INFECTION AND IMMUNITY, May 1999, p. 2103–2109 Vol. 67, No. 5
0019-9567/99/$04.00+0

Identification and Characterization of an *Escherichia coli* Invasion Gene Locus, *ibeB*, Required for Penetration of Brain Microvascular Endothelial Cells

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Received 30 September 1998/Returned for modification 30 December 1998/Accepted 27 January 1999

***Escherichia coli* K1 is the most common gram-negative organism causing neonatal meningitis, but the mechanism by which *E. coli* K1 crosses the blood-brain barrier is incompletely understood. We have previously described the cloning and molecular characterization of a determinant, *ibeA* (also called *ibe10*), from the chromosome of an invasive cerebrospinal fluid isolate of *E. coli* K1 strain RS218 (O18:K1:H7). Here we report the identification of another chromosomal locus, *ibeB*, which allows RS218 to invade brain microvascular endothelial cells (BMEC). The noninvasive Tn*phoA* mutant 7A-33 exhibited <1% the invasive ability of the parent strain in vitro in BMEC and was significantly less invasive in the central nervous system in the newborn rat model of hematogenous *E. coli* meningitis than the parent strain. The Tn*phoA* insert with flanking sequences was cloned and sequenced. A 1,383-nucleotide open reading frame (ORF) encoding a 50-kDa protein was identified and termed *ibeB*. This ORF was found to be 97% identical to a gene encoding a 50-kDa hypothetical protein (p77211) and located in the 13-min region of the *E. coli* K-12 genome. However, no homology was observed between *ibeB* and other known invasion genes when DNA and protein databases in GenBank were searched. Like the Tn*phoA* insertion mutant 7A-33, an isogenic *ibeB* deletion mutant (IB7D5) was unable to invade BMEC. A 7.0-kb locus containing *ibeB* was isolated from a LambdaGEM-12 genomic library of *E. coli* RS218 and subcloned into a pBluescript KS vector (pKS7-7B). pKS7-7B was capable of completely restoring the BMEC invasion of the noninvasive Tn*phoA* mutant 7A-33 and the *ibeB* deletion mutant IB7D5 to the level of the parent strain. More importantly, the *ibeB* deletion mutant IB7D5 was fully complemented by pFN476 carrying the *ibeB* ORF (pFN7C), indicating that *ibeB* is required for *E. coli* K1 invasion of BMEC. Taken together, these findings indicate that several *E. coli* determinants, including *ibeA* and *ibeB*, contribute to crossing of the blood-brain barrier.**

Bacterial meningitis has remained associated with high mortality and morbidity despite advances in antimicrobial chemotherapy and supportive care (16, 24). A major contributing factor is the incomplete understanding of the pathogenesis and pathophysiology of this disease. For example, most cases of bacterial meningitis develop as a result of hematogenous spread, but it is not completely understood how circulating bacteria cross the blood-brain barrier.

Escherichia coli is the most common gram-negative organism that causes meningitis during the neonatal period. Using *E. coli* as a paradigm, we have examined how circulating bacteria cross the blood-brain barrier. These studies have become feasible because of the availability of both in vitro and in vivo models of the blood-brain barrier (7, 22). For example, we have successfully isolated and cultivated brain microvascular endothelial cells (BMEC), which constitute the blood-brain barrier (22, 23). The in vivo model of the blood-brain barrier has been established by induction of hematogenous meningitis in infant rats (7, 10). In this experimental meningitis model, bacteria are injected via the subcutaneous or intracardiac route, resulting in bacteremia and subsequent entry of bacteria into the central nervous system (CNS). Since the blood-brain barrier separates the brain and cerebrospinal fluid (CSF) from the intravascular compartment, the entry of bacteria should occur at sites of the blood-brain barrier. The development of techniques for atraumatic collection of blood and CSF specimens has enabled us to use this in vivo model to examine the pathogenic mechanisms involved in crossing of the blood-brain barrier by circulating *E. coli* (10).

To facilitate the identification of the genes contributing to *E. coli* invasion of BMEC, we have used transposon Tn*phoA* and generated a collection of noninvasive *E. coli* mutants (7). Tn*phoA* is a modified transposon engineered by insertion of the *phoA* gene into one end of Tn*5* (12). The gene fusion 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 critical *E. coli* determinants involved in the invasion of BMEC in vitro and in vivo. For example, we have previously identified the *ibeA* (*ibe10*) locus via Tn*phoA* mutagenesis and screening for loss of invasiveness by use of the in vitro and in vivo models of the blood-brain barrier (7). In addition, we have shown that *E. coli* OmpA contributes to the invasion of BMEC (15). In the present study, we characterized the noninvasive mutant 7A-33, which was derived from a CSF isolate of *E. coli* K1 strain RS218 by Tn*phoA* mutagenesis. This mutant was significantly less able than the parent strain to invade BMEC in vitro and to enter the CNS in the newborn rat model of hematogenous meningitis in vivo. Similar findings were obtained with an *ibeB* deletion mutant. The DNA fragments containing *ibeB* (invasion of brain endothelial cells) were shown to restore the ability of the noninvasive mutant 7A-33 and the *ibeB* deletion mutant to invade BMEC.

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TABLE 1. *E. coli* strains and plasmids used in this study

E. coli strain or plasmid	Genotype or characteristic(s)	Reference or source
Strains		
RS218	O18:K1:H7	21
E44	Rif^r derivative of RS218	
DH5α	F$^-$ *recA1 hsdR17 thi-1 gyrA96 supE44 endA1 relA1 recA1 deoR* Δ(*lacZYA-argF*)U169 (ϕ80 *lacZ*M15)	Gibco/BRL
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (λpir) *pro endA hsdA hsdR supF*	3
7A-33	E44 *ibeB*::Tn*phoA*	This study
IB7D5	Δ*ibeB* derivative of E44 via allelic exchange	This study
HB101	K-12 *supE44 ara-14 galK12 rpsL20* (Str^r)	18
Plasmids		
pRT733	oriR6K mobRP4 Tn*phoA*	7
pCVD433	pACYC184 modified by insertion of an *Mlu*I linker	3
pCVD442	Amp^r SacB oriR6K mobRP4	4
pCD7D	pCVD442 carrying a 2.3-kb DNA fragment with an in-frame *ibeB* deletion	This study
pCRII	Amp^r Kan^r oriCol E1 oriF1	Invitrogen
pCD7A	pCVD433 carrying the *ibeB* locus with a Tn*phoA* insertion	This study
pCIB7B	*ibeB* (0.67 kb) pCRII	This study
pCR7C	pCRII carrying the *ibeB* gene (1.6 kb)	This study
pKS7-13K	pBluescript KS carrying a 13.0-kb *ibeB* locus (13K)	This study
pKS7-7B	pBluescript KS carrying a 7.0-kb *ibeB* locus (7B)	This study
pKS7-6N	pBluescript KS carrying a 6.1-kb DNA fragment with an *ibeB* deletion	This study
pKS7-5H	pBluescript KS carrying a 5.7-kb DNA fragment with *ibeB* deleted (5H)	This study
pFN476	Amp^r low copy, T7 promoter	20
pFN7C	pFN476 carrying the *ibeB* gene (1.6 kb)	This study
pGP1-2	Kan^r, low copy, T7 RNA polymerase gene	20

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* RS218 (O18:K1:H7) is a clinical isolate from the CSF of a newborn infant with meningitis (21), and E44 is a spontaneous rifampin-resistant mutant of RS218. DH5α is a host strain for subcloning and preparation of plasmids for DNA sequence determination. Strains containing plasmids were grown at 37°C in L broth (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter) with ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (20 μg/ml), or chloramphenicol (100 μg/ml) for positive selection of plasmids (Table 1). Bacteria were cultured in L broth and stored in L broth plus 20% glycerol at −70°C.

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 ultrapure quality and were obtained from National Diagnostics (Atlanta, Ga.). Reagents for DNA sequencing reactions with Sequenase and other chemicals were purchased from U.S. Biochemical Corp. (Cleveland, Ohio). DNA sequencing kits with dye terminators were obtained from PE Applied Biosystems (Foster City, Calif.).

Isolation of the noninvasive Tn*phoA* mutant 7A-33 of *E. coli* K1. The noninvasive Tn*phoA* mutant 7A-33 was generated as previously described (7). Briefly, strain SM10λpir containing the suicide vector plasmid pRT733 was used as a Tn*phoA* donor, while *E. coli* K1 strain E44 was used as a recipient. SM10λpir carrying pRT733 was mated with E44 on Luria-Bertani (LB) agar by cross-streaking and then incubation at 37°C for 6 h. The conjugants were selected on LB agar containing kanamycin and rifampin (7). Tn*phoA* mutants were screened for their ability to invade BMEC as described previously (7). Probing of the DNA blots with a ^{32}P-labeled 0.6-kb Kan^r gene fragment derived from Tn*5* identified noninvasive mutants with a single Tn*phoA* insertion (7).

Tissue cultures and invasion assays. BMEC were prepared from bovine and human brains (22, 23), and invasion assays were performed as previously described (7). Briefly, brain specimens devoid of large blood vessels were homogenized in Dulbecco minimal essential medium (DMEM) containing 2% bovine calf serum (DMEM-S) and centrifuged in 25% bovine serum albumin for bovine BMEC or in 15% dextran in DMEM-S for human BMEC. The pellets containing crude microvessels were further digested in a solution containing collagenase or dispase (1 mg/ml). Microvascular capillaries were isolated by adsorption to a column of glass beads and were recovered in growth medium (22). The resulting bovine and human BMEC were positive for factor VIII, carbonic anhydrase IV, gamma-glutamyl transpeptidase, and the ability to take up low-density lipoproteins, demonstrating their brain endothelial cell characteristics (22, 23).

Invasion assays were done with approximately 10^7 bacteria added to confluent monolayers of BMEC at a multiplicity of infection of 50 to 100. The monolayers were incubated for 1.5 h at 37°C to allow invasion to occur. The number of intracellular bacteria was determined after the extracellular bacteria were eliminated by incubation of the monolayers with experimental medium containing gentamicin (100 μg/ml) (7). Results were expressed either as percent invasion [100 × (number of intracellular bacteria recovered/number of bacteria inoculated)] or as relative invasion (invasion as a percentage of the invasion of the parent strain *E. coli* K1).

Neonatal rat model of hematogenous *E. coli* K1 meningitis. The noninvasive mutant with a single Tn*phoA* insertion (7A-33) was examined for its ability to enter the CNS in our neonatal rat model of hematogenous *E. coli* meningitis as described previously (7, 10). 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 E44 or 4.4×10^4 CFU of the mutant strain 7A-33. Our pilot experiments revealed that these bacterial inocula for strains E44 and 7A-33 produced nonlethal bacteremia of 10^5 to 10^8 CFU/ml of blood in >90% of animals within 18 h of inoculation. At 18 h after bacterial inoculation, blood and CSF specimens were obtained for quantitative cultures as described previously (10). Blood and CSF specimens obtained from animals infected with mutant 7A-33 were cultured in brain heart infusion broth and agar containing kanamycin (40 μg/ml).

Cloning of Tn*phoA* fragments. *Mlu*I-digested genomic DNA from the noninvasive mutant 7A-33 was cloned into the *Mlu*I site of pCVD433, which was derived from pACYC184 by insertion of *Mlu*I linkers into the *Eco*RV site (3). Transformation was performed by electroporation of *E. coli* DH5α 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 as previously described (7). The kanamycin-resistant transformants were identified as *Mlu*I fragments containing Tn*phoA*. The construct carrying a 13-kb *Mlu*I fragment of the noninvasive mutant 7A-33 in pCVD433 was designated pCD7A (Table 1).

PCR cloning and analysis of *ibeB*. The PCR was performed as described previously (8, 11). Briefly, 0.5 μg of genomic DNA was added to a mixture containing 1× *Taq* polymerase buffer (Cetus), 1.5 mM $MgCl_2$, 0.5 mM deoxynucleoside triphosphates (Cetus), 50 pmol of each primer, and *Taq* DNA polymerase in a final volume of 50 μl. Amplification was carried out with a PTC-100 programmed thermal controller (M. J. Research) for 40 cycles: denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and primer extension for 3 min at 70°C. The two pairs of primers (primers IB7-3a and IB7-5a and primers IB-5HB and IB7-5a) (Table 2) used for the PCR were synthesized with an Applied Biosystems (Foster City, Calif.) 380B DNA synthesizer. Two DNA fragments, a 0.67-kb fragment carrying the partial *ibeB* coding sequence and a 1.6-kb fragment containing the complete *ibeB* open reading frame (ORF), were amplified from genomic DNA of wild-type strain RS218 and subcloned into TA cloning vector pCRII (Invitrogen). The resulting constructs were designated pCIB7B and pCR7C, respectively (Table 1).

DNA sequencing and analysis. The nucleotide sequence of *ibeB* was determined by the dideoxy chain termination method of Sanger et al. (19) with a Sequenase version 2.0 kit from U.S. Biochemical Corp. and [^{35}S]dATP (1,000 to

TABLE 2. Oligonucleotides used for cloning and sequencing

Gene or transposon	Primer	Strand	Sequence	Use(s)
Tn*phoA*	Tnp5	−	5′-TCGCTAAGAGAATCACGCAGAG-3′	Sequencing
Tn*5*	Tnp3	+	5′-GCACGATGAAGAGCAGAAG-3′	Sequencing
ibeB	IB-5HB	+	5′-AGGATCCGAGCCTATGTCTCCTTG-3′	Cloning, sequencing
ibeB	IB7-5a	−	5′-GTAAAGCGCATGGTCATC-3′	Cloning, sequencing
ibeB	IB7-5b	+	5′-TTTAATGCCCAGGCTAACG-3′	Cloning, sequencing
ibeB	IB7-3a	+	5′-GCCAGCAGCGATCTGTCGTC-3′	Cloning, sequencing
ibeB	IB7-31	−	5′-CGGCGATATCCAGATTG-3′	Sequencing
ibeB	IB7-32	−	5′-TTAGCCGCCATTAACGCGTG-3′	Sequencing
ibeB	IB7-33	−	5′-TTACCCAGCGTTTCTTCG-3′	Sequencing
ibeB	IB7-3c	+	5′-ATCAGCAACTGGCGTATG-3′	Sequencing
ibeB	IB7-34	−	5′-TTAACCAGGCCGTTCTGG-3′	Sequencing

1,500 Ci/mmol) from Du Pont, NEN Research Products (Boston, Mass.). To sequence the portion of *ibeB* proximal to the Tn*phoA* insertion site, the initial DNA sequence was obtained from plasmid pCD7A 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 *ibeB* was determined with primers complementary to the *ibeB* sequence in pCD7A, pFN7C, and pKS7-7B (Table 1). Both strands of the DNA were resequenced by the automated approach with fluorescence-labeled nucleotides (Applied Biosystems 373A automated sequencer) to ensure accuracy, and the sequence data were analyzed with the DNA analysis program 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 use of the BLAST algorithm.

Construction and screening of a genomic library of *E. coli* RS218. High-molecular-weight chromosomal DNA was purified from *E. coli* K1 strain RS218 as previously described (7). Genomic DNA was partially digested with *Sau*3AI (New England Biolabs), which is compatible with *Bam*HI. Partially digested genomic DNA (15 to 23 kb) was partially filled in with dGTP and dATP. This DNA was ligated into LambdaGEM-12 arms with an *Xho*I half site. Ligation and packaging of recombinant lambda phage were performed according to the manufacturer's instructions (Promega). The *E. coli* genomic library was screened by DNA hybridization (7) to identify phage clones that contained *ibeB*. A 0.67-kb *ibeB* DNA fragment in pCIB7B was released with *Eco*RI, purified by preparative agarose electrophoresis and by use of Geneclean (Bio 101), labeled with [α-^{32}P]dCTP by use of an Oligolabeling kit (Pharmacia), and used as a probe for screening (>1 × 10^8 cpm/μg). The phage plaques were replicated onto nylon filters, UV linked, and hybridized as described previously (7, 9). Plaques hybridizing to the probe were identified by autoradiography and then purified.

Complementation analysis. A 7.0-kb *Bam*HI-*Eco*RI subclone of pKS7-13K containing the *ibeB* ORF (7B) and a 5.7-kb *Bam*HI-*Hpa*I subclone lacking this sequence (5H) were subcloned into the pBluescriptII KS vector (see Fig. 2). The resulting constructs were designated pKS7-7B and pKS7-5H, respectively. A 1.6-kb *Xba*I-*Hin*dIII subclone of pCR7C carrying the complete *ibeB* ORF was subcloned into pFN476. The resulting construct was designated pFN7C. The ligation mixture was used to transform DH5α, and selection was done on LB plates containing ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The white colonies were picked for identification of plasmids containing the insert. Cells of the noninvasive *E. coli* K1 mutants 7A-33 and IB7D5 were made competent in 10% glycerol as described previously (7). 7A-33 was transformed with the pBluescriptII KS vector and the recombinant plasmids pKS7-7B and pKS7-5H (Table 1). IB7D5 was transformed with pFN7C and pGP1-2. The expression of *ibeB* in pFN476 is driven by the T7 promoter, and pGP1-2 is a vector carrying the T7 RNA polymerase gene (20). The transformants were tested for their ability to invade BMEC.

Nucleotide sequence accession number. The nucleotide sequence of *ibeB* has been deposited in the GenBank nucleotide sequence data library under accession no. AF94824.

RESULTS

Noninvasive phenotype of the Tn*phoA* insertion mutant 7A-33. We previously showed that the mutant 7A-33, with a single Tn*phoA* insertion, retained the same phenotypic and genotypic characteristics as the parent strain RS218 or E44 (7). When mutant 7A-33 was examined for its ability to invade bovine and human BMEC, compared to that of the parent strain, its invasion capacity was <0.001%, while the parent strain exhibited an invasion frequency of 0.1%.

Prevalence of meningitis in infant rats. We next examined whether the differences in BMEC invasiveness would be biologically relevant in our well-established infant rat model of experimental hematogenous meningitis. Table 3 summarizes the prevalence of meningitis (defined as positive CSF cultures) in 5-day-old rats infected with the parent strain E44 or its noninvasive mutant with a single Tn*phoA* insertion, 7A-33. As expected, subcutaneous injections of 1.4 × 10^4 CFU of strain E44 or 4.4 × 10^4 CFU of mutant 7A-33 resulted in bacteremia of 10^5 to 10^8 CFU/ml of blood in 100% of the animals. This level of bacteremia has been shown to be sufficient to allow circulating *E. coli* to enter the CNS (10). As shown in Table 3, the magnitudes of bacteremia were similar between the two groups. However, the occurrence of meningitis was significantly lower (*P*, 0.003) in animals receiving mutant strain 7A-33 (4 of 25, or 16%) than in those receiving parent strain E44 (15 of 27, or 56%). These findings support the concept that the Tn*phoA* insertion mutant 7A-33 is truly less invasive both in vitro and in vivo, suggesting that the DNA flanking the transposon insertion in mutant 7A-33 may contain a gene(s) necessary if not sufficient for invasion of BMEC. It is also important to recognize that the BMEC invasion frequency of 0.1% is related to enhanced invasion of the CNS in vivo.

Sequence analysis of *ibeB*. In order to identify the *ibeB* gene, we sequenced the corresponding region of DNA flanking Tn*phoA*. As shown in Fig. 1, a 1,383-nucleotide open reading frame (ORF) assigned to the *ibeB* gene coded for a protein with 460 amino acids and a calculated molecular mass of 50 kDa (Fig. 1). This ORF was found to be 97% identical to a gene encoding a 50-kDa hypothetical protein (p77211) and located in the 13-min region of the *E. coli* K-12 genome (1). However, no homology was observed between *ibeB* and other known invasion genes when DNA and protein databases in GenBank were searched. Potential −10 (TATGAG) and −35 (TTGTCA) promoter regions were found at the 5′ noncoding region of *ibeB*. The Tn*phoA* insertion site was identified by sequencing the fusion joint with the 5′ primer Tnp5 and the 3′

TABLE 3. Development of bacteremia and meningitis (defined as positive CSF cultures) in two groups of newborn rats receiving *E. coli* E44 or 7A-33

E. coli strain	No. of animals	Bacteremia (log CFU/ml of blood [mean ± SD])	No. (%) of animals with positive CSF
7A-33	25	7.01 ± 1.17	4 (16)[a]
E44	27	7.06 ± 1.29	15 (56)

[a] The *P* value was 0.003 (Fisher's exact test) for animals receiving 7A-33 compared to animals receiving E44.

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1    ATTTTGTCAATTTTCCTGTC ACCGGAAAATCAGAGCCTGG CGAGTAAAGT TGGCAGCATA AAATCACCAGAAATTATGAG CCTATGTCTCCTTGTAAAAT
       '-35'                                                                          '-10'     M S P C K I

101  TTTGCCATTTTGTGTGGCCC TTGCGCTAACCGGTTGTTCA ATGGCACCGGATTATCAGCG TTCGGCAATGCCCGTGCCGC ACCAGTTCTCACTCAGCCAG
       L P F C V A L A L T G C S M A P D Y Q R S A M P V P H Q F S L S Q

201  AACGGCCTGGTTAACGCAGC AGATAACTATCGGAGCGCGG GCTGGCGCACCTTTTTTGTT GATAATCAGGTGAAGACGCT GATTAGCGAGGCGTTGGTGA
       N G L V N A A D N Y R S A G W R T F F V D N Q V K T L I S E A L V N

301  ATAACCGGGATTTGCGCATG GCGGCGCTGAAAGTGCAGGA AGCGCGGGCGCAATATCGTC TGACCGATGCCGACCGCTAC CCACAGTTCAATGGCGAGGA
       N R D L R M A A L K V Q E A R A Q Y R L T D A D R Y P Q F N G E D

401  CAGCGGCAGCTGGAGCGGCA ATCTTAAAGGCGATTCAGCC ACGACGCGGGAGTTCTCGAC TGGCCTTAACGCCAGCTTTG ATCTCGATTTTTTCGGGCGC
       S G S W S G N L K G D S A T T R E F S T G L N A S F D L D F F G R

501  TTAAAGAACATGAGCGAAGC CGAGCGCCAAAAT TATTTAG CCACTGAGGAAGCTCAGCGC GCGGTGCATATTTTGCTGGT TTTTAATGTCGCGCAAAGCT
       L K N M S E A E R Q N Y L A T E E A Q R A V H I L L V F N V A Q S Y

601  ATTTCAATCAGCAACTGGCG TATGCGCAATTGCAAATAGC CGAAGAAACGCTGGGTAATT ATCAGCAG TCATATGCGTTT GTCGAAAAACAACTGTTGAC
       F N Q Q L A Y A Q L Q I A E E T L G N Y Q Q S Y A F V E K Q L L T

701  CGGTAGCAGCAATGTTCTGG CGCTGGAACAGGCTCGCGGG GTGATAGAAAGTACCCGCAG CGACATCGCTAAACGTCGGG GGGAACTGGCGCAGGCGAAT
       G S S N V L A L E Q A R G V I E S T R S D I A K R R G E L A Q A N

801  AATGCATTGCAACTGTTATT GGGAAGCTACGGCAAGCTGC CGCAAGCGCAGACAGTAAAC AGCGACAGCCTGCAAAGCGT TAAATTACCGGCGGGCTTGC
       N A L Q L L L G S Y G K L P Q A Q T V N S D S L Q S V K L P A G L P

901  CGTCGCAAATCTTATTGCAG CGCCCTGATATTATGGAAGC TGAACACGCGTTAATGGCGG CTAATGCCAATATTGGTGCT GCGCGTGCGGCATTTTTCCC
       S Q I L L Q R P D I M E A E H A L M A A N A N I G A A R A A F F P

1001 GTCTATCAGCCTGACCAGCG GAATATCAACCGCCAGCAGC GATCTGTCGTCATTATTTAA TGCCAGCAGCGGGATGTGGA ATTTTATTCCTAAAATTGAG
       S I S L T S G I S T A S S D L S S L F N A S S G M W N F I P K I E

1101 ATCCCCATTTTTAATGCTGG ACGCAACCAGGCCAATCTGG ATATCGCCGAAATTCGCCAG CAGCAGTCGGTGGTGAATTA TGAACAGAAAATCCAGAACG
       I P I F N A G R N Q A N L D I A E I R Q Q Q S V V N Y E Q K I Q N A

1201 CCTTTAAAGAAGTGGCAGAT GCGCTGGCATTACGTCAAAG CCTGAACGATCAAATCAGCG CCCAGCAGCGTTATCTTGCG TCGCTGCAAATTACTTTGCA
       F K E V A D A L A L R Q S L N D Q I S A Q Q R Y L A S L Q I T L Q

1301 ACGGGCGCGGGCATTATATC AGCACGGCGCGGTAAGTTAT CTGGAAGTGCTGGATGCCGA GCGTTCTTTATTTGCAACCC GACAAACTTTACTTGACCTG
       R A R A L Y Q H G A V S▲Y L E V L D A E R S L F A T R Q T L L D L

1401 AATTATGCCCGTCAGGTTAA CGAAATTTCTTTATATACCG CCTTAGGCGGCGGTTGGCAG CAATAATTTT TAACTCCAGG AGAGAATAAATGAAAAAAGC
       N Y A R Q V N E I S L Y T A L G G G W Q Q *

1501 ACTGCAAGTCGCAATGTTTA GTCTGTTTACCGTTATTGGC TTTAATGCCCAGGCTAACGA ACATCATCATGAAACCATGA GCGAAGCACAACCACAGGTT

1601 ATTAGCGCCACTGGTGTGGT AAAAGGTGTTGATCTGGAAA GCAAAAAAATCACCATCCAT CACGATCCGATTGCTGCCGT GAACTGGCCGGAGATGACCA

1701 TGCGCTTTACC
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FIG. 1. Complete nucleotide sequence and deduced amino acid sequence for the *ibeB* gene from an *E. coli* K1 strain. The calculated molecular mass of the full-length protein is 50 kDa. Boxes indicate potential −10 and −35 promoter sites. The arrow indicates a Tn*phoA* insertion site in *ibeB*. Two putative transmembrane domains are underlined.

primer Tnp3, which are complementary to the two ends of Tn*phoA*. The insertion occurred in the codon corresponding to residue 419.

A restriction map and the genetic organization of the invasion gene *ibeB* in the chromosome of *E. coli* RS218 are shown in Fig. 2. The sequence of the entire *ibeB* ORF and its upstream and downstream regions is presented in Fig. 1.

Construction of an *E. coli* RS218 LambdaGEM-12 library and isolation and subcloning of invasion determinants. *E. coli* K1 strain RS218 was used as the source of DNA for cloning experiments. This virulent strain is capable of invading human and bovine BMEC and inducing meningitis in newborn rats (7). To clone the invasion determinants from RS218, a genomic library was constructed in LambdaGEM-12. By use of *Xho*I half sites in the vector and *Sau*3AI in the genomic inserts

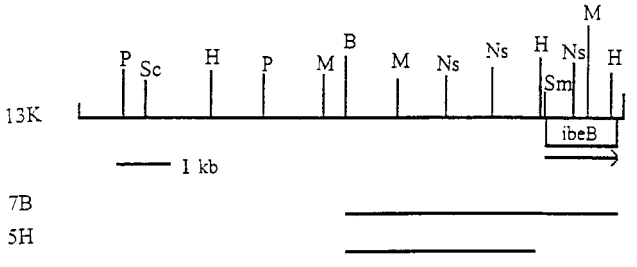

FIG. 2. Restriction map of the DNA fragments containing the *ibeB* invasion gene cloned from *E. coli* K1 strain RS218. Relevant restriction sites for *Bam*HI (B), *Hin*dIII (H), *Mlu*I (M), *Nsi*I (Ns), *Pst*I (P), *Sac*I (Sc), and *Sma*I (Sm) are shown. The horizontal arrow indicates the transcription direction for the *ibeB* ORF (box).

for library construction, self-ligation of vector and genomic sequences was eliminated, since only recombinant phages containing a single insert of the appropriate size (9 to 23 kb) were capable of being packaged. Using *ibeB* (0.67 kb) as a probe, approximately 5×10^5 recombinant phages were screened and seven phage clones for *ibeB* were identified. The recombinant phage DNAs were purified and digested with *Not*I. The sizes of the inserts were between 12 and 16 kb. A 13-kb insert containing *ibeB* was subcloned into the *Not*I site of pBluescriptII KS (pKS7-13K).

Construction of an isogenic in-frame deletion mutant. In order to determine whether or not the noninvasive phenotype of the mutant 7A-33 was due to a polar effect of the Tn*phoA* insertion, an *ibeB* in-frame deletion mutant was generated by integration of the suicide plasmid pCD7D (Table 1). pKS7-6N was constructed by removing the 0.87-kb *Sma*I-*Nsi*I N-terminal fragment of *ibeB* from pKS7-7B and religating the plasmid containing part of *ibeB* with a 30-bp *Eco*RV-*Nsi*I fragment from plasmid pZerO-2.1 (Invitrogen). A 2.3-kb *Bgl*II-*Eco*RI fragment carrying mutated *ibeB* from pKS7-6N was converted into blunt ends through a filling-in reaction (18) and subcloned into pCVD442 (4) with *Sma*I. The resulting construct (pCD7D) was confirmed by PCR and DNA sequencing as having the *ibeB* in-frame deletion.

The mutants were obtained by mating E44 with SM10λpir carrying pCD7D and selection on LB agar containing ampicillin and rifampin. A single such colony was picked and grown to the late logarithmic phase in LB broth without selection. Dilutions were plated on LB agar plates containing no NaCl and 5% sucrose. Sucrose-resistant colonies were tested for the loss of ampicillin resistance, indicative of the loss of vector sequences.

PCR was used to confirm the deletion in the desired chromosomal *ibeB* gene in the deletion mutant IB7D5 with a 5′ primer (5′-ATTTCCTCCGCATGTTGC-3′) and a 3′ primer (IB7-31). Amplification was carried out with the following cycle profile: 40 cycles at 94°C for 1 min, 55°C for 1 min, and 70°C for 3 min. The PCR DNA samples were sequenced with primers IB7-31 and IB7-32 to confirm the *ibeB* in-frame deletion (Table 2).

Complementation of noninvasive mutants. The mutagenesis experiments (Tn*phoA* insertion and isogenic deletion of *ibeB*) indicated that *ibeB* was required for invasion of BMEC by strain E44. As shown in Fig. 3, The Tn*phoA* insertion mutant (7A-33) and the *ibeB* deletion mutant (IB7D5) were significantly less invasive than E44 in BMEC. To demonstrate that the Tn*phoA* insertion and the *ibeB* deletion were truly responsible for the noninvasive phenotype, we attempted to complement the noninvasive mutants with pKS7-13K, which contained a 13-kb DNA fragment with *ibeB* in the pBluescriptII KS vector; pKS7-7B, carrying a 7-kb *Bam*HI-*Eco*RI DNA fragment with *ibeB* derived from pKS7-13K; and pFN7C, carrying the *ibeB* gene (1.6 kb). pKS7-7B was capable of completely restoring the invasive phenotype of the Tn*phoA* noninvasive mutant 7A-33 (Fig. 4A) and the *ibeB* deletion mutant IB7D5 (Fig. 4B). More importantly, pFN7C was able to fully confer invasive capability to IB7D5 (Fig. 4C). However, pKS7-13K was able to partially complement the mutants (Fig. 4A), suggesting that the expression of *ibeB* may be reduced because of a larger plasmid with a decreased copy number or some unknown repressor elements present in the larger DNA fragment (18). On the contrary, pKS7-5H, carrying a 5.7-kb *Bam*HI-*Hpa*I DNA fragment with an *ibeB* deletion derived from pKS7-7B, was unable to complement the Tn*phoA* mutant 7A-33 or the *ibeB* deletion mutant IB7D5 (Fig. 4A and B).

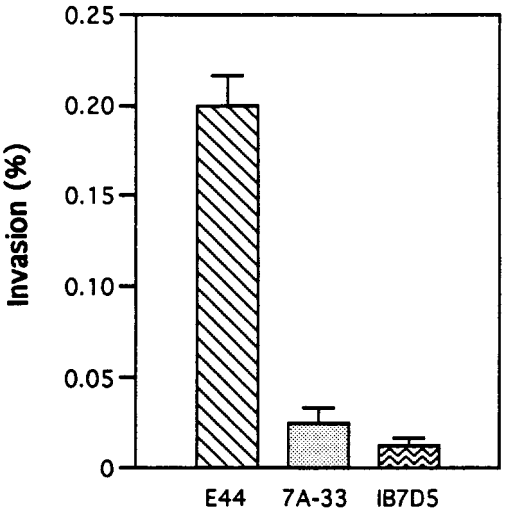

FIG. 3. BMEC invasion frequencies for *E. coli* K1 parent strain E44 and its Tn*phoA* and *ibeB* deletion mutants (7A-33 and IB7D5, respectively). Values are means of four independent assays; error bars indicate standard errors of means.

DISCUSSION

Although most cases of bacterial meningitis develop as a result of hematogenous spread, how circulating bacteria cross the blood-brain barrier is not completely understood. We have previously shown that several *E. coli*-BMEC interactions contribute to successful crossing of the blood-brain barrier by *E. coli*; these include *E. coli* binding to BMEC via S fimbriae (7, 15, 22). However, S fimbria-mediated binding to BMEC glycoproteins and glycolipids was not accompanied by invasion of BMEC (22), suggesting that binding and invasion are separate phenomena for *E. coli* translocation from blood to the CNS. We have shown that invasion of BMEC is needed for *E. coli* to cross the blood-brain barrier in vivo. We have recently described the invasion gene locus *ibeA* (*ibe10*) from *E. coli* K1 strain RS218; this locus has been shown to contribute to invasion of BMEC both in vitro and in vivo (7).

In the process of characterizing the noninvasive mutant 7A-33 derived from *E. coli* K1 strain RS218, we showed that mutant 7A-33 had a single Tn*phoA* insertion and was considerably less invasive for BMEC (the invasion frequency was 0.001%; that for the parent strain was 0.1%) and significantly less invasive for the CNS in the newborn rat model of experimental hematogenous meningitis (4 of 25, or 16%, for the mutant strain versus 15 of 27, or 56%, for the parent strain; *P*, 0.003). We have previously shown that a high degree of bacteremia is a primary determinant for meningeal invasion by *E. coli* K1 (10). The magnitudes of bacteremia between the two groups of animals receiving the parent strain and the mutant strain were similar, indicating that a decreased ability of the mutant to enter the CSF was not an artifact of the lack of a sufficient number of circulating bacteria in the bloodstream. Taken together, these findings suggest that the DNA flanking the transposon insertion in the mutant 7A-33 includes a gene(s) necessary if not sufficient for the invasion of BMEC. This gene, derived from mutant 7A-33, was termed *ibeB* (invasion of brain endothelial cells).

We have previously shown, using the hematogenous *E. coli* meningitis model, that the K1 capsule is a critical determinant needed for *E. coli* to cross the blood-brain barrier as live bacteria (10). We have also shown that OmpA contributes to the invasion of BMEC by *E. coli* K1 (15). Both the parent strain

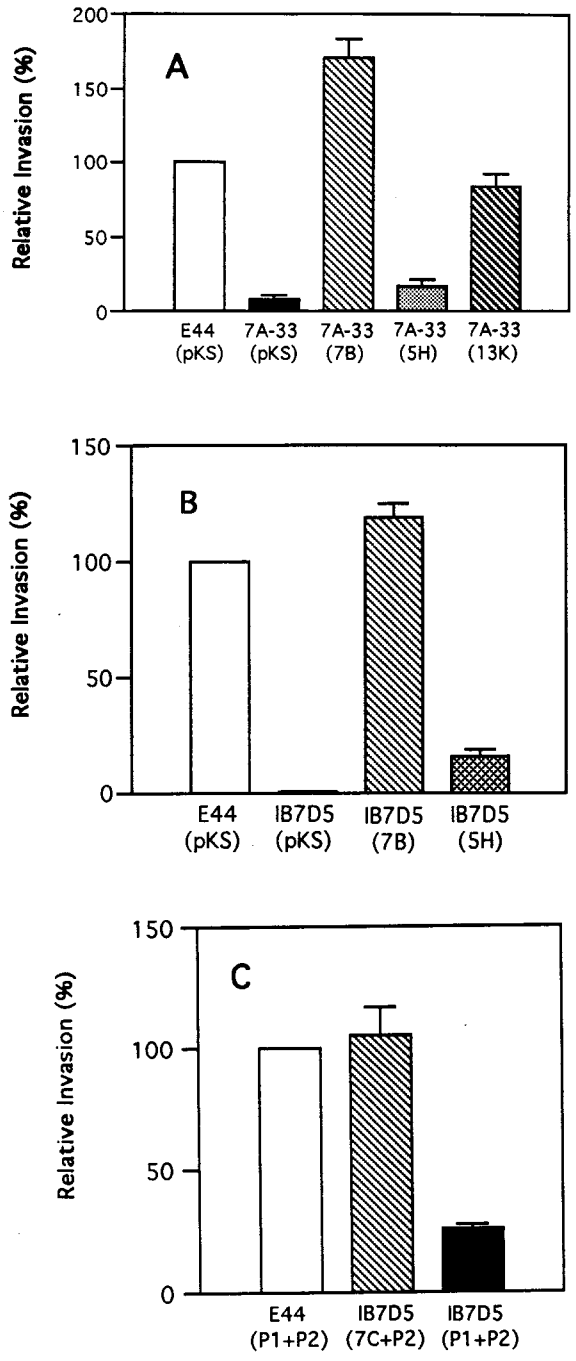

FIG. 4. Complementation of the noninvasive mutants of E44 with plasmids carrying the *ibeB* locus. The invasiveness of the mutants relative to that of the parent strain E44 is shown. Results are means of four separate experiments; error bars represent standard errors of means. (A) Complementation of the Tn*phoA* insertion mutant 7A-33 by pKS7-13K (13K), pKS7-7B (7B), pKS7-5H (5H), and pBluescript KS (pKS). (B) Complementation of the isogenic *ibeB* deletion mutant IB7D5 by pKS7-7B (7B), pKS7-5H (5H), and pBluescript KS (pKS). (C) Complementation of the isogenic *ibeB* deletion mutant IB7D5 by combinations of pFN7C (7C), pGP1-2 (P2), and pFN476 (P1).

RS218 and the mutant strain 7A-33 were found to possess the K1 capsule and OmpA. In addition, the *Eco*RV-*Mlu*I Tn*phoA* fragment of the mutant 7A-33 did not hybridize to the probes for the K1 capsule and OmpA. These findings suggest that the noninvasive property of 7A-33 is not likely to be the result of a polar effect of Tn*phoA* on the other known genes involved in invasion (e.g., those for the K1 capsule and OmpA). This concept was also supported by our demonstration that the isogenic *ibeB* deletion mutant IB7D5 was unable to invade BMEC, and its inability to invade BMEC was fully complemented by the *ibeB* ORF.

Nucleotide sequence analysis of the *ibeB* gene showed a single ORF encoding a protein of 460 amino acids and having a predicted molecular mass of 50 kDa (Fig. 1). The deduced protein displayed the characteristics of an outer membrane protein with two transmembrane domains (Fig. 1). This ORF was found to be 97% identical to a gene encoding a 50-kDa hypothetical protein (p77211) and located in the 13-min region of the *E. coli* K-12 genome. However, no homology was found with genes for any other recognized invasion proteins, suggesting that *E. coli* K1 *ibeB* results in a novel phenotype, i.e., *E. coli* invasion of BMEC. It is not clear whether the *ibeB* homologue from *E. coli* K-12 will result in the same phenotype in *E. coli* K1 strains.

The invasion of BMEC by *E. coli* represents a unique mechanism used by bacteria to gain entry into the CNS. We have previously shown that the characteristics of invasion of endothelial cells by *E. coli* K1 are specific to BMEC and that no such invasion characteristics are observed for endothelial cells of nonbrain origin, e.g., human umbilical vein endothelial cells (15). It is of interest that human BMEC have been shown to form a continuous lining of endothelial cells and to exhibit a transendothelial electrical resistance of 100 to 600 $\Omega \cdot$ cm (14, 17), a unique property of the BMEC monolayer (compared to the systemic vascular endothelium). It is important to recognize that the frequency of invasion of BMEC by the parent strain RS218 (approximately 0.1%) is considerably lower than the reported frequency of invasion of epithelial cells by other gram-negative bacteria, such as *Shigella* and *Salmonella* species (usually 1 to 10%). However, as shown here and in our previous publication (7), the BMEC invasion frequency of approximately 0.1% contributes to enhanced bacterial penetration through the blood-brain barrier in vivo and thus is biologically relevant.

We have recently shown that the invasion gene locus *ibeA* contributes to *E. coli* invasion of human BMEC (7). Here we report another chromosomal invasion locus, *ibeB*, contributing to *E. coli* invasion of BMEC. The genetic locus *ibeB* was capable of completely restoring the ability of the *ibeB* deletion and Tn*phoA* insertion mutants to invade BMEC. However, *E. coli* K-12 strain HB101 was not complemented by the *ibeB* locus (data not shown), suggesting that multiple determinants contribute to *E. coli* invasion of BMEC. Our demonstration that multiple chromosomal genes are required for *E. coli* invasion of BMEC is conceptually similar to the reported requirements for different determinants in the attachment and entry of epithelial cells by other meningitis-causing bacteria (2, 13, 25). It remains to be determined how different invasive loci of *E. coli* contribute to crossing of the blood-brain barrier.

In conclusion, we cloned and characterized the chromosomal gene locus that allows *E. coli* K1 strain RS218 to invade BMEC both in vitro and in vivo. This gene, termed *ibeB*, encoded a 50-kDa potential membrane protein. A 7-kb DNA fragment (7B) containing *ibeB* as well as the *ibeB* ORF was able to restore the ability of the in-frame *ibeB* deletion mutant to invade BMEC. Studies are in progress to define the mechanisms by which the *ibeB* locus exerts its invasive phenotype.

ACKNOWLEDGMENTS

We thank Jin Liu for restriction enzyme mapping of *ibeB* clones.

This study was supported by a research career development award from Childrens Hospital Los Angeles Research Institute (to S.-H.H.),

a grant-in-aid from the American Heart Association Great Los Angeles Affiliate (to S.-H.H.), and USPHS grants R29AI40635 (to S.-H.H.) and R01NS26310 (to K.S.K.).

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Editor: P. E. Orndorff