INFECTION AND IMMUNITY, May 2005, p. 2923–2931 Vol. 73, No. 5
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.5.2923–2931.2005

Escherichia coli K1 RS218 Interacts with Human Brain Microvascular Endothelial Cells via Type 1 Fimbria Bacteria in the Fimbriated State

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Received 20 September 2004/Returned for modification 5 November 2004/Accepted 22 December 2004

***Escherichia coli* K1 is a major gram-negative organism causing neonatal meningitis. *E. coli* K1 binding to and invasion of human brain microvascular endothelial cells (HBMEC) are a prerequisite for *E. coli* penetration into the central nervous system in vivo. In the present study, we showed using DNA microarray analysis that *E. coli* K1 associated with HBMEC expressed significantly higher levels of the *fim* genes compared to nonassociated bacteria. We also showed that *E. coli* K1 binding to and invasion of HBMEC were significantly decreased with its *fimH* deletion mutant and type 1 fimbria locked-off mutant, while they were significantly increased with its type 1 fimbria locked-on mutant. *E. coli* K1 strains associated with HBMEC were predominantly type 1 fimbria phase-on (i.e., fimbriated) bacteria. Taken together, we showed for the first time that type 1 fimbriae play an important role in *E. coli* K1 binding to and invasion of HBMEC and that type 1 fimbria phase-on *E. coli* is the major population interacting with HBMEC.**

The mortality and morbidity associated with neonatal gram-negative bacterial meningitis have remained significant despite advances in antimicrobial chemotherapy. This is mainly attributed to inadequate knowledge of the pathogenesis and pathophysiology of this disease. *Escherichia coli* K1 is the most common gram-negative bacterium that causes meningitis during the neonatal period (26).

E. coli meningitis develops as a result of hematogenous spread, but it is not clear how circulating bacteria cross the blood-brain barrier. Our laboratory has successfully isolated and cultivated human brain microvascular endothelial cells (HBMEC), which constitute the blood-brain barrier (9, 10). We showed that *E. coli* invasion of HBMEC is a prerequisite for *E. coli* penetration into the central nervous system in vivo. However, the basis of *E. coli*-HBMEC interactions involved in binding to HBMEC is incompletely understood.

Adherence of bacteria to their host cells is considered the initial step of the pathogenesis of infectious diseases including meningitis. To date OmpA is the only identified determinant that is involved in *E. coli* K 1 binding to HBMEC (8, 10). Previous reports have implied that S fimbriae might be another potential *E. coli* K1 factor involved in adherence to HBMEC (3, 16, 24, 32). However, according to our recent data, S fimbriae do not play a significant role in binding of *E. coli* K1 to HBMEC (35).

Type 1 fimbriae are filamentous surface organelles produced by *E. coli* and mediate mannose-sensitive adhesion of *E. coli* to various eukaryotic cells. In *E. coli* K1, type 1 fimbriae have been shown to be important for oropharyngeal colonization in a neonatal rat model (4). Type 1 fimbriae are encoded by a *fim* gene cluster, including at least nine genes required for their biosynthesis (20). The fimbriae are composed primarily of the major FimA protein and a small tip structure containing FimF, FimG, and FimH (12). The lectin-like adhesin, FimH, located at the tip of the fimbrial shaft is responsible for the mannose-sensitive adhesion to eukaryotic host cells (7).

Expression of type 1 fimbria is regulated by a phase variation in which each individual bacterium can alternate between fimbriated and nonfimbriated states, also called "phase on" and "phase off," respectively (1). The phase switching is determined by the orientation of a 314-bp chromosomal region that contains the promoter of *fim* structure genes and is located upstream of *fimA*. The 314-bp invertible element is flanked with 9-bp invert repeats, the left invert repeat (IRL) and the right invert repeat. By recognizing the flanking invert sequences this inversion is catalyzed by two site-specific recombinases, FimB and FimE, encoded upstream of the invertible element (13).

The present study was carried out to examine the role of type 1 fimbriae in the pathogenesis of *E. coli* meningitis, i.e., *E. coli* K1 binding to and invasion of HBMEC. We constructed a *fimH* deletion mutant and type 1 fimbria phase-locked mutants of *E. coli* K1 and compared their binding and invasion capabilities in HBMEC compared to the parent *E. coli* K1 strain. We also examined the populations of *E. coli* K1 associated with HBMEC by invertible element orientation assay and DNA microarray.

MATERIALS AND METHODS

Endothelial cell culture and bacterial strain and culture condition. HBMEC were isolated and cultured as previously described (31). HBMEC cultures were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 10% Nu-Serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), essential amino acids, and vitamins. *E. coli* K1 strain RS218 (O18:K1:H7) is a cerebrospinal fluid isolate from a neonate with meningitis. *E. coli*

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TABLE 1. Primers used

Primer	Sequence
CM27	GGGGGTGCACTCAGGGAACCATTCAGGCA
CM28	GGGGCATGCTTATTGATAAACAAAAGT
mut-fimH-F2	TACCTACAGCTGAACCCAAAGAGATGATTGTAATGAAACGAGTTATTACCCATATGAATATCCTCCTTAGT
mut-fimH-R2	TTGCGTACCAGCATTAGCAATGCCCTGTGATTTCTTTATTGATAAACAAAGTGTAGGCTGGAGCTGCTTC
flank-fimH-F	GAGCCACTCAGGGAACCATT
flank-fimH-R	TTTAGCTTCAGGTAATATTGCGTACCA
Ch1-F	AGTAATGCTGCTCGTTTTGC
Ch1-R	GACAGAGCCGACAGAACAAC
Ch2-F-SacI	TCGAGCTCAATTCGCCTGTTTCCATAC
Ch2-R-BglII	GGGAGATCTAATTGTCTTGTATTTATTTG
Lock-off-BglII	GGGGAGATCTCAACTGTCTATATCATAAATAAG
Locked-R-XbaI	GCGTCTAGATCCTGTTATCACATTATCTG
Ch3-F-XbaI	GCTCTAGAAATTCGCCTGTTTCCATAC
Ch3-R	TGAGTCAAAAGAGATCTAATTGTCTTGTATTTATTTG
Locked-on-2	TACAAGACAATTAGATCTCTTTTGACTCATAGAGGAAAG

strains were grown at 37°C overnight in brain heart infusion (BHI) broth with shaking at 200 rpm.

Antibodies and antisera. Anti-FimH antiserum was derived by using FimH recombinant protein as follows. The N-terminal *fimH* of *E. coli* K1 RS218, which encodes the amino acid residues 1 to 156, was cloned into the expression vector pBAD/Thio-TOPO (Invitrogen, Carlsbad, CA) and the C-terminal part of the DNA fragment was fused to a six-His tag from the plasmid. The recombinant FimH proteins were expressed and purified by nickel-charged Sepharose resins per the manufacturer's instructions. Anti-FimH antiserum was obtained by immunizing New Zealand White rabbits with the purified recombinant protein as described previously (33).

Anti-type 1 fimbria antiserum was derived from immunizing rabbits with purified type 1 fimbriae as previously described (29). To remove nonspecific antibodies, the antiserum was adsorbed with a *fim* gene cluster deletion mutant of *E. coli* K1 RS218. The anti-O18 and anti-OmpA monoclonal antibodies were previously described (11, 25).

Construction of *fimH* deletion mutant. The *fimH* deletion mutant of RS218 was constructed by deleting the *fimH* gene and replacing it with a chloramphenicol resistance cassette using the protocol described by Datsenko and Wanner (5). Briefly, the wild-type strain was transformed with plasmid pKD46 (5), which encodes the arabinose-inducible lambda red recombinase that promotes gene recombination between linear DNA and the host chromosome based on extremely short stretches of homology (30 to 50 nucleotides). PCR primers mut-fimH-F2 and mut-fimH-R2 (Table 1) contain 50 nucleotides of 5′-flanking portions exactly homologous to the 5′ and 3′ ends of the *fimH* gene, respectively. The 3′ ends of the primers are able to probe the plasmid, pKD3 (5), and amplify the chloramphenicol resistance cassette from it. The resultant PCR product of about 1 kb was gel purified. By electroporation, the purified PCR product was then transferred into the pKD46-containing RS218, which had been incubated with 10 mM of arabinose during the procedure of making competent cells. After cultivation in Super Optimal Catabolite medium (Invitrogen, Carlsbad, CA) for 1 h at 37°C, the bacteria were plated onto Luria-Bertani (LB) agar containing chloramphenicol (25 μg/ml) and 10 mM arabinose to select for antibiotic-resistant transformants and to cure pKD46. The resultant transformants were tested with primers flank-fimH-F and flank-fimH-R to ensure that the wild-type gene (*fimH*) had been entirely deleted and replaced with the chloramphenicol resistance cassette.

***fimH* cloning and complementation of the *fimH* mutation.** The primers CM27 and CM28 (Table 1), which contain ApaLI and SphI at their 5′ ends, respectively, were designed to amplify *fimH* from *E. coli* K1 RS218. The PCR product was cut with ApaLI and SphI and ligated to the pACYC184-derived plasmid pBG2-24 (30), which was cut with the same enzymes. The resultant plasmid was designated pCHT5. For complementation, pCHT5 was transferred into the *fimH* deletion mutant. pACYC184 was transferred into the wild-type strain and the *fimH* deletion mutant as vector control.

Construction of the locked-off and locked-on mutants. The primers Ch2-F-SacI and Ch2-R-BglII (Table 1) were used to amplify the upstream region of the invertible element. The PCR product was flanked with SacI and BglII sites on its 5′ end and 3′ end, respectively. The primers Lock-off-BglII and Locked-R-XbaI (Table 1) were used to amplify the invertible element oriented in phase-off position and the downstream sequence. The resulting product was flanked with BglII and XbaI site on its 5′ end and 3′ end, respectively. The two PCR products were sequentially cloned into a commercial cloning vector, pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA), and fused together with their BglII sites. The fused fragment, fragment OFF, contains the same sequence as the phase-off-oriented invertible element and its upstream and downstream regions with the exception that the 9-bp IRL sequence was changed from 5′-TTGGGGCCA-3′ to 5′-TTAGATCTC-3′. Fragment OFF was then cloned into the suicide vector pCVD442 by utilizing SacI and XbaI sites. The resulting plasmid, designated pCHT2, was then subjected to the allelic-exchange procedure as previously described (2, 6). The locked-off mutant was selected by DNA sequencing.

The primer pairs Ch3-F-XbaI and Ch3-R (Table 1) were used to amplify the upstream region (designated fragment A) of the invertible element oriented in phase-on position except that the 9-bp IRL sequence was changed from 5′-TTGGGGCCA-3′ to 5′-TTAGATCTC-3′ and an XbaI site was added to the 5′ end of this PCR product. The primer pairs Locked-on-2 and Locked-R-XbaI (Table 1) were utilized to amplify the DNA fragment (designated fragment B) including the whole invertible element oriented in phase-on position and the downstream sequence. Like fragment A, the IRL sequence was changed in fragment B. An XbaI site was added to its 3′ end.

Since fragments A and B have 12 bp of overlap, the two fragments were mixed as templates and subjected to PCR by using Ch3-F-XbaI and Locked-R-XbaI. The resulting PCR product, fragment ON, was flanked with XbaI sites. XbaI-digested fragment ON was cloned into NheI-digested pST76-C, which is a temperature-sensitive suicide vector containing a chloramphenicol resistance gene and a rare restriction site, I-SceI (23). The new plasmid was designated pCHT3. pCHT3 was subsequently used in an allelic-exchange procedure as previously described (22). Briefly, RS218 was transformed with pCHT3 and grown on LB plates containing chloramphenicol (25 μg/ml) at 30°C overnight. Several colonies were selected and grown on chloramphenicol-containing LB plates at 42°C for 7 h and then transferred to 37°C for an additional 12 h of incubation. The surviving bacteria contained the suicide vector integrated into the chromosome. To remove the integrated plasmid and make the locked-on mutant, another temperature-sensitive plasmid, pST76-ASceP, was transferred into the bacteria and incubated at 30°C overnight. pST76-ASceP harbors an ampicillin resistance gene and expresses I-SceI nuclease (22). The nuclease cut the I-SceI site in integrated pCHT3, which killed the partial merodiploid strain, so that only the bacteria that experienced the intramolecular recombination could survive. The surviving bacteria were either the wild-type strain or the locked-on mutant. By DNA sequencing, we selected the locked-on mutant. The selected bacteria were then incubated at 37°C overnight to remove pST76-AScep.

Western blot analysis. OmpA and FimH expression in the wild-type *E. coli* and mutant strains was examined as follows. Whole-bacterial-cell lysates were prepared from overnight cultures. Equal amounts of whole-cell lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Anti-OmpA monoclonal antibody and anti-FimH serum were used as primary antibodies at a dilution of 1:2,000 to detect OmpA and FimH, respectively. Goat horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibodies and goat horseradish peroxidase-conjugated anti-rabbit IgG antibodies were utilized as secondary antibodies at a dilution of 1:3,000 to label the anti-OmpA and anti-FimH antibodies, respectively. The signals were visualized with the Amersham ECL Western detection system (Amersham, Piscataway, NJ).

***E. coli* invasion and association assays in HBMEC.** Invasion assays were performed using gentamicin protection assay as previously described (25) with a minor adjustment. Briefly, confluent monolayers of HBMEC grown in 24-well plates were infected with 10^7 bacteria at a multiplicity of infection of 100. After 90 min of incubation at 37°C, the monolayers were washed with phosphate-buffered saline (PBS), incubated with gentamicin-containing medium (100 μg/ml) for 1 h to kill extracellular bacteria, and then washed three times with PBS. HBMEC were lysed by incubation in sterile water at room temperature for 30 min. The released intracellular bacteria were enumerated by plating on sheep blood agar. The actual invasion rates were calculated by dividing the number of bacteria invading by the amounts of the original inoculum. Results were presented as relative invasiveness, percent invasion compared with the wild-type RS218, which was arbitrarily set at 100%. Association assays were performed like the invasion assay except that the gentamicin treatment step was omitted.

Invertible element orientation assay. The invertible element orientation assay utilized the asymmetrical digestion site of SnaBI within the invertible element. A pair of primers, Ch1-F and Ch1-R (Table 1), were designed and able to amplify a 602-bp fragment containing the invertible element from both the phase-on and phase-off bacteria. When the invertible element is in the "on" position, SnaBI digestion will produce fragments of 404 and 198 bp. Otherwise, the restriction enzyme digestion will make fragments of 442 and 160 bp. The SnaBI-digested PCR products were separated on a 1.5% agarose gel. To quantify the percentage of phase-on bacteria, a standard curve was prepared as described previously (17) except that we used defined numbers of locked-on and locked-off bacteria as PCR templates. The intensities of each band of the DNA fragments were determined using the ImageJ program download from the website of the National Institutes of Health (http://rsb.info.nih.gov).

Immunofluorescence labeling of bacteria. Surface presentation of type 1 fimbriae on free bacteria was assessed by immunofluorescent microscopy with an anti-type 1 fimbria rabbit polyclonal serum as previously described (21) with a minor adjustment. Briefly, overnight bacterial cultures were harvested and washed with PBS once. Bacterial cells were fixed by mixing 200 μl of bacteria with 800 μl of a 4% (wt/vol) solution of paraformaldehyde in PBS. The mixture was incubated on ice for 20 min. The fixed bacteria were then washed twice with PBS. Fifteen microliters of each sample was placed on a poly-L-lysine-coated slide and air dried. Twenty-five microliters of a 1:10 dilution of anti-type 1 fimbria serum in PBS was placed on top of each sample, and the samples were incubated for 1 h at room temperature in a moist chamber. After washing three times with PBS, each slide was incubated with 25 μl of goat fluorescein isothiocyanate-conjugated anti-rabbit IgG antibodies at a dilution of 1:100 in PBS for 1 h at room temperature in a moist chamber.

Surface presentation of type 1 fimbriae on HBMEC-bound bacteria was examined as follows. HBMEC grown on collagen-coated glass coverslips in 24-well plates until confluence were subjected to *E. coli* association assay as described above except that the HBMEC were not lysed. The samples on the coverslips were fixed in 4% paraformaldehyde on ice for 20 min and washed with PBS twice. The fixed samples were subjected to double immunofluorescence labeling for HBMEC-bound bacteria and type 1 fimbriae on their surface. To label the HBMEC-bound bacteria, anti-O18 monoclonal antibody (primary antibodies) and goat rhodamine-conjugated anti-mouse IgG antibodies (secondary antibodies) were utilized at a dilution of 1:50 and 1:100, respectively. The expression of type 1 fimbriae was determined as described above.

Yeast aggregation assay. Yeast aggregation titers of *E. coli* strains were measured as described previously (30). Briefly, *E. coli* cultures, at an optical density of 0.4 at 530 nm, were subjected to serial twofold dilutions in PBS and mixed with commercial baker's yeast cells (5 mg/ml). Aggregation was monitored visually, and the titer was recorded as the highest dilution giving a positive aggregation result.

Bacterial RNA extraction from HBMEC-associated and nonassociated *E. coli* K1. HBMEC-associated and nonassociated *E. coli* was recovered from *E. coli* K1 incubated with HBMEC based on a differential lysis technique recently developed in our laboratory (F. Di Cello et al., submitted for publication). Briefly, confluent HBMEC were incubated with *E. coli* RS218 at a multiplicity of infection of 100 for 90 min at 37°C. Following incubation, the bacteria in supernatant were collected by centrifugation and designated as non-HBMEC associated. The HBMEC monolayer was subsequently washed twice and lysed with RLT buffer (Qiagen, Valencia, CA), and the HBMEC-associated *E. coli* RS218 was pelleted by centrifugation.

Bacterial RNA was extracted with the Ribopure Bacteria kit (Ambion, Austin, TX) according to the manufacturer's instructions. The extracted RNA mixture was purified with two silica column-based cleanup procedures to reduce protein and genomic DNA contaminations. Crude bacterial RNA was first purified with a silica filter cartridge (Ribopure Bacteria kit; Ambion), and then further cleaned up with an RNeasy minicolumn combined with on-column DNase treatments as instructed (RNeasy Mini kit; Qiagen). Finally, the purified RNA was eluted with 30 μl nuclease-free water.

Fluorescent labeling and DNA microarray hybridization. Cy3- and Cy5-labeled cDNA probes were generated with an indirect labeling procedure. Briefly, 5 μg purified bacterial total RNA was converted to aminoallyl-labeled cDNA (aa-cDNA) by Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) combined with amine-modified random hexamer and aminoallyl-dUTP/deoxynucleoside triphosphates (ratio of dTTP:aminoallyl-dUTP was 1:4). After selective degradation of template RNA molecules and single-strand aa-cDNA purification with QIAquick MinElute PCR purification kit (Qiagen, Valencia, CA), fluorescent dyes (CyDye Post-Labeling reactive dye pack; Amersham Biosciences, Piscataway, NJ) were chemically coupled to the amine group on aa-cDNA. Fluorescent dye-coupled cDNA was then purified with the QIAquick PCR purification kit (Qiagen).

The purified Cy3- and Cy5-coupled cDNAs were combined and concentrated by centrifugation in a spin filter (Nanosep; molecular weight cutoff, 30,000; Pall, Ann Arbor, MI). The concentrated cDNA was resuspended in 50 μl hybridization buffer (Pronto! universal hybridization kit; Corning, NY). The hybridization mixture was then denatured at 95°C for 5 min. After brief centrifugation, the mixture was applied onto the arrayed area under a LifterSlip coverslip (Erie Scientific, Portsmouth, NH). The assembled slide was placed in a hybridization chamber (Corning) and incubated at 42°C for 16 to 18 h. Following hybridization, slides were extensively washed for 1 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS at 42°C; 5 min in 2× SSC, 0.1% SDS at 42°C; 10 min in 1× SSC at room temperature; and 2 min in 0.1× SSC at room temperature twice.

Data acquisition and analysis. Hybridized microarray slides were scanned at 10-μm resolution (GenePix 4000B; Axon Instruments, Foster City, CA). DNA microarray images were analyzed with GenePix 4.0 software (Axon). The signal intensities between two channels were normalized with print-pin group *loess* normalization. The differential expression of genes was also determined with the Limma package from Bioconductor (http://www.bioconductor.org). The cutoff adjusted *P* value of significance for differential expression was set as 0.01.

RESULTS

Microarray analysis of gene expression profile within the *fim* cluster. We initially examined using DNA microarray analyses whether the expression levels of *fim* cluster genes differ between the HBMEC-associated bacteria and the unbound bacteria during the host-bacterium interaction. After 90 min of incubation of *E. coli* K1 RS218 with HBMEC, the HBMEC-associated and unbound bacteria were collected and subjected to mRNA extraction and *E. coli* DNA microarray analysis. The associated bacteria showed higher expression levels of the *fim* cluster genes, which encode type 1 fimbria components, than the free bacteria (Fig. 1). The differences were significant for the first three genes (*fimA*, *fimI*, and *fimC*) downstream of the *fim* promoter. The expression levels of *fimA*, *fimI*, and *fimC* of the HBMEC-bound bacteria are 216% ± 6%, 162% ± 11%, and 204% ± 4%, respectively, compared with those of the free bacteria (Fig. 1). Expression levels of the remaining downstream *fim* genes (*fimD*, *fimF*, *fimG*, and *fimH*) of the HBMEC-bound bacteria ranged from 114% to 137% compared with those of the free bacteria (Fig. 1). These results suggest that type 1 fimbria may play a role in the interaction of *E. coli* K1 with HBMEC.

The *fimH* deletion mutant of *E. coli* K1 is defective in binding to and invasion of HBMEC in vitro. To determine whether type 1 fimbriae play a role in the *E. coli* binding to and invasion of HBMEC, an isogenic *fimH* deletion mutant was generated from strain RS218. The growth characteristics of the wild-type RS218 and the RS218-Δ*fimH* isogenic mutant were similar on BHI and blood agar. Western blot analysis of the parent and the mutant strains with anti-FimH antibodies confirmed that

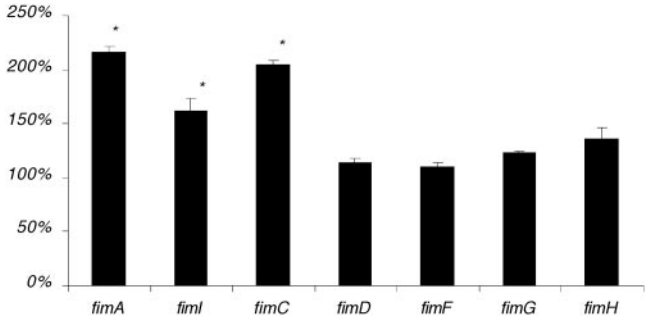

FIG. 1. The expression levels of the *fim* genes in HBMEC-bound bacteria compared to those in free bacteria after 90 min incubation of *E. coli* K1 strain RS218 with HBMEC. The expression levels in the HBMEC-bound bacteria are presented as the percentage in comparison to those in the free bacteria (which was set at 100% arbitrarily). Data shown are the means ± standard deviations derived from three independent experiments. *, $P < 0.01$ compared to free bacteria.

only the parent strain and not the mutant expressed the FimH protein (see Fig. 3B). Immunofluorescence microscopy with an anti-type 1 fimbria serum showed that a very low (<1%) percentage of the *fimH* deletion mutant cells were type 1 fimbria piliated.

The RS218-Δ*fimH* isogenic mutant was then investigated for its ability to bind and invade HBMEC. We observed that the mutant exhibited significantly less binding to HBMEC compared to the parent strain (33% ± 7% of the wild-type RS218). The mutant also showed only 7% ± 1% of invasion frequency of the wild-type RS218 (Fig. 2A). In addition, to confirm that the above results were not caused by a polar effect of the *fimH* gene deletion, complementation experiments were performed. The RS218 *fimH* was amplified with PCR and subcloned into the pACYC184-derived plasmid pGB2-24 (30). The mutant was transformed in *trans* with the *fimH*-carrying plasmid or with the vector pACYC184. The wild-type strain was also transformed with pACYC184 as a control. The above transformants were then subjected to HBMEC association and invasion assays. Both the association and invasion frequencies of the mutant harboring the *fimH*-containing plasmid were restored to the levels of the wild type, whereas the association and invasion frequencies of the mutant harboring pACYC184 were significantly lower than those of the parent strain (Fig. 2B). The results indicate that type 1 fimbria is an important determinant for *E. coli* K1 RS218 to adhere and invade HBMEC.

Construction and characterization of the type 1 fimbria locked-on and locked-off mutants of RS218. As mentioned in the introduction, type 1 fimbrial expression is phase variable (1), so the wild-type RS218 population is likely to be a heterogeneous mixture of two subpopulations: the phase-on subgroup, which expresses type 1 fimbriae, and the phase-off subgroup, which does not produce this fimbria. Therefore, the frequencies of the wild-type RS218 adherence to and invasion of HBMEC are likely to stem from a mixture of type 1 fimbriated and nonfimbriated bacteria. In order to examine the contributions of phase-on and phase-off subpopulations to interaction with HBMEC, we constructed isogenic mutants of RS218 whose *fim* promoter-containing invertible elements were fixed in either "on" or "off" orientation by changing the wild-type IRL sequence from 5′-TTGGGGCCA-3′ to 5′-TTAGATCTC-3′(see Materials and Methods). The *fim* promoter of the locked-on mutant is constitutively in the "on" orientation to allow transcription of *fim* genes, while that of the locked-off mutant is in "off" orientation and is not able to produce type 1 fimbriae. The two mutants were used to simulate the phase-on and phase-off subgroups of the wild-type strain (see the following section). Their genotypes were confirmed by the invertible element orientation assay as described in Materials and Methods. After 16 h of cultivation in BHI, the locked-on mutant and the locked-off mutant still showed their expected restriction patterns, confirming the genotypes of the phase-locked mutants and the stability of their genotypes, while the wild-type strain showed the mixed pattern of the two mutants, indicating coexistence of type 1 fimbrial phase-on and phase-off bacteria in the wild-type RS218 population (Fig. 3A).

To determine the protein expression level of type 1 fimbriae in these mutants, Western blot analysis of the two phase-locked mutants, the wild-type RS218, and the RS218-Δ*fimH* isogenic mutant with anti-FimH antibody was performed,

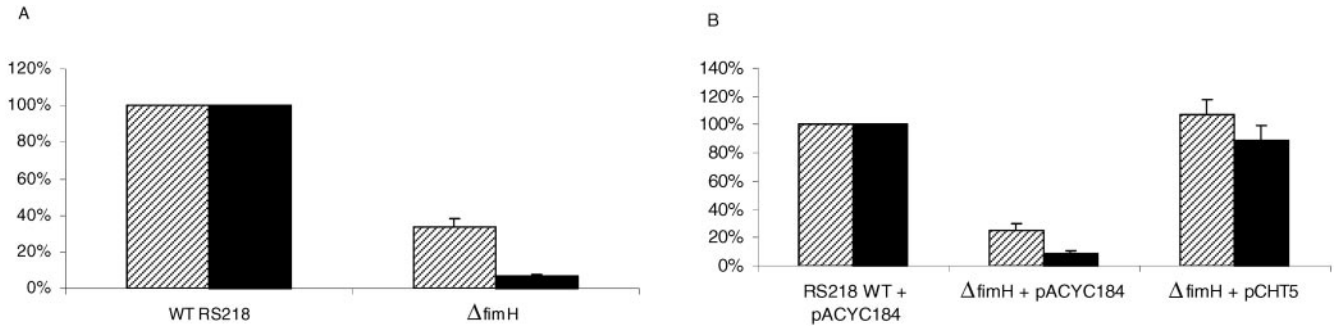

FIG. 2. The RS218-Δ*fimH* isogenic mutant showed decreased association and invasion rates in comparison to the wild-type RS218 (A). The defects of the mutant were restored by complementation with the *fimH* gene (B). pCHT5, which was derived from the vector plasmid pACYC184, harbors *fimH* from *E. coli* K1 RS218. Hatched and filled bars indicate relative association and invasion rates, respectively, compared with the wild-type strain RS218, which was set at 100%. The invasion frequencies of the wild-type RS218 (A) and the pACYC184-harboring wild-type RS218 (B) were 0.81% ± 0.11% and 0.57% ± 0.09% of the original inocula, respectively, while their association frequencies were 18% ± 4% (A) and 15% ± 3% (B) of the original inocula. Data shown are representative of three independent experiments done in triplicate. Results are shown as means ± standard deviations.

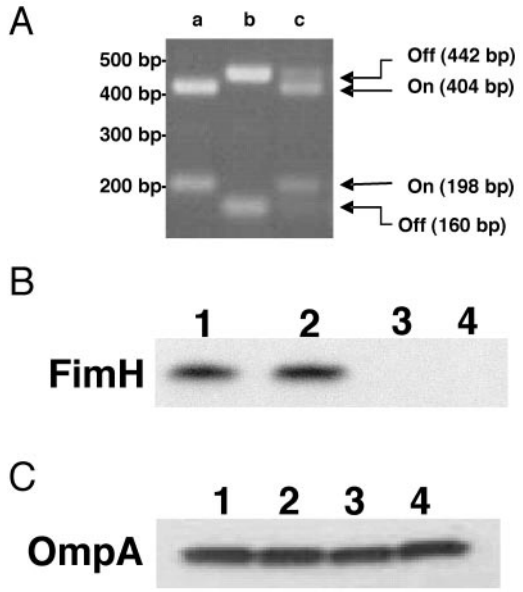

FIG. 3. Characterization of the locked-on and locked-off mutants compared to the wild-type RS218 and RS218-Δ*fimH* isogenic mutant. The genotypes of the mutants were confirmed with the invertible element orientation assay. Lane a, the locked-on mutant; lane b, the locked-off mutant; lane c, the wild-type RS218 (A). After overnight cultivation in BHI, no FimH was expressed in the locked-off and the RS218-Δ*fimH* isogenic mutants, while the locked-on mutant and the wild-type strain showed similar levels of FimH expression (B). OmpA expression was similar among the mutants and the wild-type RS218 (C). Lane 1, the wild-type RS218; lane 2, the locked-on mutant; lane 3, the locked-off mutant; lane 4, the RS218-Δ*fimH* isogenic mutant.

which revealed that the RS218-Δ*fimH* isogenic mutant and the locked-off mutant were unable to express the type 1 fimbrial adhesin FimH (Fig. 3B). In contrast, the wild-type RS218 and the locked-on mutant showed similar levels of FimH expression (Fig. 3B).

To assess type 1 fimbria piliation among the phase-locked mutants and the wild-type RS218, immunofluorescence microscopy was performed by labeling the bacteria with anti-type 1 fimbria serum. The wild-type RS218 and the locked-on mutant showed 19% ± 5% and 97% ± 3% of the bacteria expressing the fimbriae on the cell surface, respectively (Table 2). No fimbria was detected on the surface of the locked-off mutant.

TABLE 2. Yeast aggregation by RS218 and phase-locked mutants

Strain[a]	% of type 1 fimbria piliation[b]	Yeast aggregation titer without addition of mannose	Yeast aggregation titer with addition of 50 mM mannose
Wild-type RS218	19 ± 5	16[c]	—
Locked-on mutant	97 ± 3	32	—
Locked-off mutant	0 ± 0	—[d]	—

[a] The strains were grown in BHI broth overnight with shaking at 200 rpm.
[b] The results are shown as means ± standard deviations of three independent counts from different fields of the microscope. One hundred bacteria were investigated for each count.
[c] Agglutination titers were recorded as the highest twofold dilution giving a positive aggregation, starting from an optical density at 530 nm of 0.4.
[d] —, no aggregation was observed.

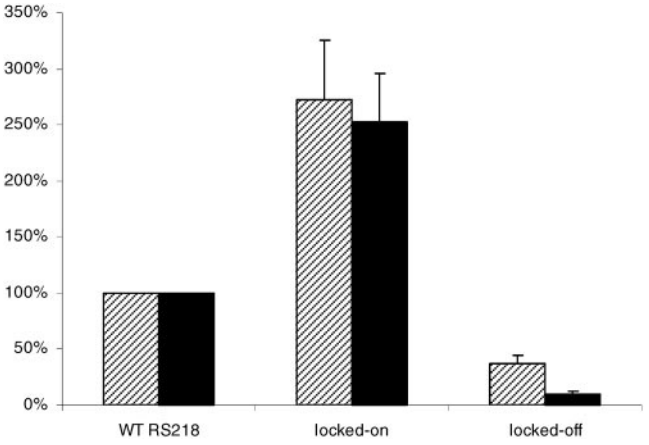

FIG. 4. The locked-on mutant exhibited significantly higher association and invasion rates than the wild-type RS218, while the locked-off mutant showed significantly lower invasion and association rates than the wild-type RS218. Hatched and filled bars indicate relative association and invasion rates, respectively, compared with the wild-type strain, which was set at 100%. The invasion and association frequencies of the wild-type RS218 were 0.7% ± 0.09% and 19% ± 3% of the original inocula, respectively. Data shown are representative of three independent experiments done in triplicate. Results are shown as means ± standard deviations of the triplicate. WT, wild type.

Type 1 fimbriated bacteria are capable of aggregating yeast cells through their ability to bind mannosylated glycoproteins present on the surface of yeast cells. The yeast aggregation assays illustrate the level of type 1 fimbria piliation (27), and mannose is shown to abolish the aggregation. We compared the yeast aggregation titers among the wild-type strain and the mutants which were grown overnight in BHI. The locked-on mutant showed twofold-higher aggregation compared to the wild-type strain, while the locked-off mutant was incapable of any yeast aggregation (Table 2). The yeast aggregations by the wild-type and locked-on mutant strains were abolished by addition of 50 mM of mannose (methyl α-D-mannopyranoside), confirming that the aggregations are due to type 1 fimbria piliation. These results are correlated with the percentages of type 1 fimbria piliated cells in the populations of these strains.

OmpA protein is the *E. coli* K1 determinant that was shown to be important for bacterial binding to and/or invasion of BMEC (8, 10). To investigate whether the type 1 fimbria expression affects the expression of OmpA, Western blot analysis of the whole-cell lysates of the phase-locked mutants, the RS218-Δ*fimH* isogenic mutant, and the wild-type strain with anti-OmpA antibodies was performed. The OmpA expression levels among these strains showed no significant difference (Fig. 3C).

The phenotype of the type 1 fimbria locked-on and locked-off mutants in binding to and invasion of HBMEC. The phase-locked mutants were examined for their association and invasion rates compared with the wild-type RS218. The locked-on mutant exhibited 273% ± 53% and 253% ± 43% of association and invasion frequencies of the wild-type strain, respectively (Fig. 4). Similar to the RS218-Δ*fimH* isogenic mutant, the locked-off mutant exhibited 37% ± 7% of association frequency of the wild-type strain and showed only 10% ± 2% of invasion frequency of the wild type (Fig. 4). These findings

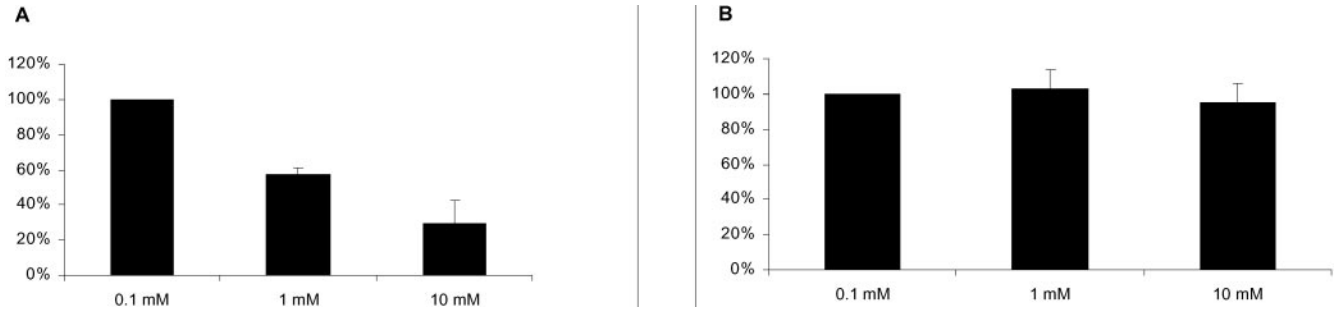

FIG. 5. Mannose (methyl α-D-mannopyranoside) dose dependently decreased association with HBMEC of the locked-on mutant (A) but did not affect the association of the locked-off mutant (B). The association rates are presented as percentages of the rates obtained with 0.1 mM of methyl α-D-mannopyranoside, which was set at 100% arbitrarily. The association frequencies of the locked-on and locked-off mutants in 0.1 mM of methyl α-D-mannopyranoside were 28% ± 2% and 8% ± 1% of their original inocula, respectively. Results shown are representative of three independent experiments done in triplicate. Results are shown as means ± standard deviations.

suggest that rates of HBMEC association and invasion are directly correlated with proportion of type 1 fimbriated bacteria in the bacterial inocula. We next examined whether increased and decreased invasion frequencies observed with the locked-on and locked-off mutants are secondary to their effects on HBMEC association by estimating invasion index (relative invasion rate/relative association rate). The values of invasion index for the locked-on, locked-off, and RS218-Δ*fimH* isogenic mutants were 0.93, 0.27, and 0.21, respectively (the ratio of the wild-type RS218 is arbitrarily set at 1). The index of the locked-on mutant is close to that of the wild type, suggesting that in comparison with the wild-type strain the increased invasion rate is likely due to the increased binding ability of this mutant. In contrast, the locked-off mutant and the RS218-Δ*fimH* isogenic mutant showed significantly lower values, suggesting that the decreased invasion rates of both mutants may be due to decreased binding ability as well as their defects in invasion ability.

Mannose treatment decreases association of the locked-on mutant but not the locked-off mutant with HBMEC. To examine whether the different invasion and association frequencies of the phase-locked mutants are related to type 1 fimbria piliation, we performed association assays of the two mutants under different concentrations of mannose. Mannose (methyl α-D-mannopyranoside) exhibited a dose-dependent decrease in the association rates of the locked-on mutant with HBMEC (Fig. 5A). In contrast, different concentrations of mannose did not significantly affect association rates of the locked-off mutant (Fig. 5B). In addition, with the addition of 10 mM of methyl α-D-mannopyranoside, the association level of the locked-on mutant became very close to that of the locked-off mutant (8.1% ± 1.1% of the original inoculum versus 7.5% ± 0.8% of the original inoculum, respectively). These results indicate that different association rates of the two mutants are mainly due to different levels of type 1 fimbria piliation.

Type1 fimbria phase-on bacteria are predominant in *E. coli* K1 RS218 cells associated with HBMEC. The above findings indicate that type 1 fimbriae are important for *E. coli* K1 to interact with HBMEC and that the type 1 fimbria locked-on mutant is more capable of binding and invading HBMEC than the locked-off mutant. We, therefore, speculated that the type 1 fimbrial phase-on bacteria are the major subgroup in the wild-type *E. coli* K1 that interact with HBMEC and performed the invertible element orientation assays to determine the percentage of cells whose *fim* promoters were in the "on" orientation in the wild-type *E. coli* K1 strain RS218 associated with HBMEC. RS218 was incubated with HBMEC for 90 min as described for the HBMEC association assays. The cell-associated bacteria and the free bacteria in the supernatant were collected and subjected to the invertible element orientation assay, as well as the total bacteria recovered after 90 min incubation with HBMEC. About 86% of HBMEC-associated bacteria had *fim* promoters which were found to be in the "on" orientation. The supernatant free bacteria and the total bacteria, which were collected after 90 min of incubation with HBMEC, showed only about 15% and 17%, respectively, of bacteria having *fim* promoters that were in the "on" orientation. In addition, type 1 fimbria piliation rates of the HBMEC-bound bacteria and unbound bacteria were assessed by using immunofluorescence microscopy with an anti-type 1 fimbria serum. Of the HBMEC bound-bacteria 91% ± 6% were piliated, while of the unbound bacteria only 11% ± 4% were piliated (Fig. 6). These findings indicate that the type 1 fimbriated bacteria are the major subgroup of *E. coli* K1 RS218 interacting with HBMEC.

DISCUSSION

E. coli K1 is the most common gram-negative bacterium that causes meningitis during the neonatal period. To cause meningitis, the pathogen needs to cross the blood-brain barrier. We have utilized the in vitro blood-brain barrier model comprised of HBMEC to study how the meningitis-causing pathogen traverses the blood-brain barrier. *E. coli* adhesion to HBMEC is considered an important first step for circulating *E. coli* to withstand the blood flow and cross the blood-brain barrier in vivo, but the microbial determinants contributing to *E. coli* adhesion to HBMEC are unclear. In the present study, we demonstrated for the first time that type 1 fimbriae contribute to the interaction between the meningitis-causing *E. coli* K1 and HBMEC and that the type 1 fimbria phase-on population is the predominant group interacting with HBMEC.

The RS218-Δ*fimH* isogenic mutant showed significantly lower adhesion and invasion rates in HBMEC compared with the wild-type RS218, and the mutant phenotypes were trans-complemented with a *fimH*-harboring plasmid. FimH is the

adhesin of type 1 fimbriae and has also been shown to be involved in the biosynthesis of type 1 fimbria (14, 15). This concept is consistent with our demonstration that very few type 1 fimbria piliated cells were found in the *fimH* deletion mutant population. These findings indicate that type 1 fimbriae play an important role in *E. coli* K1 strain RS218 adhesion to and invasion of HBMEC.

Type 1 fimbria expression is known to be phase variable, and the wild-type strain population is a heterogeneous mixture of type 1 fimbriated and nonfimbriated bacteria. The association and invasion frequencies of the wild-type *E. coli* K1 RS218 are, therefore, likely to be the net effect of the two populations. Bacteria alternate between these two states by switching the *fim* promoter between "on" orientation, which allows transcription of *fim* genes, and "off" orientation, which prevents expression of *fim* genes. It is impossible to keep the wild-type bacteria in either the fimbriated or nonfimbriated state during the host-bacterium interaction. To further assess the role of type 1 fimbriae in the interactions of RS218 with HBMEC, we constructed the type 1 fimbria phase-locked mutants to simulate the type 1 fimbria phase-on and phase-off bacteria of the wild-type strain. The phase-locked isogenic mutants were constructed by changing five out of nine nucleotides of the IRL sequence without interruption or deletion of genes, and their genotypes were confirmed with DNA sequencing and the invertible element PCR assay. After overnight cultivation the phase-locked mutants still showed the same genotypes, confirming the stability of these mutants. Western blot analysis of the phase-locked mutants to determine FimH expression level showed no FimH expression in the locked-off mutant. No type 1 fimbria piliation was detected on the locked-off mutant, and the mutant is incapable of yeast aggregation. The locked-off mutant, as expected, doesn't express FimH and type 1 fimbriae, since the *fim* promoter is not in the right orientation to allow transcription of *fim* genes. In contrast, the locked-on mutant consisted of about 97% type 1 fimbria piliation and exhibited twofold-higher yeast aggregation titer than the wild-type strain, which showed about 19% type 1 fimbria piliation. Western blot analysis with anti-FimH antiserum, however, revealed similar levels of FimH between the locked-on mutant and the wild-type strain. There are two possible explanations for different yeast aggregation and Western blot results between the locked-on mutant and the wild-type RS218. First, Western blot analysis may not be sensitive enough to differentiate the FimH protein levels between the wild-type RS218 and the locked-on mutant. Second, since FimH is only one of the minor components of type 1 fimbria structure (a copy of type 1 fimbria consists about 1,000 copies of the major subunit protein, FimA, while only a small amount of the adhesin, FimH, is needed for a fimbria [28]), FimH production in *E. coli* may not correctly reflect type 1 fimbria piliation on the cell surface. This second possibility is supported by our microarray results, which showed that the transcription level of the type 1 fimbrial major subunit gene, *fimA*, in HBMEC-bound bacteria was about twofold higher than that in the free/unbound bacteria, while the *fimH* transcription level in HBMEC-bound bacteria is only about 1.3-fold higher than that in free bacteria. Thus, the expression level of FimA may be more representative of type 1 fimbria production in *E. coli*.

The locked-on mutant showed about 2.7- and 2.5-fold-higher association and invasion rates in HBMEC than the wild-type strain, while the locked-off mutant exhibited about 36% of the association and 7% of the invasion abilities of the wild-type strain in HBMEC. The difference in HBMEC association and invasion rates of the locked-on, the locked-off, and the wild-type strains may be related to the proportion of type

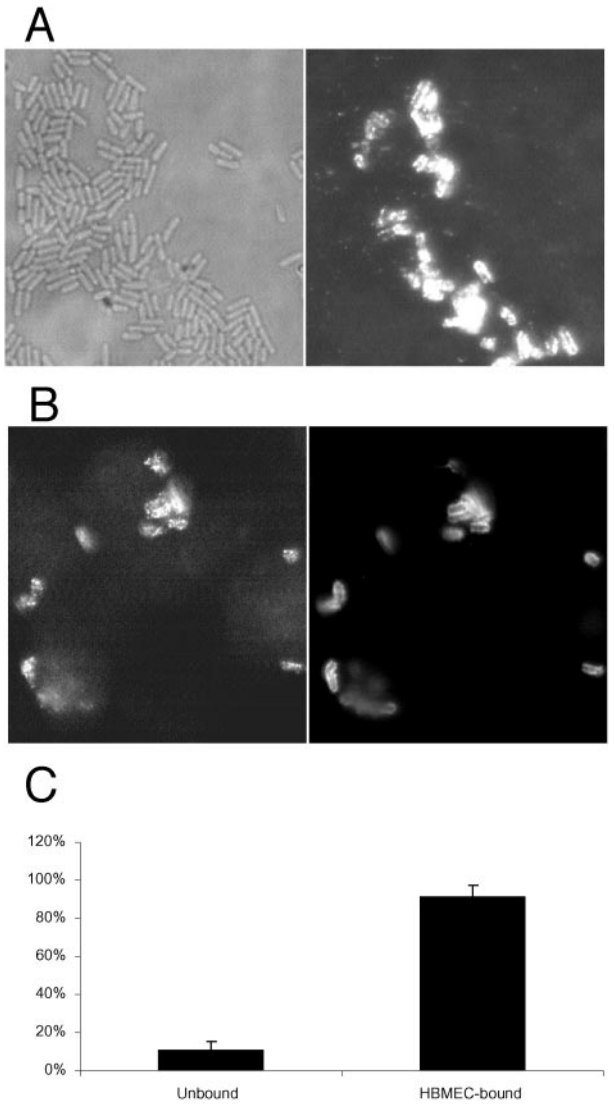

FIG. 6. Type 1 fimbria piliation of *E. coli* K1 RS218 interacting with HBMEC. (A) To assess type 1 fimbria piliation of unbound bacteria, the unbound bacteria were detected with light microscopy (left panel) and their type 1 fimbria piliation was detected with immunofluorescence microscopy with a rabbit antiserum directed against type 1 fimbria and fluorescein isothiocyanate-conjugated anti-rabbit IgG antibodies (right panel). (B) To asses type 1 fimbria piliation of HBMEC-bound bacteria, double immunofluorescence microscopy was performed. The HBMEC-bound bacteria were detected with anti-O18 antibody and rhodamine-conjugated anti-mouse IgG antibodies (left panel), and their type 1 fimbria piliation was detected as described in panel A (right panel). (C) The percentages of type 1 fimbria piliated bacteria in the unbound and HBMEC-bound bacteria population were determined. Data shown are the means ± standard deviations of three independent determinations from different fields of the microscope. One hundred bacteria were examined for each determination.

1 fimbriated bacteria present in the bacterial population at the time of association and invasion assays. Type 1 fimbriae specifically bind mannose, and addition of methyl α-D-mannopyranoside decreased the adherence to HBMEC of the locked-on mutant, not the locked-off mutant, suggesting that the difference in the adherence to HBMEC between the phase-locked mutants is mainly due to type 1 fimbrial expression. However, we cannot completely exclude the possibility that some other genes involved in *E. coli* K1 adherence to HBMEC may be affected in the phase-locked mutants. OmpA is shown to be an important determinant of *E. coli* K1 to bind and invade brain microvascular endothelial cells (8, 10). Our data indicated that the expression levels of OmpA protein did not differ among the wild-type RS218 and the phase-locked mutants. Thus, these different adherence rates of the locked-on and locked-off mutants for HBMEC are not related to different levels of OmpA expression.

The locked-on mutant was shown to have significantly greater invasion into HBMEC compared to the wild-type bacteria, while the locked-off mutant's invasion was significantly less than that of the wild-type bacteria. The difference in their invasion rates may stem from different binding and/or invasive abilities. We, therefore, compared the invasion index, calculated from relative invasive rate/relative association rate. The invasion index of the locked-on mutant was similar to that of the wild-type RS218, suggesting that the adherent locked-on mutant is as effective as the adherent wild-type strain in invading HBMEC and that the increased ability to invade HBMEC is mainly due to increased binding ability of the locked-on bacterial population.

In contrast, the locked-off mutant showed significantly lower invasion index than the locked-on mutant and the wild-type RS218. This finding suggests that the locked-off mutant has decreased binding ability for HBMEC but may also have the decreased invasion ability compared with the wild-type RS218 and the locked-on mutant. Type 1 fimbriae have been shown to activate neutrophils and mast cells (18, 34). Type 1-fimbriated *E. coli* is able to trigger phosphorylation of focal adhesion kinase, activation of phosphoinositide 3-kinase, and complex formation between focal adhesion kinase/phosphoinositide-3 kinase and vinculin/α-actin when invading bladder epithelial cells (19). These studies reveal that type 1 fimbriae are capable of triggering intracellular signaling in mammalian target cells. Type 1 fimbriae of *E. coli* K1 may also be able to stimulate intracellular signal transduction in HBMEC, which helps *E. coli* K1 internalize its host cell, and decreased invasion rate of the locked-off mutant may stem from the failure of type 1 fimbria-mediated intracellular signaling. Studies are in progress to determine whether type 1 fimbria-mediated intracellular signaling is involved in *E. coli* K1 invasion of HBMEC.

Our demonstrations of decreased association and invasion abilities of the RS218-Δ*fimH* isogenic mutant and different association and invasion rates with the phase-locked mutants indicate that type 1 fimbriae play an important role in *E. coli* K1-HBMEC interaction. Since expression of type 1 fimbriae is phase variable in wild-type *E. coli* K1, we hypothesized that, during the *E. coli* K1-host cell interaction, the type 1 fimbrial phase-on population is the dominant subgroup interacting with their target cells. To confirm this hypothesis, we used the invertible element orientation assay to determine the real-time percentage of cell population whose *fim* promoters were in the "on" orientation in the wild-type bacteria interacting with HBMEC. In comparison to only 17% of the population in the total bacteria collected after 90 min incubation with HBMEC and 15% of the population in the bacteria not associated with HBMEC having their *fim* promoters "on," the *fim* promoters of 86% of the HBMEC-associated bacteria were found to be in the "on" orientation. In addition, we demonstrated that about 91% of the HBMEC-bound bacteria expressed type 1 fimbria on the cell surface, but only about 11% of the unbound bacteria were type 1 fimbria piliated. These findings support the concept that the type 1 fimbria phase-on bacteria are the predominant subpopulation interacting with HBMEC.

In summary, our data demonstrated for the first time that type 1 fimbriae play an important role in adherence to and invasion of HBMEC, and the type 1 fimbria phase-on bacteria represent the predominant population interacting with HBMEC by *E. coli* K1 strain RS218. These findings imply that type 1 fimbriae are important in the pathogenesis of *E. coli* K1 meningitis. Studies are in progress to further elucidate the mechanism(s) involved in type 1 fimbria-mediated *E. coli* K1 binding to and invasion of HBMEC.

ACKNOWLEDGMENTS

This work was supported by NIH grants.

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Editor: J. N. Weiser