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 CNF5 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) 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 (Marshburn-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 OMVs into host cells in a receptor-dependent manner (Kesty et al., 2004).
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 DH5α was used as the host for plasmids, and EC100D pir116+ (Epigenic Biotechnologies) as the host for R6K<sup>γ</sup> origin plasmid. *E. coli* strains were routinely grown at 37 °C in Luria Broth (LB). Where appropriate, the medium was supplemented with ampicillin (100 µg ml<sup>-1</sup>), spectinomycin (100 µg ml<sup>-1</sup>), tetracycline (10 µg ml<sup>-1</sup>) or chloramphenicol (20 µg ml<sup>-1</sup>).

### TEM 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 5 min at 37°C.

### 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 (Epigenic Biotechnologies) for 1 h at 37 °C. Transposomes were electroporated into competent NBC cells. Transposon insertion mutants were selected with spectinomycin.

#### Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
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<tr>
<td>RS218</td>
<td>O18:K1:H7 strain isolated from the cerebrospinal fluid of a neonate with <em>E. coli</em> meningitis</td>
<td>Khan et al. (2002)</td>
</tr>
<tr>
<td>EC100D</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 gaiU galk λ&lt;sup&gt;−&lt;/sup&gt; rpsL nupG pir&lt;sup&gt;+&lt;/sup&gt; (DHFR)</td>
<td>Epicentre Biotechnologies</td>
</tr>
<tr>
<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; Φ80dlacZΔM15 Δ(laclZYA-argF)U169 deoR recA1 endA1 hsdR17(τ&lt;sub&gt;λ&lt;/sub&gt; m&lt;sub&gt;+&lt;/sub&gt;) phoA supE44 λ&lt;sup&gt;−&lt;/sup&gt; thi&lt;sup&gt;−&lt;/sup&gt;1</td>
<td>Lab stock</td>
</tr>
<tr>
<td>NBC</td>
<td>β-Lactamase reporter gene translationally fused to the C-terminus of the <em>cnf1</em> gene in the chromosome of strain RS218</td>
<td>Yu &amp; Kim (2010)</td>
</tr>
<tr>
<td>NBC-1E6</td>
<td>NBC with Tn5 inserted in the <em>ygfZ</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td>YK6</td>
<td>RS218 (O18:K1:H7) <em>ygfZ</em> deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>HNS</td>
<td>RS218 (O18:K1:H7) <em>hns</em> deletion mutant</td>
<td>Lab stock</td>
</tr>
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<td>YK7</td>
<td>RS218 (O18:K1:H7) <em>ygfZ</em> deletion mutant complemented with <em>ygfZ</em> under the control of its native promoter</td>
<td>This study</td>
</tr>
<tr>
<td>YK14</td>
<td>NBC-1E6 complemented with <em>ygfZ</em> under the control of its native promoter</td>
<td>This study</td>
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<td>YK15</td>
<td>RS218 (O18:K1:H7) <em>ygfZ</em> and <em>hns</em> double deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>YK16</td>
<td>RS218 (O18:K1:H7) <em>ygfZ</em> and <em>hns</em> double deletion mutant complemented with <em>ygfZ</em> under the control of its native promoter</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pFLAG-CTC</td>
<td>5348 bp <em>E. coli</em> expression vector used for cytoplasmic expression of a properly inserted ORF as a C-terminal FLAG fusion protein</td>
<td>Sigma</td>
</tr>
<tr>
<td>pFLAG-CTS-BAP</td>
<td><em>E. coli</em> plasmid used for efficient and controlled periplasmic expression of C-terminal FLAG-BAP fusion protein</td>
<td>Sigma</td>
</tr>
<tr>
<td>pCTC-YgfZ</td>
<td>Expression vector of <em>YgfZ</em> fused with FLAG at the C-terminus</td>
<td>This study</td>
</tr>
<tr>
<td>pCXN</td>
<td>CNF1 coding region cloned into the <em>KpnI</em> site of PCX340, tetracycline resistant</td>
<td>Yu &amp; Kim (2010)</td>
</tr>
<tr>
<td>pKD3</td>
<td>Containing chromomycin-resistance gene and R6K&lt;sup&gt;γ&lt;/sup&gt; replication origin</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pKD47</td>
<td>Derivative of pKD46 (Datsenko &amp; Wanner, 2000), with the only modification that <em>blaM</em> was replaced by spectinomycin-resistance gene</td>
<td>This study</td>
</tr>
<tr>
<td>pGRG36</td>
<td>Tn7 insertion vector, ampicillin resistance, temperature sensitive</td>
<td>McKenzie &amp; Craig (2006)</td>
</tr>
<tr>
<td>pSR</td>
<td>Tn5 vector, spectinomycin resistant, R6K&lt;sup&gt;γ&lt;/sup&gt; replication origin</td>
<td>Yu &amp; Kim (2010)</td>
</tr>
<tr>
<td>pG-ygfZ</td>
<td>DNA fragment containing <em>ygfZ</em> gene obtained by PCR amplification from genomic DNA, and ligated into AvrII and <em>Pvu</em> sites of pGRG36</td>
<td>This study</td>
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20 h. Nitrocefin, as the chromogenic substrate of Bla, undergoes a distinctive colour change from yellow ($\lambda_{\text{max}}$ 390 nm at pH 7.0) to red ($\lambda_{\text{max}}$ 486 nm at pH 7.0) as the amide bond in the $\beta$-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 µl genomic DNA (0.5 µg µl$^{-1}$) and 12 µl sequencing primer SR-Seq (8 µM) 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 allelic 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 PstI sites of pGRG36. The resulting plasmid was designated pG-ygfZ. pG-ygfZ was then electroporated into NBC-1E6 (Tn1E6 s and 1E6-a (Table 2), and then ligated into ApaI and PstI sites of pGRG36. The resulting plasmid was designated pG-ygfZ, pG-ygfZ was then electroporated into NBC-1E6 (Tn5::ygfZ), the ΔygfZ mutant, or ΔygfZΔhns, 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 $\beta$-lactam ring, which produces an easily detectable colour change in CCF4 fluorescence from green to blue emission (Charpentier & Oswald, 2004; Yu & Kim, 2010). 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 Ndel and SalI 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 µm 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-CNFI monoclonal antibody (DD1) (Meyrick et al., 2001), alkaline phosphatase (AP or PhoA) monoclonal antibody (Millipore), $\beta$-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, $\beta$-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 10$^{8}$ 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 % CO$_2$ 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 µg ml$^{-1}$) for 1 h.

### Table 2. Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)*</th>
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<tbody>
<tr>
<td>1E6-KOF</td>
<td>TCTTTTACATTAGTTAATATACGCGCGTAAAGCAGAAGATCTCGAGATCTTTGAGCTGCTTCT</td>
</tr>
<tr>
<td>1E6-KOR</td>
<td>GACACCACTGTGCAATCACTACGGATGAAATGCTGTGAGATCAGATAGATAATATCTCTGTTAG</td>
</tr>
<tr>
<td>1E6CKF</td>
<td>GAGGCTCTATCACGCTATCC</td>
</tr>
<tr>
<td>1E6CKR</td>
<td>GGGCAAAGCTTCTGAGGTTCT</td>
</tr>
<tr>
<td>1E6-a</td>
<td>CCACATCGCGATACAGAGATTACTCTTC</td>
</tr>
<tr>
<td>1E6-s</td>
<td>CCGCGCTAGGTGTCTCAGAAAAATCGGAT</td>
</tr>
<tr>
<td>1E6-s2</td>
<td>ACCGTGCTATACGCTTACACGTTTACCATAC</td>
</tr>
<tr>
<td>1E6-a2</td>
<td>GCCGTGCTGTTCTCAGG</td>
</tr>
<tr>
<td>SPC-SeqR</td>
<td>GCCGTGCTGTTCTCAGG</td>
</tr>
<tr>
<td>Tn7-ckf</td>
<td>AGGCGCGAAGCTGAGAC</td>
</tr>
<tr>
<td>Tn7-ckr</td>
<td>TGAGAGCCCGGCTAACCCT</td>
</tr>
</tbody>
</table>

*Restriction sites for cloning are underlined.
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 ΔygfZ, 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 ΔygfZ was, however, restored in the complemented strain YK7 (ΔygfZ::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 ΔygfZ, we compared the subcellular distribution of CNF1 between the ΔygfZ mutant and wild-type strain RS218. The amounts of CNF1 in the cytoplasmic and periplasmic fractions were similar between RS218 and the ΔygfZ mutant (Fig. 3a), suggesting that the defect in CNF1 translocation into HBMEC in the ΔygfZ 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-lauryl sarcosine 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 ΔygfZΔhns 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

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[**Fig. 2.** Analysis of the translocation of CNF1 into HBMEC. HBMEC were pre-loaded with the Bla substrate CCF4/AM dye 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, ΔygfZ 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.]
note that the amount of OmpA was similar between the OMVs isolated from Δhns and ygfZ/Δhns mutants (Fig. 3c), suggesting that the failure to demonstrate CNF1 in OMVs in the ΔygfZ 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 ΔygfZ 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 ΔygfZ/Δhns mutant and the ΔygfZ/Δhns 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 ΔygfZ 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.
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 ΔygfZ mutant was significantly defective in invasion of HBMEC compared to the parent strain RS218 (Fig. 6). We then complemented the ΔygfZ 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

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

**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 μg 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 OD_{620}). M, molecular size markers. (b) Western blot analysis of different fractions of pCTC-ygfZ/RS218 with different antibodies as listed on the left.
YgfZ in CNF1 secretion

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 C$_1$ units (Teplyakov et al., 2004). Recent studies showed that YgfZ is involved in methyliating 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 Escherichia coli meningitis.

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Figure 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 ± 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 B$_5$)-binding capability of YgfZ is reminiscent of the involvement of BtuB (the receptor of vitamin B$_{12}$) 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


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