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Escherichia coli Invasion of Brain Microvascular Endothelial Cells In Vitro and In Vivo: Molecular Cloning and Characterization of Invasion Gene *ibe10*

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Most cases of neonatal *Escherichia coli* meningitis develop as a result of hematogenous spread, but it is not clear how circulating *E. coli* crosses the blood-brain barrier. In an attempt to identify *E. coli* structures contributing to invasion into the central nervous system (CNS), Tn*phoA* mutagenesis was performed with an invasive CSF isolate of *E. coli* K1 strain RS218 (O18:K1:H7), and Tn*phoA* mutants were examined for their noninvasive capability in brain microvascular endothelial cells (BMEC). The noninvasive mutants exhibited the invasive ability of <1% of the parent strain. One of the noninvasive mutants (10A-23) with a single Tn*phoA* insertion and no changes in phenotypic characteristics was found to be significantly less invasive into the CNS in the newborn rat model of hematogenous *E. coli* meningitis. The Tn*phoA* inserts with flanking sequences were cloned and sequenced. A novel open reading frame (8.2 kDa) was identified. Open reading frame analysis indicated that the 8.2-kDa protein (Ibe10) contained multiple transmembrane domains. *ibe10* was cloned into an expression vector, pQE30, and the purified Ibe10 was shown to inhibit invasion of BMEC by strain RS218. These findings indicate that *ibe10* is one of the *E. coli* genes involved in the invasion of BMEC in vitro and in vivo.

The mortality and morbidity associated with neonatal gram-negative bacillary meningitis have remained significant despite advances in antimicrobial chemotherapy and supportive care (12, 36). A contributing factor is the incomplete understanding of the pathogenesis and pathophysiology associated with this disease. For example, most cases of neonatal *Escherichia coli* meningitis develop as a result of hematogenous spread; however, it is not clear how circulating *E. coli* invades into the central nervous system (CNS).

Several investigators have made use of cultured mammalian cells as a model to study bacterial pathogenesis (1, 11, 18–20). Successful isolation and cultivation of brain microvascular endothelial cells (BMEC), which constitute the blood-brain barrier, enabled us to dissect the pathogenetic mechanisms of *E. coli* meningitis in vitro (28, 32). Using this in vitro system of the blood-brain barrier, we have previously shown that S fimbriae mediate *E. coli* binding to sialoglycoproteins and glycolipids of BMEC (28, 32). However, binding via S fimbriae was not accompanied by invasion into BMEC (27).

To facilitate the identification of the genes contributing to *E. coli* invasion of BMEC, we used the transposon Tn*phoA* (26, 35, 39) to generate a collection of noninvasive *E. coli* mutants. Tn*phoA* is a modified transposon which is engineered by inserting the *phoA* gene (encoding *E. coli* alkaline phosphatase) into one end of Tn*5* (26). The gene fusions can be randomly generated by Tn*phoA* insertion into the target gene in the chromosome or plasmid. The Tn*phoA* approach has led to the discovery of virulence factors in gram-negative bacteria, including *E. coli* (8, 9, 14, 35, 37, 39).

In the present paper, we describe molecular analysis and characterization of a mutant (10A-23) which was derived from a cerebrospinal fluid (CSF) isolate of *E. coli* K1 by Tn*phoA* mutagenesis. This mutant was unable to invade cultured BMEC in vitro and the CNS in the newborn rat model of hematogenous meningitis in vivo. An *E. coli* gene (*ibe10* [termed after invasion of brain endothelial cells from 10A-23]) encoding an 8.2-kDa protein was shown to be associated with *E. coli* K1 invasion of BMEC in vitro and in vivo.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacteria were cultured in L broth (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter) and stored in L broth plus 20% glycerol at −70°C. *E. coli* RS218 (O18:K1:H7) is a clinical isolate from the CSF of a newborn infant with meningitis (37). DH5αMCR, which has mutations in *mcr* regions (13), was used as the host strain in cloning Tn*phoA* DNA fragments and preparation of plasmids for DNA sequence determinations. Strains with antibiotic resistance were grown at 37°C in L broth with ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (20 μg/ml), rifampin (100 μg/ml), or chloramphenicol (100 μg/ml) for positive selection of plasmids (Table 1).

Chemicals and enzymes. Restriction endonucleases, T4 DNA ligase, and other enzymes were purchased from New England Biolabs (Beverly, Mass.) unless otherwise noted. Chemicals were purchased from Sigma (St. Louis, Mo.). All isotopes were obtained from New England Nuclear Corp. (Boston, Mass.). Reagents for preparation of DNA sequencing gels were of ultrapure quality from National Diagnostics (Atlanta, Ga.). The reagents for DNA sequencing reactions and other chemicals were purchased from U.S. Biochemical Corp. (Cleveland, Ohio).

Tissue cultures. BMEC were isolated from bovine and human brains as described previously (32). On the basis of factor VIII and carbonic anhydrase IV expression and the ability to take up low-density lipoproteins, the BMEC were >99% pure (32). The BMEC were plated at 2×10^3 cells per 0.32-cm^2 well in 96-well plates and at 2×10^4 cells per 2.0-cm^2 well in 24-well culture plates containing Dulbecco modified Eagle-Ham F12 medium with 10% fetal bovine serum.

Tn*phoA* transposon mutagenesis of *E. coli* K1 RS218. Strain SM10 containing the suicide plasmid pRT733 was used as a Tn*phoA* donor, while *E. coli* K1 strain RS218 was used as a recipient. Strain SM10 carrying pRT733 was mated with a spontaneous rifampin-resistant mutant of RS218 on Luria-Bertani (LB) agar by cross-streaking and then incubating at 37°C for 6 h. Conjugants were selected on LB agar containing kanamycin and rifampin. The indicator XP (5-bromo-4-chloro-3-indolyl phosphate) was included to select for those transpositions with PhoA fusion protein expression (blue colonies). However, blue and white colo-

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TABLE 1. Bacterial strains and plasmids used in the present study

Name	Genotype or characteristic(s)	Reference or source
E. coli		
RS218	O18:K1:H7	34
C5	O18:K1:H7	19
DH5αMCR	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) θ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 phoA supE44*λ^-*thi-1 gyrA96 relA1*	13
HB101	F^- *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20*(Sm^r) xyl5λ^- *leu mtl1*	Gibco-BRL
E91	RS218 Rif^r Tet^r $OmpA^-$	28
M15	Nal^s Str^s Ril^s *lac ara gal mtl* F^- *recA*$^+$ *uvr*$^+$ pREP4	Qiagen
SM10 (λpir)	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (λpir) *pro endA hsdA hsdR supF*	34
Plasmids		
pRT733	Amp^r *oriR6K mobRP4* Tn*phoA*	31
pRD87	Amp^r *oriColE1*; cloned ompA	34
pWG123	Amp *oripBR322* ptac 5S rrnBT_1T_2	23
pCVD433	Modified pACYC184 by inserting an *Mlu*I linker	8
pCRII	Amp^r Kan^r *oriColE1 oriF1*	Invitrogen
pQE30	Amp^r *oriColE1 cat*	Qiagen
pCD10A	pCVD433 carrying 10A-23 locus and Tn*phoA*	This study
pCIB10A	Ibe10 (0.25 kb) pCRII	This study
pCIB10B	Ibe10 (0.58 kb) pCRII	This study
pQIB10	Ibe10 (0.25 kb) pQE30	This study

nies were used for screening of noninvasive property to exclude the possibility that PhoA fusion proteins also had invasive activity.

Screening of Tn*phoA* mutants for invasive ability. All 704 Tn*phoA* mutants that appeared blue on medium containing XP and 234 white colonies were tested for invasive ability. Invasion assays were performed by a modification of the method described by Tang et al. (34). To a confluent monolayer of BMEC in each well of a 96-well plate, approximately 2×10^6 mid-log-phase bacteria were added, and the plate was incubated for 2 h at 37°C to allow invasion to occur. The wells were washed and incubated with medium containing gentamicin (100 μg/ml) at 37°C for 1 h to eliminate extracellular bacteria. Subsequently, the BMEC were washed and lysed with Triton X-100, and 10 μl from each well was plated onto sheep blood agar for determination of surviving CFU. The results were read qualitatively, e.g., whether mutants were invasive or heavy growth (>100 CFU) or whether mutants were noninvasive or few isolated colonies (usually <10 CFU). This screening assay always included strain RS218 as a positive control and strains E91 and HB101 as negative controls (noninvasive K1 and K-12 strains, respectively). These mutants were derived from different matings, thus reducing the possibility of the mutants being derived from siblings.

The mutants identified as noninvasive by the screening assay described above were verified for their noninvasive property by the standard invasion assay as described below. Briefly, approximately 10^7 bacteria were added to confluent BMEC grown in each well of a 24-well plate, which was incubated at 37°C for 1.5 h. The monolayers were washed four times and incubated with medium containing gentamicin (100 μg/ml) at 37°C for 1 h to eliminate extracellular bacteria. Subsequently, BMEC were again washed four times and lysed with 0.5% Triton X-100, and viable bacteria were enumerated by plating onto sheep blood agar. Bacterial viability was not affected by 0.5% Triton X-100 treatment for all strains tested. Each assay was run in triplicate and also repeated at least three times.

Southern blot analysis of noninvasive mutants. Total chromosomal DNAs were prepared by sodium dodecyl sulfate (SDS) lysis, lysozyme and protease K digestion, phenol-chloroform extraction, and ethanol precipitation. Strain RS218 and the noninvasive mutants were found to possess plasmids (data not shown), and plasmid DNAs were also isolated by the method of alkali lysis (30). About 4 μg of chromosomal and plasmid DNAs was digested with the restriction endonucleases *Eco*RV and *Mlu*I, which do not cut Tn*phoA*, was transferred to nylon membranes, and was probed with a ^{32}P-labeled 0.6-kb Kan^r gene fragment derived from Tn*5* (26, 35). Probes were labeled by the random oligonucleotide-labeling kit from Pharmacia with [α-^{32}P]dATP. Hybridization was performed under stringent conditions as described previously (16).

Characterization of noninvasive mutants with single Tn*phoA* insertion. Because a possible polar effect resulted from the transposon mutagenesis, noninvasive mutants with single insertions were first examined for their phenotypic characteristics in comparison with those of their parent strain RS218. The presence of the K1 capsule was verified by the antiserum agar technique with antiserum to group B meningococci as described previously (21). O18 lipopolysaccharide (LPS) was examined by colony blot analysis with anti-O18 monoclonal antibodies (22). LPS was also extracted by the hot-phenol water method (38), and the size distribution of LPS was assayed by silver staining of SDS-polyacrylamide gel electrophoresis (PAGE) gels as described previously (4, 5). Strain RS218 was found to possess type 1 and S fimbriae, and the presence of type 1 and S fimbriae was examined by mannose-sensitive and mannose-resistant hemagglutination tests as well as by colony blot assays with anti-type 1 fimbria antibody and anti-S fimbria monoclonal antibodies (28). Members of the outer membrane protein (Omp) complex were isolated by sonication and selective detergent solubilization, and their patterns were examined by SDS-PAGE as described previously (4, 5). OmpA was examined by colony blot assay as well as by Western blotting (immunoblotting) of Omp complex members with anti-OmpA antibody (27). We also examined whether the noninvasive mutants have an insertion in the open reading frame of the known virulent factors (K1 capsule and OmpA) by Southern hybridization. Probes for K1 (1.3 kb) and OmpA (1.2 kb) were obtained from pWG123 and pRD87, respectively (25, 37) and used for Southern blot analysis as described above.

Cloning of Tn*phoA* fragments. *Mlu*I-digested genomic DNAs from noninvasive mutants were ligated into the *Mlu*I site of pCVD433, which was derived from pACYC184 by insertion of *Mlu*I linkers into the *Eco*RV site (8). Transformation was performed by electroporation of *E. coli* DH5αMCR in 10% glycerol with 0.1-cm cuvettes and an *E. coli* gene pulser (Bio-Rad Laboratories, Richmond, Calif.) set at 1.8 kV, 200 Ω, and 25 μF. The kanamycin-resistant transformants were identified as *Mlu*I-fragments containing Tn*phoA*. The construct carrying a 13-kb *Mlu*I fragment of a noninvasive mutant 10A-23 in pCVD433 was designated pCD10A.

PCR cloning and analysis of *ibe10*. PCR was performed as described previously (6, 17). Briefly, 0.5 μg of genomic DNA was added to a mixture of 1× *Taq* polymerase buffer (Cetus), 1.5 mM $MgCl_2$, 0.5 mM deoxynucleoside triphosphate (Cetus), 50 pmol of each primer, and *Taq* DNA polymerase in a final volume of 50 μl. Amplification was carried out in a thermocycler (M.J. Research) for 40 cycles consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and primer extensions for 3 min at 70°C. The three primers (IB10-5a, IB10-5b, and IB10-3a) (Table 2) used for PCR were synthesized on an Applied Biosystems (Foster City, Calif.) 380B DNA synthesizer. Two fragments containing *ibe10*-coding sequences (250 and 580 bp) flanking the two ends of Tn*phoA* were amplified from genomic DNA of wild-type strain RS218 and subcloned into TA cloning vector pCRII (Invitrogen). The two constructs were designated pCIB10A (0.25-kb insert) and pCIB10B (0.58-kb insert). For examining whether *ibe10* is unique to CSF isolates of *E. coli*, PCR amplification was performed with genomic DNA samples from CSF isolates and laboratory strains of *E. coli*. The conditions and profiles described above were used for the amplification of a 0.58-kb DNA fragment with the primers IB10-5b and IB10-3a (Table 2).

DNA sequencing and analysis. The DNA sequence of *ibe10* was determined by the dideoxy chain termination method described by Sanger et al. (31) with the Sequenase kit (version 2.0) from U.S. Biochemicals Corp. DNA sequence analysis was performed with ^{35}S-dATP (1,000 to 1,500 Ci/mmol) obtained from Du Pont NEN Research Products (Boston, Mass.). To sequence the portion of *ibe10* flanking the Tn*phoA* insertion site, the initial DNA sequence was obtained from plasmid pCD10A with the 5′ primer Tnp5 and the 3′ primer Tnp3 (Table 2). The two primers are complementary to the two ends of Tn*phoA*. The remaining DNA sequence of *ibe10* was determined with primers complementary to the *ibe10* DNA sequence in pCD10A. Both strands of the DNA were sequenced to ensure accuracy, and the sequence data were analyzed with the DNA analysis program

TABLE 2. Oligonucleotides used for cloning and sequencing

Gene	Primer	Strand	Sequence	Use
PhoA	Tnp*5*	−	5′-TCGCTAAGAGAATCACGCAGAG-3′	Sequencing
Tn*5*	Tnp*3*	−	5′-GCACGATGAAGAGCAGAAG-3′	Sequencing
Ibe10	IB10-5a	+	5′-TGGCGGAATGATGACAAC-3′	Cloning and sequencing
Ibe10	IB10-5b	+	5′-CCCGCTGTAATATACCTG-3′	Cloning and sequencing
Ibe10	IB10-3a	−	5′-AAAGCTTGTCATACACATTAGCTC-3′	Cloning and sequencing
Ibe10	IB10-3b	−	5′-AGTCCACCTGACTCTACCG-3′	Sequencing

developed by the Genetics Computer Group of the University of Wisconsin. DNA and deduced protein sequences were used to search the DNA and protein databases at the National Center for Biotechnology Information (National Library of Medicine, Washington, D.C.) by using the BLAST algorithm. Hydrophobicity analysis of the deduced amino acid sequence of Ibe10 was performed by the method described by Kyte and Doolittle with a window of 7 (23). Positive values indicate hydrophobic sequences, and negative numbers indicate hydrophilic regions.

Expression constructs. The following constructs were made by standard procedures (30). The *Bam*HI-*Hin*dIII fragment (250 bp) from pCIB10A, which encodes Ibe10, was eluted from 2% agarose gel after digestion and then ligated to the same restriction sites of pQE30 (Qiagen). This construct (pQIB10) encodes a 9-kDa protein with a histidine tag at the N terminus. M15 was the host strain for pQIB10. Electrocompetent *E. coli* strains were made in 10% glycerol and then electrotransformed as described above. Transformants were identified by the predicted phenotypes (ampicillin and kanamycin resistance). Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C.

Purification of Ibe10 protein. Small-scale expression and purification of the recombinant Ibe10 protein was carried out according to the instructions of the manufacturer (Qiagen). The protein preparations isolated from the supernatants and pellets were resolved by SDS–16% PAGE (24). The results indicated that Ibe10 predominately resided in the insoluble fraction. Insoluble Ibe10 protein with a histidine tag was purified by binding to nickel-nitrilotriacetic acid (Ni-NTA) resin in 6 M guanidine-HCl (Qiagen). The eluted proteins containing 8 M urea were refolded by sequential dialysis against decreasing concentrations of urea in 25 mM Tris-HCl (pH 8.0)–0.2 M NaCl–1 mM EDTA–10 mM β-mercaptoethanol. The purity of the final product was assessed by subjecting the indicated amounts of protein to SDS-PAGE (16%). The protein was assayed by using the Bio-Rad protein assay reagents according to the manufacturer's instructions. The protein was examined for the effect on invasion of BMEC by strain RS218. Briefly, BMEC were incubated with the protein (10 to 50 μg/well) for 1 h at room temperature before addition of bacteria, and invasion assays were carried out as described above.

Neonatal rat model of hematogenous *E. coli* K1 meningitis. One of the noninvasive mutants, 10A-23, was examined for its ability to enter the CNS by using our neonatal rat model of hematogenous *E. coli* meningitis as described previously (21). Briefly, at 5 days of age, all members of each litter were randomly divided into two groups to receive subcutaneously 1.4×10^4 CFU of the parent strain RS218 or 6.0×10^4 CFU of the mutant 10A-23. Our pilot experiments revealed that these bacterial inocula for strains RS218 and 10A-23 produced nonlethal bacteremia of 10^5 to 10^8 CFU/ml of blood in approximately 90% of the animals within 18 h of inoculation. At 18 h after bacterial inoculation, blood and CSF specimens were obtained for quantitative cultures as described previously (21). Blood and CSF specimens obtained from animals infected with the mutant 10A-23 were cultured in brain heart infusion broth and agar containing kanamycin (40 μg/ml).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper were submitted to GenBank, and the sequence was assigned accession number L42624.

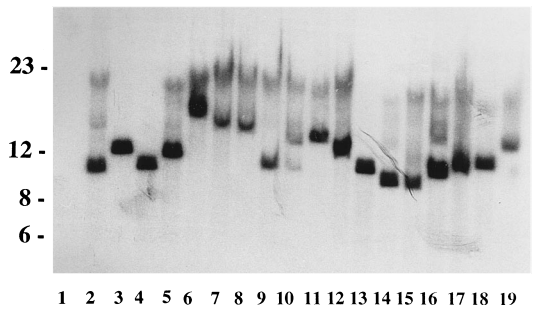

FIG. 1. Southern hybridization of noninvasive Tn*phoA* mutants. Genomic DNAs (10 μg) from RS218 (lane 1) and its Tn*phoA* mutants (lanes 2 to 19) were digested with *Eco*RV and *Mlu*I (which do not cut Tn*phoA*), separated on 0.5% agarose gels at 25 V overnight, transferred to a Hybond-N membrane, and probed with a ^{32}P-labeled Kan^r gene. Because Tn*phoA* is 7.7 kb long, fragments smaller than this would not be expected to be seen. The following three mutants were found to have single Tn*phoA* insertions: 7A-33 (lane 3), 8A-4 (lane 4), and 10A-23 (lane 13). Molecular sizes are given in kilobases.

RESULTS

Construction of noninvasive mutants of *E. coli* K1. A total of 938 PhoA(+) and PhoA(−) transconjugants were screened for their ability to invade BMEC. The screening assay identified 186 mutants which exhibited qualitatively decreased invasion abilities similar to those of negative control strains. These noninvasive mutants were reexamined by the standard invasion assay. A total of 169 mutants were identified, which demonstrated an invasion capacity of <0.0001%, compared with approximately 0.1% for the parent strain RS218 and invasive mutants.

Southern analysis. To determine the number of Tn*phoA* insertions in each of the noninvasive mutants and to get an estimate of the sizes of chromosomal loci contributing to the invasion phenotype, Southern hybridization analysis of the noninvasive mutants was carried out by using a probe containing the kanamycin resistance gene internal to Tn*phoA* (Fig. 1). The parent strain RS218 (lane 1) had no sequences recognized by this probe, indicating that the hybridization observed in the mutants was specific to Tn*phoA* sequences. Hybridization of *Mlu*I- and *Eco*RV-digested chromosomal DNA from each of the 169 noninvasive mutants with this probe identified 52 mutants with single Tn*phoA* insertions. The 52 mutants were found to have single *Eco*RV-*Mlu*I fragments of 0.6 to 11.3 kb into which the 7.7-kb Tn*phoA* DNA was inserted (Fig. 1). The kanamycin resistance gene probe was also used to probe the plasmid DNAs from the noninvasive mutants. The probe did not hybridize to the plasmid DNAs, indicating that there were no Tn*phoA* insertions.

Characteristics of noninvasive mutants with single Tn*phoA* insertions. The phenotypic characteristics of the noninvasive mutants for the K1 capsule, O18-LPS, LPS and Omp profiles, OmpA, and type 1 and S fimbriae were identical to those of the parent strain RS218. In addition, none of the *Eco*RV-*Mlu*I

TABLE 3. Development of meningitis (defined as positive CSF culture) in newborn rats with various degrees of bacteremia

Bacteremia (CFU/ml of blood)	No. of animals with positive CSF culture/no. of animals with positive blood culture (%)[a]	
	Strain RS218	Mutant 10A-23
$<10^5$	0/3	0/3
10^5 to $<10^6$	1/8 (13)	0/5
10^6 to $<10^7$	0/1	0/5
$>10^7$	11/13 (85)	3/11 (27)

[a] See Materials and Methods for description of strains.

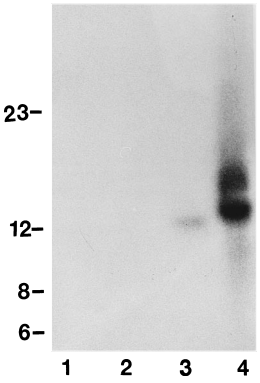

FIG. 2. Southern blot analysis of *E. coli* chromosomal DNA. DNA samples (5 μg) from various *E. coli* strains were digested with *Mlu*I and hybridized with a 0.58-kb *Eco*RI fragment (containing *ibe10*) derived from pCIB10B. The *ibe10* probe did not hybridize to the K-12 strains DH5α (lane 1) and SM10 (lane 2), while the *ibe10* gene was detected in CSF isolates of *E. coli* K1, strains C5 (lane 3) and RS218 (lane 4). Molecular sizes are given in kilobases.

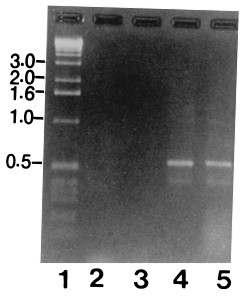

FIG. 3. PCR analysis of *ibe10*. The DNA samples that were used for Southern hybridization (Fig. 2) were used as templates for PCR. PCR was performed as described in Materials and Methods. Lanes: 1, DNA marker (1-kb ladder); 2, DH5α (K-12 strain); 3, SM10 (K-12 strain); 4, RS218 (K1 strain); and 5, C5 (K1 strain).

Tn*phoA* fragments hybridized to the probes for the K1 capsule and OmpA.

Prevalence of meningitis in infant rats. Table 3 summarizes the prevalence of meningitis (defined as positive CSF cultures) in 5-day-old rats with various degrees of bacteremia caused by the parental strain RS218 or its noninvasive mutant with a single Tn*phoA* insertion, 10A-23. As expected, subcutaneous injections of 1×10^4 CFU of strain RS218 or 6×10^4 CFU of mutant 10A-23 resulted in bacteremia of 10^5 to 10^8 CFU/ml of blood in approximately 90% of the animals. This level of bacteremia has been shown to be sufficient for allowing circulating *E. coli* to enter the CNS (21). A total of 12 of 22 animals (55%) infected with RS218 and developing bacteremia of 10^5 to 10^8 CFU/ml of blood were found to have meningitis. In contrast, only 3 of the 21 animals (14%) infected with the mutant 10A-23 developed meningitis despite the induction of a similar level of bacteremia, and this rate of meningitis was significantly ($P < 0.02$) less than the rate observed with RS218. These findings support the concept that the single insertion mutant 10A-23 is truly less invasive both in vitro and in vivo, suggesting that the DNA flanking the transposon insertion in the mutant 10A-23 encodes a gene(s) that is necessary if not sufficient for invasion into BMEC.

Southern and PCR analysis of *ibe10*. To examine whether *ibe10* is unique to *E. coli* derived from CSF, genomic DNA samples from CSF isolates (strains C5 and RS218) and laboratory strains (DH5α and SM10) were digested with *Mlu*I and hybridized a 0.58-kb *Eco*RI fragment which is bigger than and contains the entire *ibe10* open reading frame. The *ibe10* probe did not hybridize to laboratory strains or noninvasive *E. coli* K1 (e.g., E412 [data not shown]), whereas it was detected in CSF isolates of *E. coli* K1 (Fig. 2). We also performed PCR amplification of genomic DNA samples from the same *E. coli* strains. The results indicate that *ibe10* is present in CSF isolates of *E. coli* K1 (e.g., RS218 and C5) (Fig. 3). This finding is consistent with that of Southern blotting analysis (Fig. 2). We also used a 0.58-kb *ibe10* probe containing the open reading frame for screening of 52 noninvasive mutants by Southern hybridization. The *Eco*RV-*Mlu*I Tn*phoA* fragments from three noninvasive mutants hybridized with the *ibe10* probe.

Sequence analysis of *ibe10*. A potential TATA box (TAT TAA) and ribosome-binding site (CGGA) were found at 30 and 3 bp upstream from the putative ATG start site of the open reading frame, respectively. The 225-nucleotide open reading frame assigned to the *ibe10* gene coded for a protein with 75 amino acids and a calculated molecular weight of 8,229 (Fig. 4). The insertion site was identified by sequencing the fusion joint with the 5′ primer Tnp5 and the 3′ primer Tnp3, which are complementary to the two ends of Tn*phoA*, respectively. The insertion occurred in the codon corresponding to residue 15. The size of Ibe10 is similar to that of InvX (6.5 kDa), a protein which is required for entry of enteroinvasive *E. coli* into Hep-2 cells (15). However, no homology was observed between *ibe10* and other invasion genes, including *invX*, by searching DNA and protein databases in GenBank. The deduced protein is hydrophobic overall but contains several small hydrophilic domains.

Predicted structure of the Ibe10 protein. The sequence of *ibe10* reveals that this gene encodes a very hydrophobic protein containing 38 hydrophobic residues (51% of the total) and 4 potential transmembrane domains separated by less hydrophobic stretches. The proposed transmembrane regions start at the N terminus and extend to residue 61. A small stretch of the C terminus of Ibe10 (residues 62 to 75) shows high hydrophilicity. Ibe10 showed partial sequence homology and similarity with several membrane-associated proteins (Table 4). The N terminus of Ibe10 (residues 9 to 48) shows 42% identity with and 50% similarity to a transmembrane protein, the *cmcT* gene product-cephamycin export protein in *Nocardia lactamdurans* (2). A region near the C terminus of Ibe10 (residues 28 to 61) exhibits 29% identity with and 50% similarity to the multidrug resistance (MDR) protein homolog and antigen peptide transporter 1, which are known to be capable of transporting proteins (7). The functional significance of these alignments is not clear. Expression of *ibe10* in the pQE30 system showed that almost 100% of the recombinant protein (9 kDa) resides in insoluble inclusion bodies by checking the histidine *tag*-containing protein, suggesting that this protein is hydrophobic.

Purification of recombinant Ibe10. Ibe10 was affinity purified from the pQE30 construct. More than 30 mg of pure protein was obtained from 600 ml of cell cultures. Figure 5 shows that the purified insoluble Ibe10 migrates as one major band in SDS-PAGE with an apparent molecular mass of 9 kDa (lanes 2 and 3). Refolding resulted in a soluble Ibe10 migrating with a single band of the same size (lanes 4 and 5).

Inhibition of RS218 invasion of BMEC by purified Ibe10 gene products. A purified recombinant Ibe10 protein was examined for its ability to inhibit invasion of BMEC by strain RS218. The purified Ibe10 at 10 μg was ineffective in blocking BMEC invasion by RS218, but 25 and 50 μg exhibited approximately 30 and 80% inhibition, respectively.

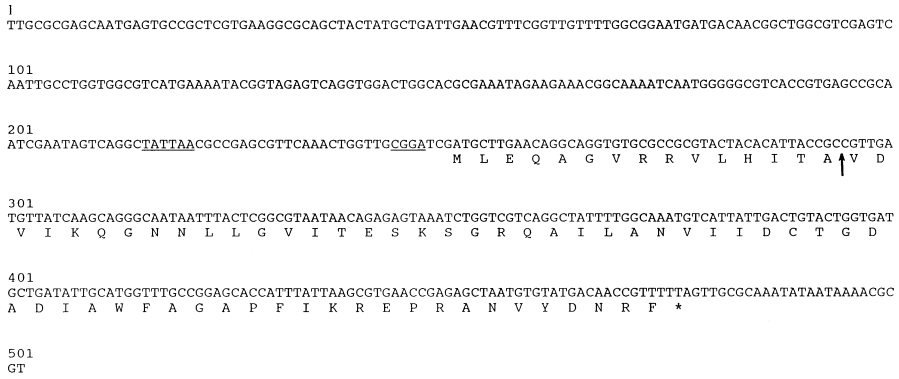

FIG. 4. Complete nucleotide sequence and deduced amino acid sequence of the gene *ibe10*. The potential TATA box and the putative ribosome-binding site are underlined. The calculated molecular mass of the full-length protein is 8.2 kDa. The arrow indicates a Tn*phoA* insertion site in *ibe10.*

DISCUSSION

Inadequate knowledge of its pathogenesis and pathophysiology has contributed to the significant mortality and morbidity associated with neonatal bacterial meningitis. *E. coli* is the most common gram-negative organism that causes meningitis during the neonatal period, but no information exists as to what traits of *E. coli* contribute to invasion of BMEC.

In the present study, we identified a Tn*phoA* mutant (10A-23) which lost its ability to invade BMEC in vitro. More importantly, this mutant was significantly less invasive into the CNS in vivo than the parent strain in the newborn rat model of hematogenous meningitis. In our experimental hematogenous meningitis model, bacteria were injected subcutaneously, resulting in bacteremia and subsequent meningitis. Because the blood-brain barrier separates the brain and CSF from the intravascular compartment, a genetic locus in the mutant 10A-23 which was affected by Tn*phoA* mutagenesis is most likely to confer the ability to invade BMEC in vitro and in vivo. This gene was termed *ibe10*. We have previously shown in the hematogenous *E. coli* meningitis model that the K1 capsule is a critical determinant for *E. coli* to cross the blood-brain barrier as live bacteria (21). We have also shown that OmpA contributes to invasion of BMEC by *E. coli* K1 (29). Both the parent strain RS218 and its mutant 10A-23 were found to possess the K1 capsule and OmpA. In addition, the *Eco*RV-*Mlu*I Tn*phoA* fragment of the mutant 10A-23 did not hybridize to the DNA probes for the K1 capsule and OmpA. These findings suggest that a noninvasive property of 10A-23 is less likely a result of a polar effect of Tn*phoA* on the other genes known to be involved in invasion (e.g., those for the K1 capsule and OmpA). This concept was also supported by our demonstration that a recombinant Ibe10 protein was able to inhibit invasion of BMEC by strain RS218.

TABLE 4. Comparison of protein sequence homologies to Ibe10

Protein	Region	Identity (%)	Similarity (%)	Reference
MDR protein homolog	55–64	70	80	7
	153–186	29	50	
Antigen peptide transporter 1	70–79	70	80	7
	168–201	29	50	
Outer membrane porin F precursor (*Pseudomonas* aeruginosa)	14–28	33	60	10
	120–139	25	65	
	220–233	42	57	
Toxin Anthopleura Fuscoviridis (AF) II	14–22	55	77	33
	21–28	62	62	
Cephamycin export protein (CMCT)	146–185	42	50	2
Sialoadhesin	425–438	35	71	3
	1028–1041	42	57	
	1154–1198	24	37	
	1288–1302	33	53	
	1471–1479	44	69	

The predicted protein displayed the characteristics of an integral membrane protein and was hydrophobic overall, with four transmembrane domains. This topology is supported by the location of the Ibe10 protein in *E. coli* M15 with pQIB10. Ibe10 showed partial sequence homology and similarity with several membrane-associated proteins; however, no homology was found with any other recognized invasion proteins, suggesting that Ibe10 is a novel protein contributing to invasion of *E. coli* into BMEC. In addition, *ibe10* did not hybridize to the *eaeA* gene (data not shown). Its hydrophobic nature suggests that Ibe10 plays a role in mediating interactions between invasive bacteria and cerebral endothelial cells.

The invasive determinant encoded by *ibe10* appeared to be unique to CSF isolates of *E. coli* K1. Southern blotting analysis revealed that *ibe10* was detected in CSF isolates of *E. coli* K1 (e.g., C5 and RS218), while laboratory strains of *E. coli* K-12 (e.g., DH5α and M294) as well as noninvasive *E. coli* K1 (e.g., E412) had no sequences recognized by the *ibe10* probe. PCR amplification of genomic DNA samples also indicated that *ibe10* is present in CSF isolates of *E. coli* K1. Additional studies are needed to determine the prevalence of *ibe10* in clinical isolates of *E. coli* from CSF and also to verify that *ibe10* is unique to CSF isolates of *E. coli* K1. Of interest is the fact that a probe containing *ibe10* hybridized to only 3 of 52 noninvasive mutants, suggesting that other potentially different genes also contribute to invasion of BMEC. Studies are in progress to identify and characterize other genes involved in entry of circulating *E. coli* into the CNS.

In conclusion, we cloned and characterized a genetic locus which allows *E. coli* K1 strain RS218 to invade BMEC both in vitro and in vivo. This gene, termed *ibe10*, was novel and encoded an 8.2-kDa protein. A recombinant Ibe10 protein was able to block the invasion of BMEC by RS218.

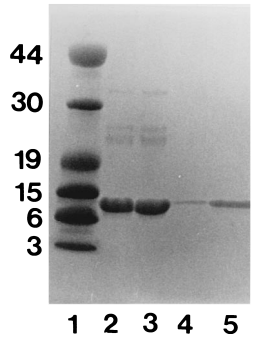

FIG. 5. Expression of a recombinant Ibe10 protein. The *ibe10* gene was subcloned into pQE30. After induction with IPTG, *ibe10* gene products were purified with affinity columns and then refolded by sequential dialysis as described in Materials and Methods. The proteins were resolved on an SDS-polyacrylamide gel and then stained with Coomassie brilliant blue. Lanes: 1, positions of molecular mass markers (in kilodaltons); 2 and 3, proteins expressed in pQE30 and purified by an Ni-NTA column under denaturing conditions and eluted with 6 M GuHCl–0.2 M acetic acid (lane 2) or eluted with 8 M urea–0.1 Na-phosphate–0.01 M Tris-HCl (pH 6.3)–250 mM imidazole (lane 3); 4 and 5, 4 and 9.5 μg of Ibe10 protein, respectively, after refolding.

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REFERENCES

1. **Bliska, J. B., J. E. Galan, and S. Falkow.** 1993. Signal transduction in the mammalian cell during bacterial attachment and entry. Cell **73:**903–920.
2. **Coque, J. J. R., P. Liras, and J. F. Martin.** 1993. Genes for a β-lactamase, a penicillin-binding protein and a transmembrane protein are clustered with the cephamycin biosynthetic genes in *Nocardia lactamdurans*. EMBO J. **12:**631–639.
3. **Crocker, P. R., S. Mucklow, V. Bouckson, A. M. William, A. C. Willis, S. Gordon, G. Milon, S. Kelm, and P. Bradfield.** 1994. Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. EMBO J. **13:**4490–4503.
4. **Cross, A. S., K. S. Kim, and D. C. Wright.** 1986. Role of LPS and capsule in serum resistance of bacteremic strains of *E. coli*. J. Infect. Dis. **154:**497–503.
5. **Cross, A., J. Sadof, P. Gemski, L. Asher, and K. S. Kim.** 1988. The relative role of lipopolysaccharide and capsule in the virulence of *E. coli*, vol. H24, p. 319–333. *In* F. Cabello and C. Pruzzo (ed.), Bacteria complement and phagocytic cell. Proceedings of NATO Workshop. NATO ASI Series, Springer-Verlag, Berlin.
6. **Delidow, B. C., J. P. Lynch, and A. White.** 1993. Polymerase chain reaction: basic protocols. Methods Mol. Biol. **15:**1–29.
7. **Deverson, E. V., I. R. Gow, W. J. Coadwell, J. J. Monaco, G. R. Butcher, and J. C. Howard.** 1990. MHC class II region encoding proteins related to the multidrug resistance family of transmembrane transporters. Nature (London) **348:**738–741.
8. **Donnenberg, M. S., S. B. Calderwood, A. Donohue-Rolfe, G. T. Keusch, and J. B. Kaper.** Construction and analysis of Tn*phoA* mutants of enteropathogenic *Escherichia coli* unable to invade Hep-2 cells. Infect. Immun. **58:**1565–1571.
9. **Donnenberg, M. S., J. Yu, and J. B. Kaper.** 1993. A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. J. Bacteriol. **175:**4670–4680.
10. **Duchene, M., A. Schweizer, F. Lottspeich, G. Krauss, M. Marget, K. Vogel, B. U. von Specht, and H. Domdey.** 1988. Sequence and transcriptional start site of the *Pseudomonas aeruginosa* outer membrane porin protein F gene. J. Bacteriol. **170:**155–162.
11. **Falkow, S., R. R. Isberg, and D. A. Portnoy.** 1991. The interaction of bacteria with mammalian cells. Annu. Rev. Cell Biol. **8:**333–363.
12. **Gladstone, I. M., R. A. Ehrenkranz, S. C. Edberg, and R. S. Baltimore.** 1990. A ten-year review of neonatal sepsis and comparison with the previous fifty-year experience. Pediatr. Infect. Dis. J. **9:**819–825.
13. **Grant, S. G., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
14. **Gutierrez, C., J. Barondess, C. Manoil, and J. Beckwith.** 1987. The use of transposon TnphoA to detect genes for cell envelope proteins subject to a common regulatory stimulus. J. Mol. Biol. **195:**289–297.
15. **Hsia, R.-C., P. L. C. Small, and P. M. Bavoil.** 1993. Characterization of virulence genes of enteroinvasive *Escherichia coli* by Tn*phoA* mutagenesis: identification of *invX*, a gene required for entry into HEp-2 cells. J. Bacteriol. **175:**4817–4823.
16. **Huang, S. H., A. Tang, B. Drisco, S.-Q. Zhang, R. Seeger, C. Ching Li, and A. Y. Jong.** 1994. Human dTMP kinase: gene expression and enzyme activity coinciding with cell cycle progression and cell growth. DNA Cell Biol. **13:** 461–471.
17. **Huang, S. H., C. H. Wu, B. Cai, and J. Holcenberg.** 1993. cDNA cloning by inverse polymerase chain reaction. Methods Mol. Biol. **15:**349–356.
18. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature (London) **317:**262–264.
19. **Isberg, R. R., and J. M. Leong.** 1990. Multiple β_1 chain integrins are receptors for invasion, a protein that promotes bacterial penetration into mammalian cells. Cell **60:**861–871.
20. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasion: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
21. **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 *E. coli* meningitis in the rat. J. Clin. Invest. **90:**897–905.
22. **Kim, K. S., J. H. Kang, A. S. Cross, B. Kaufman, W. Zollinger, and J. Sadoff.** 1988. Functional activities of monoclonal antibodies to the O side chain of *Escherichia coli* lipopolysaccharides in vitro and in vivo. J. Infect. Dis. **157:** 47–53.
23. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of protein. J. Mol. Biol. **157:**105–132.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
25. **Liu, J. L.-C., G.-J. Shen, Y. Ichikawa, J. F. Rutan, G. Zapata, W. F. Vann, and C. H. Wong.** 1992. Overproduction of CMP-sialic acid synthetase for organic synthesis. J. Am. Chem. Soc. **114:**3901–3910.
26. **Manoil, C., and J. Beckwith.** 1985. TnphoA: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
27. **Prasadarao, N. V., M. F. Stins, C. A. Wass, and K. S. Kim.** 1994. Role of S fimbriae and outer membrane protein A in *E. coli* binding to and invasion of endothelial cells, abstr. 406, p. A70. Abstr. Annu. Meet. Exp. Biol. Anaheim, Calif.
28. **Prasadarao, N. V., C. A. Wass, J. Hacker, K. Jann, and K. S. Kim.** 1993. Adhesion of S-fimbriated *E. coli* to brain glycolipids mediated by *sfaA* gene-encoded protein of S-fimbriae. J. Biol. Chem. **268:**10356–10363.
29. **Prasadarao, N. V., C. A. Wass, J. N. Weiser, M. F. Stins, S. H. Huang, and K. S. Kim.** Outer membrane protein A (OmpA) of *E. coli* contributed to invasion of brain microvascular endothelial cells. Submitted for publication.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
32. **Stins, M. F., N. V. Prasadarao, L. Ibric, C. A. Wass, P. Luckett, and K. S. Kim.** 1994. Binding characteristics of S fimbriated *E. coli* to isolated brain microvascular endothelial cells. Am. J. Pathol. **145:**1228–1236.
33. **Sunahara, S., K. Muramoto, and H. Kamiya.** 1987. Amino acid sequence of two sea anemone toxins from anthopleura fuscoviridis. Toxicon **25:**211–219.
34. **Tang, P., V. Foubister, M. Garciela-Pucciarelli, and B. B. Finaly.** 1993. Methods of bacterial invasion. J. Microbiol. Methods **18:**227–240.
35. **Taylor, R. K., C. Manoil, and J. J. Mekalanos.** 1989. Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. **171:**1870–1878.
36. **Unhanan, M., M. M. Mustafa, G. H. McCraken, Jr., and J. D. Nelson.** 1993. Gram-negative enteric bacillary meningitis: a twenty-one-year experience. J. Pediatr. **122:**15–22.
37. **Weiser, J. N., and E. C. Gotschlich.** 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect. Immun. **59:**2252–2258.
38. **Westphal, O., O. Luderitz, and F. Bister.** 1952. Uber die Extraction von Bakterien mit Phenol-Wasser. Z. Naturforsch. Teil B **7:**148–155.
39. **Wilmes-Riesenberg, M. R., and B. L. Wanner.** 1992. TnphoA and TnphoA′ elements for making and switching fusions for study of transcription, translation, and cell surface localization. J. Bacteriol. **174:**4558–4575.

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