioM2 monoclonal antibody (Sigma) and OmpA monoclonal antibody (Yu & Kim, 2010). The membrane was washed with 0.5% Tween 20 in TBS and subsequently incubated for 60 min at room temperature with horseradish-peroxidase-linked secondary antibodies. The membrane was developed with Amersham ECL Advance (for detection of CNF1, β -Gal and NuoCD) or the Amersham ECL detection system (for detection of FLAG, AP and OmpA) (GE Healthcare).

E. coli invasion of HBMEC. *E. coli* invasion assays were performed in HBMEC monolayers as previously described (Khan *et al.*, 2002; Yu & Kim, 2010). Briefly, confluent HBMEC grown in 24-well plates were incubated with 10⁷ c.f.u. of *E. coli* (m.o.i. 100) in experimental medium. The plates were incubated for 90 min at 37 °C in 5% CO₂ to allow bacterial invasion to occur. The number of intracellular bacteria was determined by culturing on blood agar plates after killing extracellular bacteria by incubation of the HBMEC monolayer with experimental medium containing gentamicin (100 μ g ml⁻¹) for 1 h.

Table 2. Primers

Primer	Sequence (5'–3')*
1E6-KOF	TCTTTTACCATTAGTTATTAATACGCCGTTAAGCAACTCAGGACTCTATTGTGTAGGCTGGAGCTGCTTC
1E6-KOR	GACACACCAGTGTGCGCATCAGGCGATAAATGCCTGATGCGATACAGAGATCATATGAATATCCTCCTTAG
1E6CKF	GACGCTCTATCACGCCATTCC
1E6CKR	GGGCAACTGTTTCGTGGGTCT
1E6-a	CCACGATCGCGATACAGAGATTTACTCTTC
1E6 s	CCAGCCTAGGTGTCTCACGAAAATCCAGTA
1E6-s2	ACCTGTCATATGGCTTTTACACCTTTTCC
1E6-a2	GCCAGTTCGACCTTTCAGCGAATATGGC
Spc-SeqR	GCCTTGCTGTTCTTCTACGG
Tn7-ckf	ACGGTCGGGAAGTGAAC
Tn7-ckr	TGACCAGCCGCGTAACCT

*Restriction sites for cloning are underlined.

Fig. 1. Identification of the *ygfZ* gene as a genetic requirement for CNF1 secretion. (a) Bla activity in the culture supernatant of strains RS218, NBC and NBC-1E6 (Tn5:: *ygfZ*). Bacteria were grown overnight in brain-heart infusion broth (static at 37 °C), the culture supernatant was obtained by centrifugation at 5000 *g* for 10 min, and Bla activity was determined based on the A₄₈₆. The Bla activity shown represents the mean ± SD of results from three experiments in triplicate. (b) Transposon insertion within the *ygfZ* gene in the mutant strain NBC-1E6 (Tn5:: *ygfZ*). (c) Schematic representation of the chromosomal structure of *ygfZ* mutants constructed in this study. Cm^r, chloramphenicol resistance gene. YK6 is the *ygfZ* deletion mutant; YK7 is a complemented strain of the *ygfZ* deletion mutant with *ygfZ*; YK14 was derived from NBC-1E6 by complementing the *ygfZ* gene in the Tn7 attachment site. YK15 and YK16 are not listed in this figure; they represent the *ygfZ* deletion mutant and the complemented strain, respectively (i.e. identical to YK6 and YK7, respectively), in the background of *hns* deletion.

Assays were performed in triplicate and repeated at least three times. Results were expressed as relative invasion frequencies (percentage invasion compared to that of the parent strain, RS218).

RESULTS

Tn5 mutagenesis screen identifies the *ygfZ* gene

To investigate the genetic requirement for CNF1 secretion in strain RS218, we performed mini-Tn5 *in vitro* mutagenesis and constructed a mutant library of strain NBC, in which β-lactamase (Bla) was fused to the C-terminus of CNF1 in the chromosome of strain RS218. In strain NBC, Bla secretion is

entirely dependent on the CNF1 secretion machinery (Yu & Kim, 2010), and the secretion of CNF1-Bla fusion protein into the culture supernatant was monitored by measuring the Bla activity. For Bla assay, strain NBC was used as a positive control, and strain RS218 was used as a negative control. We identified a mutant (NBC-1E6) that exhibited barely detectable Bla activity based on both visual colour change and the spectrometric reading. The mutant was significantly defective in secretion of CNF1 into the culture medium, based on the Bla activity (Fig. 1a). We determined the location of the transposon insertion as being within the *ygfZ* gene by direct DNA sequencing of the mutant's genomic DNA (Fig. 1b).

CNF1 translocation is impaired in *ygfZ* deletion mutant

We next deleted the entire *ygfZ* gene from the chromosome of strain RS218, and examined the ability of the resulting strain to translocate CNF1 into HBMEC using CNF1-Bla fusion protein expressed from plasmid pCXN, as previously described (Yu & Kim, 2010). In pCXN, CNF1 was translationally fused with Bla, and controlled by the *trc* promoter. *E. coli* transformants harbouring pCXN were pre-induced with 1 mM IPTG and then added to HBMEC pre-loaded with CCF4/AM dye. After 45 min incubation, translocation of CNF1 into HBMEC was visualized by fluorescence microscopy. As expected, wild-type strain RS218 exhibited successful translocation of CNF1-Bla protein into HBMEC, as shown by the emission of bright blue fluorescence (Fig. 2). In screening a $\times 10$ view of the HBMEC monolayer, 147 bright blue cells were observed compared to 332 green cells. However, the translocation of CNF1-Bla into HBMEC was impaired in strain $\Delta ygfZ$, as only 6 weakly blue cells were observed, compared to 445 green cells (Fig. 2). The translocation of CNF1-Bla fusion into HBMEC in strain $\Delta ygfZ$ was, however, restored in the complemented strain YK7 ($\Delta ygfZ::ygfZ$), which demonstrated 163 blue cells and 392 green cells (Figs 1c and 2).

YgfZ contributes to secretion of CNF1 into OMVs

In order to determine the steps that are involved in defective CNF1 secretion in strain $\Delta ygfZ$, we compared the subcellular distribution of CNF1 between the $\Delta ygfZ$ mutant and wild-type strain RS218. The amounts of CNF1 in the cytoplasmic and periplasmic fractions were similar between RS218 and the $\Delta ygfZ$ mutant (Fig. 3a), suggesting that the

defect in CNF1 translocation into HBMEC in the $\Delta ygfZ$ strain is likely to occur at the step of crossing the outer membrane from the periplasmic space. To test this possibility, we fractionated strains RS218, NBC, NBC-1E6 (Tn5::*ygfZ*) and the genetically complemented strain YK14 (NBC-1E6 complemented with *ygfZ* by Tn7 transposition, Fig. 1c). CNF1-Bla was present in the cytoplasmic, periplasmic and membrane fractions of strain NBC-1E6, but not in culture supernatant; in strain NBC and the complemented strain YK14, CNF1-Bla was present in all four fractions (Fig. 3b). The membrane fraction was not separated into the inner and outer membranes because *N*-laurylsarcosine would denature CNF1-Bla fusion protein and interfere with the Bla activity. Since there is no detectable CNF1 in the inner membrane (Kouokam *et al.*, 2006), the Bla activity detected in the membrane fraction is likely to represent the amount of CNF1 in the outer-membrane fraction.

It has been shown that CNF1 is downregulated by H-NS (histone-like nucleoid structuring protein) in uropathogenic *E. coli* strain J96, and *hns* deletion led to increased CNF1 expression in this strain (Kouokam *et al.*, 2006), suggesting that *hns* deletion is a useful tool for demonstrating CNF1 in cell fractions. We therefore examined CNF1 secretion in the H-NS deletion background of strain RS218. As indicated by markers of each fraction, the different cell fractions exhibited their expected protein profile. In the Δhns mutant, the presence of CNF1 was detected in the cytoplasmic, periplasmic, outer-membrane and OMV fractions (Fig. 3c, lanes 1). In contrast, in the $\Delta ygfZ/\Delta hns$ double mutant, CNF1 was detected in the cytoplasmic, periplasmic and outer-membrane fractions, but not in the OMVs (Fig. 3c, lanes 2). It is important to

	Green channel	Blue channel	Merged	Number
RS218				B: 147 G: 332
$\Delta ygfZ$				B: 6 G: 445
YK7				B: 163 G: 392

Fig. 2. Analysis of the translocation of CNF1 into HBMEC. HBMEC were pre-loaded with the Bla substrate CCF4/AM for 1 h, and then infected with *E. coli* strains bearing plasmid pCXN. pCXN expresses the CNF1-Bla fusion. After 45 min incubation, the translocation of CNF1 into HBMEC, indicated by blue emission, was visualized by fluorescence microscopy. RS218 is the wild-type strain, $\Delta ygfZ$ is a *ygfZ* deletion mutant, and YK7 is the *ygfZ* deletion mutant complemented with *ygfZ*. The numbers of green (G) and blue (B) cells from a $\times 10$ view are listed on the right.

Fig. 3. YgfZ contributes to CNF1 secretion into OMVs. (a) Western blot analysis of periplasmic (P) and cytoplasmic (C) distribution of CNF1 in RS218 and $\Delta ygfZ$. Alkaline phosphatase (AP) was used as a marker for the periplasm. (b) Relative Bla activity in the cytoplasmic (C), periplasmic (P), membrane (M) and culture supernatant (S) fractions of strains RS218, NBC, NBC-1E6 (Tn5::ygfZ) and YK14 (NBC-1E6 complemented with ygfZ). Assays were performed in triplicate and repeated at least three times. Results (mean $\pm$ sd) are expressed as relative Bla activity (percentage activity compared to that of strain NBC, which was set at 100 %). *, $P < 0.05$ %. (c) Western blot analysis of proteins from the cytoplasmic (C), periplasmic (P), outer-membrane (OM) and OMV fractions from Δhns (lanes 1) and $\Delta hns/\Delta ygfZ$ mutant (lanes 2). For cytoplasmic protein, 40 μ g total cellular proteins from crude bacterial extracts were loaded in each well. Alkaline phosphatase (AP) and OmpA were used as the loading markers for periplasm and outer membrane (including OMVs), respectively. CNF1, AP, OmpA and β -Gal were detected by their respective specific antibodies, and different ECL substrates were used as described in Methods.

note that the amount of OmpA was similar between the OMVs isolated from Δhns and $ygfZ/\Delta hns$ mutants (Fig. 3c), suggesting that the failure to demonstrate CNF1 in OMVs in the $\Delta ygfZ$ mutant is not generic to all proteins and/or related to technical issues of OMV preparation. The ability to demonstrate CNF1 in OMVs in the $\Delta ygfZ/\Delta hns$ mutant was restored by complementation with ygfZ containing its own promoter (strain YK16) (Fig. 4b). Taken together, these findings demonstrate that YgfZ is likely to be involved in CNF1 secretion into OMVs in *E. coli* K1.

It was of interest that SDS-PAGE-based comparison of the protein profiles of the OMVs isolated from the Δhns mutant, the $\Delta ygfZ/\Delta hns$ mutant and the $\Delta ygfZ/\Delta hns$ mutant complemented with ygfZ revealed a different pattern of protein bands (Fig. 4a). The dominant protein in the band position of CNF1 was α -haemolysin as revealed by mass spectrometry analysis, and the secretion of α -haemolysin into OMVs was also affected when ygfZ was deleted (Fig. 4a). α -Haemolysin is a known protein secreted into OMVs, and α -haemolysin associated with OMVs is more stable than free α -haemolysin (Aldick *et al.*, 2009). However, deletion of the type I secretion system responsible for the secretion of α -haemolysin did not affect CNF1 secretion (data not shown), suggesting that different secretion systems are likely to be involved in secretion of α -haemolysin and CNF1. YgfZ is, therefore, likely to affect a shared secretion machinery for selective

loading of cargo proteins, including CNF1 and α -haemolysin, into the OMVs. Taken together, our results demonstrate that secretion of CNF1 as well as α -haemolysin into OMVs is diminished in the $\Delta ygfZ$ mutant, and it occurred without any detectable decrease of OmpA in OMVs.

YgfZ is predominantly localized in the periplasmic space

In order to understand how YgfZ affects CNF1 secretion, we cloned ygfZ into pCTC-FLAG (Sigma), and expressed the resulting plasmid pCTC-YgfZ in RS218. SDS-PAGE analysis demonstrated that YgfZ is predominantly located in the periplasmic space (54 %), but it is also detected in the cytoplasmic (41 %) and inner-membrane (5 %) fractions (Fig. 5a). As shown in Fig. 5(b), the cytoplasmic fraction contained β -Gal protein, but was devoid of periplasmic protein PhoA (alkaline phosphatase, AP). In contrast, the periplasmic fraction contained PhoA, but was devoid of β -Gal, outer-membrane protein OmpA and inner-membrane protein NuoCD, suggesting that our cell fractions exhibited their expected protein profile. The predominant demonstration of YgfZ in the periplasmic space, therefore, is not due to contamination of the periplasmic fraction by cytoplasmic and inner-membrane proteins.

Fig. 4. Complementation of strain $\Delta hns/\Delta ygfZ$ with *ygfZ* restores CNF1 secretion. (a) SDS-PAGE analysis of the protein profile in OMVs prepared from Δhns (lane 1), $\Delta hns/\Delta ygfZ$ (lane 2) and YK16 ($\Delta hns/\Delta ygfZ$ mutant complemented with *ygfZ*) (lane 3). M, molecular size markers. (b) Demonstration of CNF1 in OMVs isolated from Δhns (lane 1), $\Delta hns/\Delta ygfZ$ (lane 2) and YK16 ($\Delta hns/\Delta ygfZ$ mutant complemented with *ygfZ*) (lane 3).

YgfZ promotes invasion of HBMEC by *E. coli* K1

CNF1 is known to contribute to *E. coli* K1 invasion of HBMEC (Khan *et al.*, 2002), and we next examined whether YgfZ contributes to the HBMEC invasion by virtue of affecting CNF1 secretion. HBMEC invasion assays revealed that the $\Delta ygfZ$ mutant was significantly defective in invasion of HBMEC compared to the parent strain RS218 (Fig. 6). We then complemented the $\Delta ygfZ$ mutant with *ygfZ* using Tn7 insertion into the second benign site of the chromosome, resulting in strain YK7. The HBMEC invasion of strain YK7 was restored to the level of the parent strain (Fig. 6).

DISCUSSION

In this study, we have demonstrated that YgfZ is involved in secretion of CNF1 into OMVs in meningitis-causing *E. coli* K1. This is shown by the fact that YgfZ deletion resulted in a decreased amount of CNF1 in the OMVs without affecting the amount of CNF1 expression in the cytoplasm, periplasm and outer membrane, and that the amount of CNF1 was restored to that of the parent strain by complementation with *ygfZ*.

YgfZ is a folate-binding protein (Teplyakov *et al.*, 2004; Waller *et al.*, 2010), but exogenous folate did not enhance CNF1 secretion (data not shown). YgfZ belongs to the COG0354 group, which was suggested to have a conserved, folate-dependent function in the activity of certain Fe-S cluster enzymes (Waller *et al.*, 2010). Previously, we

Fig. 5. YgfZ is predominantly localized in the periplasm. (a) SDS-PAGE analysis of the protein profiles of different fractions [cytoplasmic (C), periplasmic (P), inner membrane (IM) and outer membrane (OM)]. The gel was stained with Coomassie blue. pCTC-YgfZ is the expression plasmid of FLAG-tagged YgfZ; pCTS-BAP represents pFLAG-CTS-BAP (Sigma), which is a 6735 bp *E. coli* plasmid used for efficient and controlled periplasmic expression of C-terminal FLAG-BAP fusion protein. For the C fraction, 20 μ g total protein was loaded. For the P, IM and OM fractions, samples were loaded in a proportional manner; the amount of periplasmic protein loaded was equal to the total periplasmic protein that was collected from 3×10^9 bacteria (the number of bacteria was estimated from the OD₆₂₀). M, molecular size markers. (b) Western blot analysis of different fractions of pCTC-ygfZ/RS218 with different antibodies as listed on the left.

Fig. 6. The *ygfZ* deletion mutant of *E. coli* strain RS218 exhibits significantly decreased invasion of HBMEC. To determine whether YgfZ plays a role in *E. coli* invasion of HBMEC, invasion assays were performed using the *ygfZ* deletion mutant and the complemented strain YK7, and the results were expressed as relative invasion frequency (%) compared to the invasion frequency of wild-type strain RS218, which was set at 100 %. *, $P < 0.05$. The data represent the mean $\pm$ SD of three independent assays performed in triplicate.

identified that ferredoxin (Fe–S assembly protein) is involved in CNF1 secretion across the bacterial inner membrane (Yu & Kim, 2010), and comparative genomics studies suggest that COG0354 genes cluster with genes encoding Fe–S enzymes or related proteins (Waller *et al.*, 2010). These findings suggest that Fe–S protein may be involved with YgfZ in the CNF1 secretion system. This concept is suggested by the demonstration that YgfZ is reported to be in complex with IscA (Hu *et al.*, 2009) and our identification of another transposon mutant of *hscA* (located within the *iscA* operon, downstream of the *fdx* gene), which is also defective in secretion of CNF1 across the outer membrane (unpublished data). Additional studies are needed to elucidate whether and/or how Fe–S proteins are involved in CNF1 secretion.

We analysed the subcellular distribution of YgfZ, and showed that this protein is predominantly localized in the periplasm (Fig. 5). This is not consistent with previous prediction that YgfZ is a cytoplasmic protein; the discrepancy might stem from our insufficient information on the secretion signal peptide of YgfZ. The folate (vitamin B₉)-binding capability of YgfZ is reminiscent of the involvement of BtuB (the receptor of vitamin B₁₂) in translocation of colicins, in which colicins bind to BtuB, recruit the porin OmpF or OmpC and then traverse the lumen of the β -barrel of the porin (Housden *et al.*, 2010; James *et al.*, 1996). However, our pull-down experiment

did not support the idea that CNF1 may interact with YgfZ (data not shown).

YgfZ was proposed to have a role in one-carbon metabolism, such as transferring C₁ units (Teplyakov *et al.*, 2004). Recent studies showed that YgfZ is involved in methylating tRNA and plumbagin (an active ingredient of anti-bacterial herbs) (Chen *et al.*, 2006; Lin *et al.*, 2010; Ote *et al.*, 2006). Another recent study showed that a lipopolysaccharide modification enzyme in *Porphyromonas gingivalis* contributes to selective sorting of cargo proteins into OMVs (Haurat *et al.*, 2011). These findings prompt us to hypothesize that the deficiency of sorting CNF1 into OMVs in the YgfZ mutant may stem from its failure to modify certain component(s) of the CNF1 secretion machinery. Additional studies on the CNF1 secretion machinery are likely to support and/or disprove this hypothesis.

Despite the availability of crystal structure information on YgfZ (Teplyakov *et al.*, 2004), the working mechanism of this protein is unclear. Our current hypothesis is that YgfZ together with Fe–S assembly complex modifies CNF1 or certain component(s) of the bacteria (e.g. lipopolysaccharide or outer-membrane protein), which can specifically sort CNF1 as a cargo protein of OMVs. Studies are in progress to examine this hypothesis. Further elucidation of the mechanisms involved in the CNF1 secretion network affected by YgfZ is likely to enhance our knowledge of the pathogenesis of *E. coli* meningitis.

ACKNOWLEDGEMENTS

This work was supported in part by the NIH (grant NS26310-22). The authors thank Dr Allison O'Brien for providing the CNF1 monoclonal antibody, Dr Nancy L. Craig for providing pGRG36 plasmid, Dr Takao Yagi for providing NuoCD antibody, and Dr Fred Heffron for providing plasmid pMini-Tn5-cycler. We would also like to thank Dr George Niemann for helpful suggestions and discussions.

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Edited by: D. L. Gally