INFECTION AND IMMUNITY, Feb. 2010, p. 838–844 Vol. 78, No. 2
0019-9567/10/$12.00 doi:10.1128/IAI.00674-09

Ferredoxin Is Involved in Secretion of Cytotoxic Necrotizing Factor 1 across the Cytoplasmic Membrane in *Escherichia coli* K1[▽]

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Received 12 June 2009/Returned for modification 16 July 2009/Accepted 5 November 2009

We previously showed that cytotoxic necrotizing factor 1 (CNF1) contributes to *Escherichia coli* K1 invasion of human brain microvascular endothelial cells (HBMEC) and interacts with the receptor on the surface of HBMEC. CNF1 is the cytoplasmic protein, and it remains incompletely understood how CNF1 is secreted across the inner and outer membranes in *E. coli* K1. In order to investigate the genetic determinants for secretion of CNF1 in *E. coli* K1, we performed Tn*5* mutagenesis screening by applying β-lactamase as a reporter to monitor secretion of CNF1. We identified a Tn*5* mutant that exhibited no β-lactamase activity in the culture supernatant and in which the mutated gene encodes a ferredoxin gene (*fdx*). In the *fdx* deletion mutant, there was no evidence of translocation of CNF1 into HBMEC. Western blot analysis of the *fdx* deletion mutant revealed that ferredoxin is involved in translocation of CNF1 across the cytoplasmic membrane. The *fdx* mutant exhibited significantly decreased invasion of HBMEC, similar to the decreased HBMEC invasion observed with the CNF1 mutant. The failures to secrete CNF1 and invade HBMEC of the *fdx* mutant were restored to the levels of the parent strain by complementation with *fdx*. These findings demonstrate for the first time that ferredoxin is involved in secretion of CNF1 across the inner membrane in meningitis-causing *E. coli* K1.

Neonatal *Escherichia coli* meningitis is associated with high mortality and morbidity, and a major contributing factor is our incomplete knowledge on the pathogenesis of *E. coli* meningitis (15, 16). Most cases of neonatal *E. coli* meningitis develop as a result of hematogenous spread (8, 14), but it is incompletely understood how circulating bacteria cross the blood-brain barrier and cause meningitis.

We have shown that cytotoxic necrotizing factor 1 (CNF1) contributes to *E. coli* K1 invasion of human brain microvascular endothelial cells (HBMEC) and penetration into the central nerve system (CNS) via the interaction with its receptor, 37 laminin receptor precursor (37LRP)/67 laminin receptor (67LR) (4, 12, 13). CNF1 is a cytoplasmic protein, and its secretion is a strategy utilized by meningitis-causing *E. coli* K1 to invade the blood-brain barrier (12). CNF1 is the paradigm of the RhoGTPase-activating bacterial toxins (2, 19). The CNF1 secretion pathway, however, remains incompletely understood. No typical signal peptide is found in the CNF1 sequence. A previous study by Kouokam et al. showed that CNF1 is tightly associated with outer membrane vesicles (18). Outer membrane vesicles from a number of bacterial species have been found to contain virulence factors, exhibit immunomodulatory effects, and adhere to and intoxicate host cells (20).

In order to study the genetic determinants for secretion of CNF1 in meningitis-causing *E. coli* K1, we designed a Tn*5* mutational screening strategy by applying TEM β-lactamase as the reporter. Using this approach, we identified a mutant which was defective in CNF1 secretion into HBMEC, and this mutant is characterized in this report.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids are shown in Table 1. *E. coli* K1 strain RS218 (O18:K1:H7) is a cerebrospinal fluid isolate from a neonate with meningitis (12). *E. coli* K-12 strain DH5α was used as the host for plasmids, and EC100D *pir*116$^+$ (Epicentre Biotechnologies, Madison, WI) was the host for the R6kγ origin plasmid. *E. coli* strains were routinely grown at 37°C in Luria broth. Where appropriate, the medium was supplemented with ampicillin (100 μg/ml), spectinomycin (100 μg/ml), tetracycline (10 μg/ml), or chloramphenicol (20 μg/ml).

Construction of CNF1-Bla hybrid in the chromosome of RS218. To integrate the TEM-1 *blaM* mature form DNA in frame with CNF1 into strain RS218 genomic DNA, *blaM* together with its upstream multiple cloning sites was cloned from pCX340 into pRS (a derivative of pSR, and the difference is that in pRS the R6kγ replication origin is upstream of the spectinomycin resistance gene), yielding pFBI (*f*use *B*la *i*n frame). Then, *cnf1* was amplified from RS218 genomic DNA with primers (Cnf1-s3 and Cnf1-a [Table 2]) and cloned into the KpnI site of plasmid pFBI, which gave pFBI-CNF1. After construction of pFBI-CNF1, the *cnf1* downstream DNA fragment was amplified from RS218 genomic DNA with primers (NC-a3 and NC-s3 [Table 2]), digested with XbaI and NcoI, and cloned into pFBI-CNF1, and the resulting plasmid was designated as pNFB. The chloramphenicol resistance gene (obtained by digesting plasmid pKD3 with XbaI) (6) was then cloned into the XbaI site of pNFB, yielding pNBC.

Primers (NN-s and NC-a [Table 2]) were used to amplify the DNA fragment from pNBC, and PCR products were digested with DpnI and gel purified. The PCR products were then electroporated into competent cells of strain RS218 containing pKD47 (a derivative of pKD46, with *blaM* in pKD46 replaced by spectinomycin resistance gene), allowing recombination to occur in the presence of arabinose. The temperature-sensitive pKD47 was cured by incubation at 37°C with agitation. The integration of *blaM* after *cnf1* into the chromosome of strain RS218 was verified by PCR using primers (NFB-CKF and NFB-CKR [Table 2]), and the resulting strain was designated as strain NBC.

Transposome formation and transposition mutagenesis. Transposon DNA was released by digesting plasmid pSR (Fig. 1C, modified from the pMini-Tn*5* cycler [9]) with PvuII (New England Biolabs, Beverly, MA) and then gel purified (QIAquick gel extraction kit; Qiagen, Valencia, CA). Transposon DNA (Fig. 1C; up to 50 μg/ml) was incubated with 10 μg/ml hyperactive Tn*5* transposase (Epicentre Technologies) for 1 h at 37°C in a 20-μl reaction volume. Transpo-

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▽ Published ahead of print on 16 November 2009.

TABLE 1. Strains and plasmids used in the current study

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. coli strains		
RS218	O18:K1:H7, isolated from cerebrospinal fluid of neonate with *E. coli* meningitis	12
EC100D	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG pir*$^+$ (DHFR)	Epicentre Biotechnologies
DH5α	F′ ϕ80d*lacZ* ΔM15Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+)*phoA supE44* λ$^-$ *thi-1*	Lab stock
Δ*cnf1* mutant	RS218 (O18:K1:H7) *cnf1* deletion mutant	12
Δ*fdx* mutant	RS218 (O18:K1:H7) *fdx* deletion mutant	This study
CΔ*fdx* mutant	RS218 (O18:K1:H7) *fdx* deletion mutant complemented with *fdx* under control of arabinose promoter; complementation achieved by Tn*7* site-specific insertion into second benign site in chromosome	This study
Plasmids		
pBC-KS	Cloning vector with chloramphenicol resistance gene	Stratagene
pBAD-*Myc*/*His*	Arabinose promoter expression vector, ampicillin resistance	Invitrogen
pCX340	PBR322 derivative, cloning vector used to fuse CNF1 to the mature form of TEM-1 β-lactamase, tetracycline resistance	3
pCX311	Negative control, fusion of MBP to TEM-1, tetracycline resistance	3
pCXN	CNF1 coding region cloned into KpnI site of pCX340, tetracycline resistance	This study
pKD3	Containing chloramphenicol resistance gene, R6kγ replication origin	6
pKD47	Derivative of pKD46 (5); the only modification is that *blaM* in pKD46 is replaced by the spectinomycin resistance gene	This study
pGRG36	Tn*7* insertion vector, ampicillin resistance, temperature sensitive	21
pFBI	Contains β-lactamase coding region (signal peptide genetically deleted); spectinomycin resistance; R6kγ replication origin	This study
pFBI-CNF1	CNF1 translationally fused with β-lactamase in pFBI	This study
pNFB	DNA fragment downstream of *cnf1* in RS218 genome was PCR amplified and cloned in pFBI-CNF1	This study
pNBC	Chloramphenicol resistance gene was inserted right after β-lactamase in pNFB	This study
pSR	Tn*5* vector; spectinomycin resistance; R6kγ replication origin	This study
pGRGM	Multiple cloning site of pGRG36 ligated into PvuII site of pBC-KS; chloramphenicol resistance	This study
pGAP	AraC and arabinose promoter (pBAD) cloned into AvrII and XhoI site of pGRGM	This study
pGAP-*fdx*	Coding region of *fdx* cloned into NdeI and NotI site of pGAP	This study
pG-*fdx*	DNA fragment containing *fdx* obtained from pGAP-*fdx* by digestion with AvrII and PacI; ligated into same sites of pGRG36	This study

TABLE 2. Primers used in the study

Primer	Sequence (5′–3′)[a]
NCHKs	CGACCTGTCCTGGTGATGC
Cnf1-s3	GCGCGGTACCATGGGTAACCAATGGCAA
Cnf1-a	GGATCCGGTACCAAATTTTTTTGAAATACC TTCA
NC-s3	GGCGTCTAGATTTTGATTCGGGAAATTATT
NC-a3	GGCGCCATGGACTCTGCCCGATGATTTTC
NN-s	GTTGAAGTACTGGCTGTGGTT
NFB-CKS	GCTACTGAGGAAGAAGCATGGAA
NFB-CKA	TCGCAGGTGAGCCGAAACT
GRGM-f	TTTCACTTATCTGGTTGGTCG
GRGM-r	CGAGGCTTGTCAGTACATCA
AraCP-s	CCGGCCTAGGCTGATTCGTTACCAATTA TGAC
AraCP-a	CCGGCTCGAGCATGGTTAATTCCTCCT GTTA
fdx-KOF	TTCGCCAATTTCGCGGCTATCCGTCCACTT AAGTCCCATACTAACCTCTGGTGTAGGC TGGAGCTGCTTC
fdx-KOR	CCAGTCGGTTCGTCGTGCGCTGAAAGGCC ATTCCGTGGACGAGGTTTAATCATATGA ATATCCTCCTTAG
FdxCKF	AGGATTTTCTCGTTGGATG
FdxCKR	AAGACTCAATGAGCTATGCC
fdx-a	CGCGCGGCCGCACTTAAGTCCCATACTAA CCTC
fdx-s	GCGCGCATATGCCAAAGATTGTTATTTTG
Spc-SeqR	GCCTTGCTGTTCTTCTACGG
Tn7-ckf	ACGGTCGGGAACTGGAAC
Tn7-ckr	TGACCAGCCGCGTAACCT

[a] Restriction sites for cloning are underlined.

somes (1 μl) were electroporated into competent NBC cells. Transposon insertion mutants were selected with spectinomycin.

β-Lactamase activity assay. Bacteria were grown in 96-well plates at 37°C overnight without agitation and then centrifuged at 3,200 rpm for 10 min, and 95 μl of supernatant from each clone was added to 5 μl of nitrocefin stock solution (Calbiochem, Gibbstown, NJ), which was incubated at room temperature for up to 24 h to allow the red color to develop. Nitrocefin is a chromogenic β-lactamase substrate that undergoes a distinctive color 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 hydrolyzed by β-lactamase. Nitrocefin stock solution (2 mM; Calbiochem) was prepared by dissolving 10.3 mg of nitrocefin in 0.5 ml of dimethyl sulfoxide and then adding 25 mM HEPES buffer (pH 7.3) to a final volume of 10 ml. Bla activity was read as positive if the color change to red occurred. Spectrophotometric assays for Bla using nitrocefin were also carried out by measuring changes in absorbance at 486 nm.

Genomic DNA isolation and sequencing. Genomic DNA was isolated from individual Tn*5* mutants as described previously (25). Chromosomal DNA was quantified with the a Quant-iT dsDNA BR assay kit (Invitrogen, Carlsbad, CA). Twelve microliters of genomic DNA (0.5 μg/μl) and 12 μl of sequencing primer SR-Seq (8 μM) were sent to the DNA Synthesis and Sequencing Facility (Johns Hopkins University School of Medicine) for sequencing.

***fdx* gene deletion and complementation.** To delete the *fdx* gene, a chloramphenicol resistance cassette was amplified from pKD3 (6) using primers *fdx*-KOF and *fdx*-KOR (Table 2). The PCR product was inserted into the chromosome by Lambda Red-mediated allele replacement (6). The correct insertion was verified by PCR.

For gene complementation, we applied Tn*7* site-specific gene insertion into the second benign site in the chromosome of the mutant as described previously (21). Since the *fdx* gene is within the *isc* operon and does not have its own promoter, we used an arabinose promoter to initiate the transcription of the *fdx* gene. We amplified the multiple cloning site of pGRG36 (21) into the PvuII site of pBC-KS (the primers used for this purpose were GRGM-f and GRGM-r [Table 2]), yielding plasmid pGRGM. Next, araC and the arabinose promoter were obtained by PCR amplification from plasmid pBAD/*Myc-His* (Invitrogen)

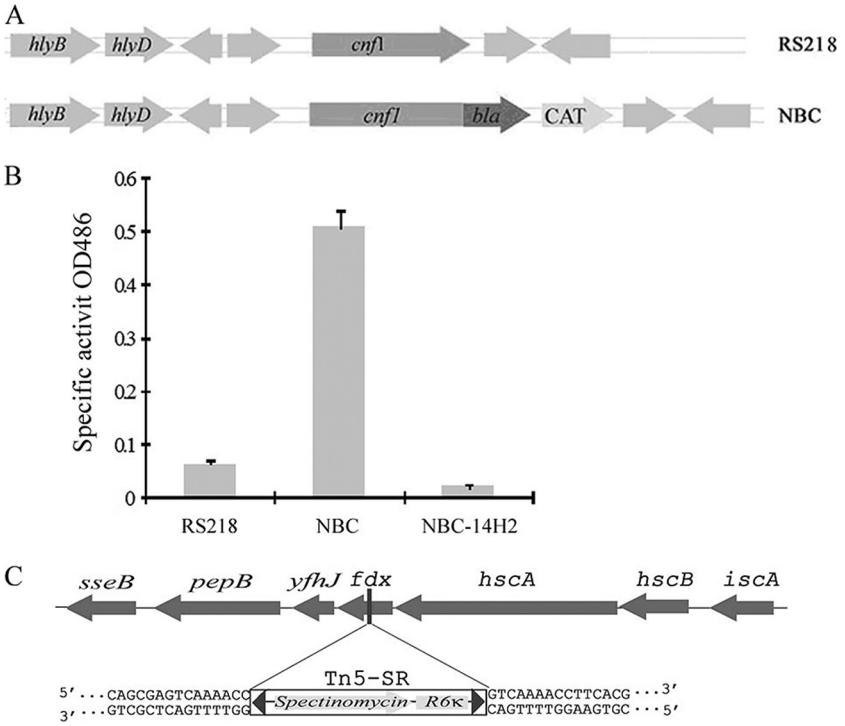

FIG. 1. Identification of the *fdx* gene as a genetic requirement for CNF1 secretion. (A) Schematic representation of the chromosomal structure of strains RS218 and NBC. In strain NBC, the *cnf1-blaM* translational chromosomal fusion was made by insertion of the *bla* gene after the *cnf1* gene. (B) Strains RS218, NBC, and transposon mutant NBC-14H2 were grown overnight in brain heart infusion medium (static at 37°C). Bacterial culture supernatant was obtained by centrifugation at 5,000 × *g* for 10 min, and specific β-lactamase activity was determined based on the absorbance at 486 nm. The represented Bla activity data (means ± standard deviations) represent the results from three experiments in triplicate. (C) Transposon insertion within the *fdx* gene in the mutant strain NBC-14H2.

with primers AraPs and AraPa (Table 2) and then ligated into AvrII and XhoI sites of pGRGM, and the resulting plasmid was designated as pGAP. The coding region of the *fdx* gene was amplified from the genomic DNA of strain RS218 with primers *fdx*-s and *fdx*-a (Table 2) and ligated into the NdeI and NotI site of pGAP, yielding plasmid pGAP-fdx. The DNA fragment containing araC, the arabinose promoter, and the *fdx* gene was obtained by digesting pGAP-*fdx* with restriction enzymes AvrII and PacI and was subsequently ligated into the same sites of pGRG36. Finally, the ligation product was electroporated into the *fdx* mutant, and transformants were selected on LB plates containing ampicillin at 32°C. The transformant was streaked once on an LB plate containing ampicillin to ensure that the bacteria carried the plasmid and then grown without antibiotic selection in LB at 32°C, and 0.1% arabinose was added to induce expression of TnsABCD (Tn*7* transposition machinery). Subsequently, the transformants were incubated at 42°C to prevent replication of the plasmid, and the insertion of Tn*7* in the attachment site was verified by PCR with primers Tn7-ckf and Tn7-ckr (Table 2).

Assessment of CNF1 translocation into HBMEC. HBMEC were cultured in clear-bottom 96-well plates (Becton Dickinson, Franklin Lakes, NJ) at 20,000 cells per well in experimental medium (M199–Ham F-12 [1:1] containing 5% fetal bovine serum, 2 mM glutamine, and 1 mM pyruvate) and incubated at 37°C in 5% CO_2. Bacterial strains were grown overnight in brain heart infusion broth at 37°C, and the expression of the CNF1-Bla fusion from pCXN was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). On the day of infection, HBMEC were preloaded with CCF4/AM dye (final concentration, 1 μM; Invitrogen) as described previously (3, 23) and incubated with bacteria. The nonfluorescent esterified CCF4/AM substrate, upon entry into HBMEC, is rapidly converted to fluorescent green CCF4 by cellular esterases. Translocation of CNF1-Bla induces catalytic cleavage of the CCF4 β-lactam ring, which produces an easily detectable change in CCF4 fluorescence from green to blue emission (3, 28). After 45 min of infection, the translocation of the CNF1-Bla hybrid into HBMEC was observed under a Nikon fluorescence microscope.

Cell fractionation. Cytoplasmic and periplasmic fractions were obtained by the method of osmotic shock as described previously (27). Periplasmic suspension was filtered through a 0.22-μm filter to remove any residual bacterial cells and then precipitated by the Na-deoxycholate–trichloroacetic acid method (5).

Western blot assays. Cell fractions were separated by SDS-PAGE, and then protein samples were transferred to a nitrocellulose membrane. The blots were blocked with 5% skim milk in Tris-buffered saline (TBS; 25 mM Tris, pH 7.4, 150 mM NaCl) for 60 min at 22°C. The membrane was incubated for 2 h at 22°C with primary antibody. Primary antibodies used in this study were anti-CNF1 monoclonal antibody (DD1) (22), alkaline phosphatase (PhoA) monoclonal antibody (Millipore), and β-galactosidase (β-Gal) antiserum (Millipore), and disulfide oxidoreductase (DsbA) antiserum was also used (1). 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 an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The *E. coli* proteins located in the cytoplasm (e.g., β-Gal protein) and periplasm (e.g., PhoA and DsbA) were used as markers for cytoplasmic and periplasmic fractions, respectively.

***E. coli* invasion assays in HBMEC.** *E. coli* invasion assays were performed in HBMEC as previously described (13). Briefly, confluent cultures of HBMEC (grown in 24-well plates) were incubated with 10^7 CFU of *E. coli* (multiplicity of infection of 100) in experimental medium. Plates were incubated for 90 min at 37°C in 5% CO_2 to allow invasion to occur. The number of intracellular bacteria was determined by culturing on blood agar plates after extracellular bacteria were killed by incubation of the HBMEC monolayers with experimental medium containing gentamicin (100 μg/ml) for 1 h. Assays were performed in triplicate and repeated at least three times. Results are expressed as relative invasion frequencies (percent invasion compared to that of the parent strain, RS218).

RESULTS

Screening of genetic determinants for secretion of CNF1. To investigate the secretion of CNF1 in meningitis-causing *E. coli* K1, we applied β-lactamase (Bla) as the reporter gene, which

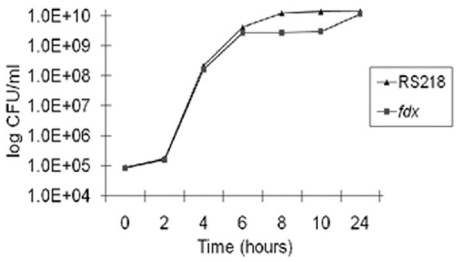

FIG. 2. Growth curves of the parent strain RS218 and its *fdx* mutant in brain heart infusion (BHI) medium. Overnight cultures of RS218 and the *fdx* mutant were diluted 1:10,000 into fresh BHI medium, and bacteria were cultured at 37°C with shaking for up to 24 h. The CFU were determined at different time intervals as indicated in the figure. The experiment was repeated three times.

was translationally fused to the C terminal of the *cnf1* gene in the chromosome of strain RS218. The resulting strain was designated as strain NBC (C*N*F1-*B*la-*C*AT) (Fig. 1A). In the NBC strain, Bla's secretion is entirely dependent on CNF1's secretion machinery. We were able to visualize the color change of nitrocefin in the culture supernatant of NBC (yellow to red), which was induced by Bla activity, compared to no detectable color change with the parent strain, RS218. These findings demonstrated that CNF1 secretion can be detected by Bla activity in strain NBC, and strain NBC is suitable for screening of genes involved in CNF1 secretion in *E. coli* K1 strain RS218.

We next performed mini-Tn*5 in vitro* mutagenesis and constructed a mutant library of strain NBC. Those mutants with the transposon being inserted within the *cnf1-bla* coding region or the promoter region of *cnf1* were excluded by PCR (with primers NCHKs and NC-a [Table 2]). For β-lactamase assays, the NBC strain was used as a positive control, while the wild-type strain RS218 was used as a negative control. We identified a mutant (NBC-14H2) that exhibited negative Bla activity based on visual color change and the spectrometric reading, and the characterization of this mutant is the purpose of this report.

Ferredoxin is essential for CNF1 secretion. The transposon mutant was significantly defective in secretion of CNF1 into the culture medium, based on the β-lactamase activity (Fig. 1B). We determined the location of the transposon insertion by direct DNA sequencing of the mutant's genomic DNA. The insertion was shown to occur within the *fdx* gene, encoding ferredoxin, which is located within the *isc* operon, downstream of the *hscA* and *hscB* genes (Fig. 1C). Genes located within *isc* operon have been shown to be involved in iron sulfur protein assembly and may operate *in vivo* as a complex (26). However, the function of ferredoxin has not been biochemically determined.

We deleted the *fdx* gene from the RS218 genome. The growth rates of the *fdx* mutant were similar to those of the parent strain, RS218, and both strains had similar numbers of bacteria after 24 h of incubation (Fig. 2). We subsequently analyzed its capability for translocating CNF1 into HBMEC by using a CNF1-Bla fusion protein expressed from the plasmid pCXN. *E. coli* transformants harboring pCXN were preinduced with 1 mM IPTG and then added to HBMEC preloaded with CCF4/AM dye. After 45 min of incubation, the CNF1 translocation into HBMEC was visualized under fluorescence microscopy. As expected, the wild-type strain successfully translocated CNF1-Bla hybrid protein into HBMEC, as shown by the emission of blue fluorescence, while the *fdx* mutant failed to do so (Fig. 3). The failure to translocate the CNF1-Bla fusion in the *fdx* mutant was restored in the complemented strain CΔ*fdx* (Fig. 3).

Bacterial lysates of the Δ*fdx*/pCXN construct containing the CNF1-Bla fusion protein, induced by 1 mM IPTG, however, was capable of translocation into HBMEC, as shown in a CCF4/AM assay (data not shown). These findings demonstrate that the CNF1-Bla fusion expressed in strain Δ*fdx* is functionally active, and the failure to translocate CNF1 into HBMEC in the *fdx* mutant is due to the lack of its secretion.

Ferredoxin is required for secretion of CNF1 at the step of crossing the cytoplasmic membrane. We next examined the secretion of CNF1 across the cytoplasmic membrane by comparing the location of CNF1 expression in cytoplasmic and periplasmic fractions derived from the parent strain, RS218, and its *fdx* mutant. As shown in Fig. 4A, the cytoplasmic fraction contained β-Gal protein but was devoid of periplasmic proteins (PhoA and DsbA). In contrast, the periplasmic fraction contained PhoA and DsbA but was devoid of β-Gal, suggesting that our cell fractions exhibited the expected protein profiles. As shown by Western blotting (Fig. 4A), the presence of CNF1 in both the cytoplasmic and periplasmic fractions was demonstrated in the parent strain, RS218. In contrast, CNF1 expression was evident in the cytoplasm but was not detectable in the periplasmic fraction in the *fdx* mutant (Fig. 4A). The absence of CNF1 in the periplasmic fraction of the *fdx* mutant was, however, restored by complementation of the *fdx* mutant with the *fdx* gene (strain CΔ*fdx*). These findings suggest that CNF1 secretion was blocked at the step of crossing the inner membrane in the Δ*fdx* mutant.

To determine whether or not the failure to secrete CNF1 across the inner membrane is unique to CNF1 in the *fdx* mutant, we examined and compared the patterns of cytoplasmic and periplasmic proteins between strain RS218 and its *fdx* mutant. SDS-PAGE and Coomassie blue staining revealed that the *fdx* mutant exhibited somewhat different patterns of the periplasmic proteins compared with those of RS218, while the patterns of the cytoplasmic proteins were similar between strain RS218 and the *fdx* mutant (Fig. 4B). Taken together, these findings suggest that ferredoxin may be involved in the secretion of CNF1 and several other proteins across the inner membrane in *E. coli* K1.

Ferredoxin promotes *E. coli* K1 invasion of HBMEC. We previously showed that CNF1 contributes to *E. coli* K1 invasion of HBMEC (12). Because CNF1 secretion was impaired in the *fdx* mutant, our next experiment was to examine the HBMEC invasion abilities of the *fdx* mutant and its complemented strain, compared to those of the wild-type strain, RS218, and the CNF1 mutant. The *in vitro* HBMEC assays revealed that the *fdx* mutant was significantly defective in invasion of HBMEC compared to the parent strain (Fig. 5), while the invasion frequency was restored to the level of the parent strain by complementation with *fdx*. To initiate the transcription of the *fdx* gene in the complemented strain CΔ*fdx*, 0.1% arabinose was added to both brain heart infusion medium and HBMEC invasion assay medium, but arabinose did not affect HBMEC

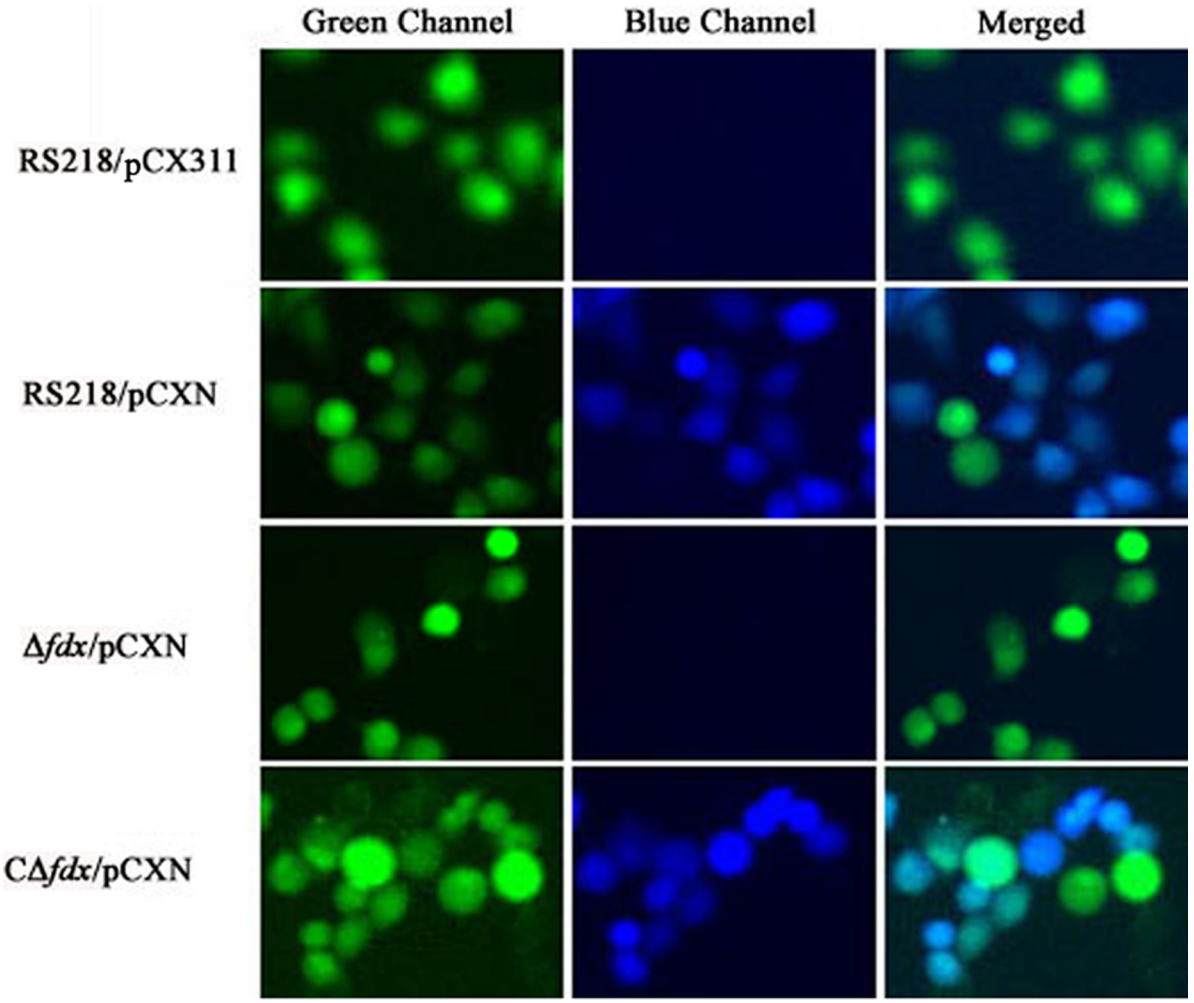

FIG. 3. Analysis of translocation of CNF1 into HBMEC. HBMEC were preloaded with the BlaM substrate CCF4/AM and then infected with *E. coli* strains bearing different plasmids as indicated on the left. Plasmid pCX311 expresses a maltose-binding protein-Bla fusion and was used as the negative control, and pCXN expresses a CNF1-Bla fusion. RS218 is the wild-type strain, Δ*fdx* is a *fdx* deletion mutant, and CΔ*fdx* is a complemented strain of the *fdx* deletion mutant with *fdx*. For strain CΔ*fdx*, 0.1% arabinose was added to promote the transcription of the complemented *fdx* gene. The software ImageJ was used to merge the green and blue channels (24).

invasion frequencies except for strain CΔ*fdx*. As expected, the CNF1 mutant was significantly defective in invasion of HBMEC, which is consistent with our previous data (12).

DISCUSSION

We previously showed that CNF1, a cytoplasmic protein, contributes to *E. coli* K1 invasion of HBMEC and penetration into the CNS, and we identified the HBMEC receptor for CNF1, 37LRP/67LR (4, 12, 13), but it remains incompletely understood how CNF1 is transported across the inner and outer membranes in *E. coli* K1. Recent studies have shown that CNF1 from uropathogenic *E. coli* strains J96 and CP9 is transported to the culture supernatant in a complex with outer membrane vesicles (7, 18).

In order to investigate the secretion and/or translocation of CNF1 from the cytoplasm of *E. coli* K1, we constructed a recombinant *E. coli* strain fused with β-lactamase in the C terminus of the *cnf1* gene in the chromosome of RS218 (strain NBC). In the NBC strain, Bla's secretion is entirely dependent upon CNF1's secretion machinery, and we initially used Bla activity to monitor CNF1 secretion from the cytoplasm. From screening of the Tn*5* library of strain NBC, we identified the mutant that exhibited no detectable Bla activity in the culture supernatant and did not have the transposon inserted into the *cnf1-bla* coding region or the promoter region of *cnf1*.

We subsequently identified that the transposon insertion occurred within the *fdx* gene, and a CNF1 translocation assay with the *fdx* deletion mutant demonstrated that ferredoxin is essential for CNF1 secretion into HBMEC. Moreover, we showed that ferredoxin was involved in secretion of CNF1 across the inner membrane of strain RS218. This was shown by our demonstrations that (i) deletion of *fdx* resulted in a failure to detect the presence of CNF1 in the periplasmic fraction of strain RS218, while the known periplasmic proteins, PhoA and DsbA, were present in the periplasmic fraction, and (ii) complementation of the *fdx* mutant with *fdx* restored the secretion of CNF1 in the periplasmic fraction. These findings

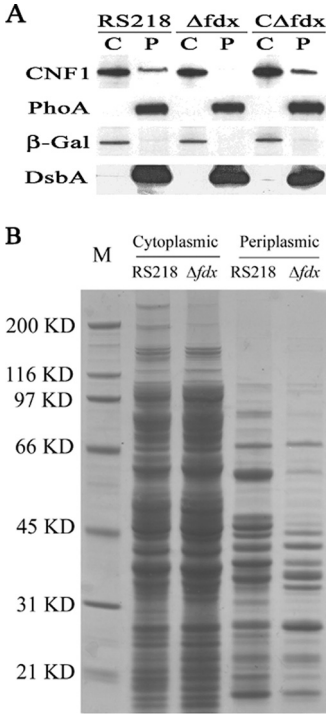

FIG. 4. (A) CNF1 subcellular localization and secretion in *E. coli* RS218. Western blot analysis was carried out with the cytoplasmic (C) and periplasmic (P) fractions from RS218, Δ*fdx*, and CΔ*fdx* (with 0.1% arabinose to promote the transcription of complemented *fdx* gene). The amount of cytoplasmic protein loaded was 40 μg, and 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 optical density at 620 nm [OD_{620}]). CNF1, PhoA, β-Gal, and DsbA were detected by their respective specific antibodies, as described in Materials and Methods. (B) SDS-PAGE analysis of the protein profile in the cytoplasmic and periplasmic fractions prepared from RS218 and Δ*fdx* as indicated in the figure. M, molecular marker; size positions are indicated along with the masses on the left. The amount of cytoplasmic protein loaded was 15 μg. The loaded periplasmic protein was equal to the total periplasmic protein that was collected from 10^{10} bacteria cells (the number of bacteria was estimated from the OD_{620}).

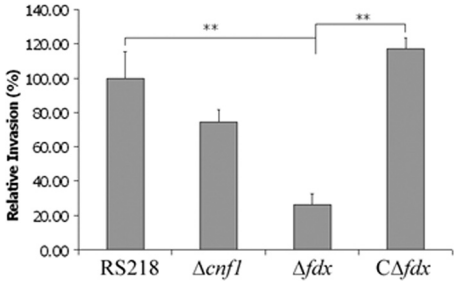

FIG. 5. The *fdx* deletion mutant of *E. coli* strain RS218 exhibits significantly decreased invasion of HBMEC. To determine whether ferredoxin plays a role in *E. coli* invasion of HBMEC, invasion assays were performed using the *fdx* deletion mutant and the complemented strain CΔ*fdx*. In strain CΔ*fdx*, the *fdx* gene is under the control of the pBAD promoter. **, $P < 0.01$. The data (means ± standard deviations) represent assay results with 0.1% arabinose.

demonstrate that ferredoxin affects the secretion of CNF1 across the inner membrane in *E. coli* K1 strain RS218.

More importantly, the *fdx* deletion mutant exhibited significantly decreased invasion of HBMEC compared to the parent strain RS218, and this invasion defect was restored to the level of the parent strain by complementation with *fdx.* CNF1 has been reported to contribute to *E. coli* K1 invasion of HBMEC, as shown by significantly decreased HBMEC invasion of the CNF1 mutant compared to the parent strain RS218 (4, 12, 13), and it is likely that the HBMEC invasion defect of the *fdx* mutant is related to the failure to secrete CNF1. Of interest, the decreased invasion frequency was significantly greater with the *fdx* mutant than with the CNF1 mutant, e.g., 26% versus 74% relative invasion frequency, respectively, compared to the invasion frequency of the parent strain RS218, suggesting that ferredoxin may affect secretion of CNF1 as well as other *E. coli* K1 determinants involved in invasion of HBMEC. Our comparison of periplasmic protein profiles showed different patterns of proteins between the parent strain and the *fdx* mutant, and it is tempting to speculate that *fdx* may also involve secretion of other *E. coli* K1 determinants involved in HBMEC invasion. Additional studies are needed to clarify this issue.

Ferredoxins are small iron-sulfur proteins that mediate electron transfer and have either a [4Fe-4S], [3Fe-3S], or [2Fe-2S] clusterwhose reduction potential is highly negative (−300 mV or lower) (10, 11). *E. coli* ferredoxin is an adrenodoxin-type [2Fe-2S] ferredoxin, and it does not mediate electron transport in the NADP photoreduction system of spinach and is incapable of replacing the *Pseudomonas putida* ferredoxin in camphor hydroxylation (10, 17). The genetic localization of the *fdx* gene suggests that it may be involved in biogenesis of Fe-S proteins (26). However, the exact physiological role of *E. coli* ferredoxin has not yet been genetically or biochemically determined. The electronic versatility of Fe-S clusters make it possible that ferredoxin may be involved in transmitting energy that is required for CNF1 secretion. There might also be other yet-undetermined mechanisms for the involvement of ferredoxin in CNF1 secretion.

In summary, we have demonstrated for the first time that ferredoxin affects the secretion of CNF1 across the inner membrane in meningitis-causing *E. coli* K1, and the *fdx* mutant was defective in secretion of CNF1 into HBMEC as well as invasion of HBMEC. Studies are needed to understand how ferredoxin affects the CNF1 secretion and also whether ferredoxin affects secretion of other virulence factors that are involved in *E. coli* K1 invasion of HBMEC.

ACKNOWLEDGMENTS

This work was supported in part by an NIH grant (NS26310-22).

We thank Alison O'Brien for providing the CNF1 monoclonal antibody, K. Ito for providing DsbA antiserum, Nancy L. Craig for providing the pGRG36 plasmid, Fred Heffron for providing the plasmid pMini-Tn*5* cycler, and Eric Oswald for providing plasmids pCX311 and pCX340. We also thank George Niemann and Bo Ma for suggestions and discussions.

REFERENCES

1. **Akiyama, Y., S. Kamitani, N. Kusukawa, and K. Ito.** 1992. In vitro catalysis of oxidative folding of disulfide-donded proteins by the *Escherichia coli dsbA (ppfA)* gene product. J. Biol. Chem. **267:**22440–22445.
2. **Boquet, P.** 2001. The cytotoxic necrotizing factor 1 (CNF1) from *Esherichia coli*. Toxicon **39:**1673–1680.
3. **Charpentier, X., and E. Oswald.** 2004. Identification of the secretion and translocation domain of the Enteropathogenic and Enterohemorrhagic

Escherichia coli effector Cif, using TEM-1 β-lactamase as a new fluorescence-based reporter. J. Bacteriol. **186:**5486–5495.
4. **Chung, J. W., S. J. Hong, K. J. Kim, D. Goti, M. F. Stins, S. Shin, V. L. Dawson, T. M. Dawson, and K. S. Kim.** 2003. 37-kDa laminin receptor precursor modulates cytotoxic necrotizing factor 1-mediated RhoA activation and bacterial uptake. J. Biol. Chem. **278:**16857–16862.
5. **Cold Spring Harb. Protoc.** 2006. doi:10.1101/pdb.prot4258.
6. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
7. **Davis, J. M., H. M. Carvalho, S. B. Rasmussen, and A. D. O'Brien.** 2006. Cytotoxic necrotizing factor type 1 delivered by outer membrane vesicles of uropathogenic *Escherichia coli* attenuates polymorphonuclear leukocyte antimicrobial activity and chemotaxis. Infect. Immun. **74:**4401–4408.
8. **Dietzman, D. E., G. W. Fischer, and F. D. Schoenknecht.** 1974. Neonatal *Escherichia coli* septicemia: bacterial counts in blood. J. Pediatr. **85:**128–130.
9. **Geddes, K., M. Worley, G. Niemann, and F. Heffron.** 2005. Identification of new secreted effectors in *Salmonella enterica* serovar Typhimurium. Infect. Immun. **73:**6260–6271.
10. **Holden, H. M., B. L. Jacobson, J. K. Hurley, G. Tollin, B. H. Oh, L. Skjeldal, Y. K. Chae, H. Cheng, B. Xia, and J. L. Markley.** 1994. Structure-function studies of [2Fe-2S] ferredoxins. J. Bioenerg. Biomembr. **26:**67–88.
11. **Kakuta, Y., T. Horio, Y. Takahashi, and K. Fukuyama.** 2001. Crystal structure of *Escherichia coli* Fdx, an adrenodoxin-type ferredoxin involved in the assembly of iron-sulfur clusters. Biochemistry **40:**11007–11012.
12. **Khan, N. A., Y. Wang, K. J. Kim, J. W. Chung, C. A. Wass, and K. S. Kim.** 2002. Cytotoxic necrotizing factor-1 contributes to *Escherichia coli* K1 invasion of the central nervous system. J. Biol. Chem. **277:**15607–15612.
13. **Kim, K. J., J. W. Chung, and K. S. Kim.** 2005. 67-kDa laminin receptor promotes internalization of cytotoxic necrotizing factor 1-expressing *Escherichia coli* K1 into human brain microvascular endothelial cells. J. Biol. Chem. **280:**1360–1368.
14. **Kim, K. S., H. Itabashi, P. Gemski, J. Sadoff, R. L. Warren, and A. S. Cross.** 1992. The K1 capsule is the critical determinant in the development of *Escherichia coli* meningitis in the rat. J. Clin. Invest. **90:**897–905.
15. **Kim, K. S.** 2003. Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. Nat. Rev. Neurosci. **4:**376–385.
16. **Kim, K. S.** 2008. Mechanisms of microbial traversal of the blood-brain barrier. Nat. Rev. Microbiol. **6:**625–634.
17. **Knoell, H. E., and J. Knappe.** 1974. Escherichia coli ferredoxin, an iron-sulfur protein of the adrenodoxin type. Eur. J. Biochem. **50:**245–252.
18. **Kouokam, J. C., S. N. Wai, M. Fällman, U. Dobrindt, J. Hacker, and B. E. Uhlin.** 2006. Active cytotoxic necrotizing factor 1 associated with outer membrane vesicles from uropathogenic *Escherichia coli*. Infect. Immun. **74:** 2022–2030.
19. **Lemonier, M., L. Landraud, and E. Lemichez.** 2007. Rho GTPase-activating bacterial toxins: from bacterial virulence regulation to eukaryotic cell biology. FEMS Microbiol. Rev. **31:**515–534.
20. **Mashburn-Warren, L. M., and M. Whiteley.** 2006. Special delivery: vesicle trafficking in prokaryotes. Mol. Microbiol. **61:**839–846.
21. **McKenzie, G. J., and N. L. Craig.** 2006. Fast, easy and efficient: site-specific insertion of transgenes into enterobacterial chromosomes using Tn7 without need for selection of the insertion event. BMC Microbiol. **6:**39.
22. **Meysick, K. C., M. Mills, and A. D. O'Brien.** 2001. Epitope mapping of monoclonal antibodies capable of neutralizing cytotoxic necrotizing factor type 1 of uropathogenic *Escherichia coli*. Infect. Immun. **69:**2066–2704.
23. **Mills, E., K. Baruch, X. Charpentier, S. Kobi, and I. Rosenshine.** 2008. Real-time analysis of effector translocation by the type III secretion system of enteropathogenic Escherichia coli. Cell Host Microbe **3:**104–113.
24. **Rasband, W. S.** 2009. Image J, 1997–2009. National Institutes of Health, Bethesda, MD. http://rsb.info.nih.gov/ij/.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Takahashi, Y., and M. Nakamura.** 1999. Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in *Escherichia coli*. J. Biochem. **126:**917–926.
27. **Wai, S. N., B. Lindmark, T. Söderblom, A. Takade, M. Westermark, J. Oscarsson, J. Jass, A. Richter-Dahlfors, Y. Mizunoe, and B. E. Uhlin.** 2003. Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. Cell **115:**25–35.
28. **Zlokarnik, G., P. A. Negulescu, T. E. Knapp, L. Mere, N. Burres, L. Feng, M. Whitney, K. Roemer, and R. Y. Tsien.** 1998. Quantitation of transcription and clonal selection of single living cells with β-lactamase as reporter. Science **279:**84–88.

Editor: B. A. McCormick