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Effects of *ompA* Deletion on Expression of Type 1 Fimbriae in *Escherichia coli* K1 Strain RS218 and on the Association of *E. coli* with Human Brain Microvascular Endothelial Cells

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We have previously shown that outer membrane protein A (OmpA) and type 1 fimbriae are the bacterial determinants involved in *Escherichia coli* K1 binding to human brain microvascular endothelial cells (HBMEC), which constitute the blood-brain barrier. In investigating the role of OmpA in *E. coli* K1 binding to HBMEC, we showed for the first time that *ompA* deletion decreased the expression of type 1 fimbriae in *E. coli* K1. Decreased expression of type 1 fimbriae in the *ompA* deletion mutant was largely the result of driving the *fim* promoter toward the type 1 fimbrial phase-OFF orientation. mRNA levels of *fimB* and *fimE* were found to be decreased with the OmpA mutant compared to the parent strain. Of interest, the *ompA* deletion further decreased the abilities of *E. coli* K1 to bind to and invade HBMEC under the conditions of fixing type 1 fimbria expression in the phase-ON or phase-OFF status. These findings suggest that the decreased ability of the OmpA mutant to interact with HBMEC is not entirely due to its decreased type 1 fimbrial expression and that OmpA and type 1 fimbriae facilitate the interaction of *E. coli* K1 with HBMEC at least in an additive manner.

Escherichia coli K1 is one of the major causes of neonatal meningitis (22). Despite advances in antimicrobial chemotherapy and supportive care, the mortality and morbidity associated with this disease have remained high, due to the incomplete understanding of the pathogenesis of this disease. For example, traversal of *E. coli* K1 across the blood-brain barrier (BBB) is required for the development of meningitis, but the underlying mechanisms involved in *E. coli* K1 traversal of the BBB remain incompletely understood. We have established an in vitro BBB model with human brain microvascular endothelial cells (HBMEC) to study the interaction of meningitis-causing bacteria, including *E. coli* K1, with the BBB (7, 11, 17, 33).

We have previously shown that the type 1 fimbria is an important determinant involved in *E. coli* K1 binding to and invasion of HBMEC (32). The type 1 fimbria is encoded in the *fim* gene cluster, which includes at least nine genes required for its biosynthesis (19). The fimbriae are composed of a polymer with the major subunit protein, FimA, and a tip structure containing FimF, FimG, and FimH (13). The adhesin, FimH, is responsible for the mannose-sensitive binding ability of type 1 fimbriae (8). The *fim* promoter, located in a 314-bp fragment (invertible element or *fim* switch) upstream of the major-subunit-encoding gene, *fimA*, controls the expression of type 1 fimbriae. The orientation of the 314-bp invertible element can be switched by site-specific recombination catalyzed by two recombinases, FimB and FimE, encoded upstream of this DNA fragment (14). By switching the orientation of the invertible element, the *fim* promoter can be directed toward the downstream *fim* structural genes to express type 1 fimbriae (phase ON) or toward the opposite direction to stop the type 1 fimbria expression (phase OFF) (1). Since type 1 fimbria expression is phase variable, wild-type *E. coli* organisms are a heterogeneous mixture containing both phase-ON and phase-OFF populations of type 1 fimbriae. We constructed type 1 fimbria locked-ON and locked-OFF mutants of *E. coli* K1 strain RS218, whose *fim* promoters were fixed in the "ON" and "OFF" orientations, respectively. The locked-ON mutant, which constitutively expresses type 1 fimbriae, exhibited significantly greater abilities to bind to and invade HBMEC than the wild-type strain, whereas the locked-OFF mutant, which exhibits no type 1 fimbria expression, showed significantly decreased abilities to bind to and invade HBMEC in comparison with the wild-type strain (32). These findings clearly indicate that type 1 fimbriae contribute to *E. coli* K1 binding to and invasion of HBMEC.

We have also shown that outer membrane protein A (OmpA), one of the major outer membrane proteins, is important in *E. coli* K1 RS218 binding to and invasion of HBMEC (9, 12, 27). In characterizing the role of OmpA in *E. coli* K1 binding to and invasion of HBMEC, we showed that deletion of *ompA* decreased the expression of type 1 fimbriae. The purposes of the present study were to characterize of the effect of *ompA* deletion on type 1 fimbria expression and to elucidate how OmpA contributes to *E. coli* K1 binding to and invasion of HBMEC, e.g., via its interaction with HBMEC and/or its effect on type 1 fimbriae.

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MATERIALS AND METHODS

Bacterial strains and culture condition. *E. coli* K1 strain RS218 (O18:K1:H7) is a cerebrospinal fluid isolate from a neonate with meningitis (2, 28). All bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown

TABLE 1. Strains used

Strain	Relevant information	Reference(s)
RS218	*E. coli* K1 isolated from cerebrospinal fluid of a neonate with meningitis	2, 27
Locked-ON mutant	RS218 mutant with *fim* promoter fixed in the ON orientation	31
Locked-OFF mutant	RS218 mutant with *fim* promoter fixed in the OFF orientation	31
ompA mutant	RS218 mutant with *ompA* deletion	This study
CHT059	Locked-ON mutant with *ompA* deletion	This study
CHT062	Locked-OFF mutant with *ompA* deletion	This study
CHT126	*ompA* mutant complemented with the *ompA* gene inserted in the *lacZ* locus	This study

at 37°C overnight statically in brain heart infusion broth unless otherwise specified.

Construction of mutants and complementation. The *ompA* deletions from the wild-type RS218 and from the type 1 fimbria locked-ON and locked-OFF mutants were constructed by using a PCR-based method as previously described (5, 32). The *ompA* deletion mutants in the locked-ON and locked-OFF backgrounds were designated CHT059 and CHT062, respectively (Table 1).

To insert the *ompA* gene into the *lacZ* locus of the *ompA* deletion mutant of *E. coli* K1 RS218, the primers OmpA-F and CM52 (Table 2) were designed to amplify the *ompA* gene and its promoter region. The suicide vector pJRins*lacZ*, which is a pCVD442 derivative, contains a *lacZ* gene (26), and the PCR product was cloned into the EcoRV site of the *lac*Z gene in the plasmid. The resulting plasmid, designated pR10, was then subjected to the allelic exchange procedure as described previously (3, 6). The resulting *E. coli* K1 strain was designated CHT126 (Table 1).

Microarray analysis of *ompA* deletion mutant gene expression profile. One milliliter of an overnight culture of the wild-type RS218 or its *ompA* deletion mutant was added to 9 ml of fresh LB broth and grown statically at 37°C for 1 h. The bacteria were collected and subjected to RNA extraction. The RNA samples were then subjected to *E. coli* microarray analysis as described previously (32).

Invertible-element orientation assay. The invertible-element orientation assay utilized the asymmetrical digestion site of SnaBI within the invertible element as previously described (32). A pair of primers, Ch1-F and Ch1-R (Table 2), were designed to amplify a 602-bp fragment containing the invertible element from both phase-ON and phase-OFF bacteria. When the invertible element is in the ON position, SnaBI digestion produces fragments of 404 and 198 bp, whereas for the OFF position, the restriction enzyme digestion results in 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 (16, 32), except that defined numbers of the type 1 fimbria locked-ON and locked-OFF bacteria were used as PCR templates. The intensity of each band of the DNA fragments was determined using the ImageJ program downloaded from the NIH website (http://rsb.info.nih.gov).

Yeast aggregation assay. Yeast aggregation titers of *E. coli* strains were measured as described previously (30, 32). Briefly, *E. coli* cultures at an optical density of 0.4 at 530 nm were subjected to serial twofold dilutions in phosphate-buffered saline (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.

Immunofluorescence labeling of bacteria. Type 1 fimbria antiserum was derived from immunizing rabbits with purified type 1 fimbriae as previously described (29, 32). To remove nonspecific antibodies, the antiserum was absorbed with the type 1 fimbria locked-OFF mutant of *E. coli* K1 RS218.

TABLE 2. Primers used

Primer	Sequence
CM52	CAGACGAGAACTTAAGCCTG
OmpA-F	GTAAATTTAGGATTAATCC
16S-F	CACATGCAAGTCGAACG
16S-R	CCTCTTTGGTCTTGCGA
FimB-F	CCTGACCCATAGTGAAATCG
FimB-R	CTTTGCCTTAAGATCAATATC
FimE-F	GGCATATCGGCATGGGATGC
FimE-R	CGTTCCTGGGTCCAGCGTTC
Ch1-F	AGTAATGCTGCTCGTTTTGC
Ch1-R	GACAGAGCCGACAGAACAAC

Surface presentation of type 1 fimbriae on whole bacteria was assessed by immunofluorescent microscopy with the absorbed type 1 fimbriae antiserum as previously described (20, 32), with a minor modification. Briefly, overnight bacterial cultures were harvested and washed once with PBS. Bacterial cells were fixed by mixing 200 μl of bacterial suspension 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 being washed three times with PBS, each slide was incubated with 25 μl of goat fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibodies at a dilution of 1:100 in PBS for 1 h at room temperature in a moist chamber and examined by fluorescence microscopy as described previously (32).

Immunodot blot assay. To assess the levels of type 1 fimbriae presented on the *E. coli* surface, immunodot blot assays were performed as previously described (4). Briefly, 1 ml of overnight bacterial culture was centrifuged, and the pelleted bacteria were resuspended with 100 μl of PBS. Five microliters of the bacterial suspension was spotted onto nitrocellulose membrane. After blocking with 2% bovine serum albumin in PBS, the membrane was subjected to antibody labeling. The rabbit type 1 fimbria antiserum was used as the primary antibody at a dilution of 1:2,000, and goat horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody was utilized as the secondary antibody at a dilution of 1:3,000. The signals were visualized with the Amersham ECL Western detection system (Amersham, Piscataway, NJ).

Real-time quantitative PCR (qPCR) analysis. Total RNA was extracted using a RiboPure-Bacteria kit (Ambion, Austin, TX) according to the manufacturer's instructions. Purified RNA was cleaned up and concentrated using the RNeasy minikit (QIAGEN, Valencia, CA) with on-column DNase treatment according to the manufacturer's instructions. The amount and quality of the RNAs were verified by measuring the absorbance at 260 and 280 nm and by native agarose gel electrophoresis.

Random-primed reverse transcription of RNA was performed with the SuperScript first-strand synthesis system for reverse transcription-PCR (Invitrogen, Carlsbad, CA), using 200 ng of RNA for each reaction. Samples without reverse transcriptase were concurrently prepared and analyzed to verify the absence of contaminating genomic DNA.

Real-time qPCR analysis was performed in a LightCycler system using the LightCycler FastStart DNA MasterPLUS SYBR green I kit (Roche Applied Science, Indianapolis, IN). The cDNA, obtained as described above, was diluted 10-fold in nuclease-free distilled water; 5 μl was used for qPCR. The PCR program consisted of one activation/denaturation step at 95°C for 10 min and 40 amplification/quantification cycles of 94°C for 10 s, 60°C for 5 s, and 72°C for 10 s, with signal acquisition at the end of each cycle. PCR amplification was followed by melting curve analysis to verify the identity of the PCR products and the absence of primer dimers. Amplification products were further verified by agarose gel electrophoresis.

For *fimB* qPCR analysis, a 157-bp PCR fragment was amplified using primers FimB-F and FimB-R (Table 2). For *fimE* qPCR analysis, a 170-bp PCR fragment was amplified using primers FimE-F and FimE-R (Table 2). Expression of *fimB* and *fimE* was normalized against the expression of the 16S rRNA; for the analysis of 16S rRNA expression, a 150-bp PCR fragment was amplified using primers 16S-F and 16S-R.

Efficiency-corrected, calibrator-normalized relative quantification was performed as described in Roche Applied Science technical note no. LC 13/2001. The Relative Expression Software Tool was used to analyze the statistical significance of the data by pairwise fixed reallocation randomization (21).

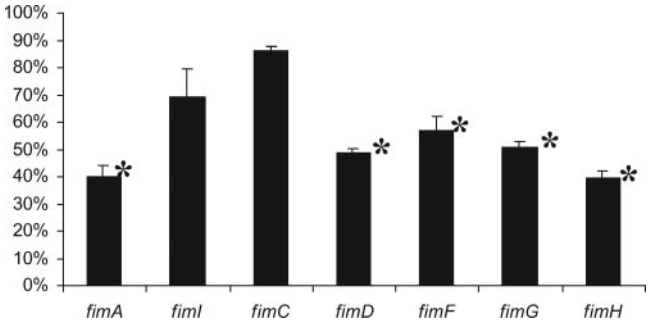

FIG. 1. Expression levels of the *fim* genes in the *ompA* isogenic mutant compared to those in the wild-type RS218. The expression levels in the *ompA* mutant are presented as percentages in comparison to those in the wild-type strain (which were set at 100%). Data are shown as means ± standard deviations from three independent experiments. *, $P < 0.01$ compared to the wild-type strain.

Assays of *E. coli* invasion of and association with HBMEC. 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. Invasion assays were performed using the gentamicin protection assay as previously described (32). Briefly, confluent monolayers of HBMEC grown in 24-well plate 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 PBS, incubated with medium containing gentamicin(100 μg/ml) for 1 h to kill extracellular bacteria, and then washed three times with PBS. HBMEC were lysed by incubation with sterile water at room temperature for 30 min. The released intracellular bacteria were enumerated by plating on sheep blood agar. The invasion rates were calculated by dividing the number of internalized bacteria by the number in the original inoculum. Results are presented as relative invasiveness, i.e., the percent invasion compared with the invasion rate of the wild-type RS218, which was arbitrarily set at 100%. Association assays were performed like the invasion assay described above except that the gentamicin treatment step was omitted.

RESULTS

Gene expression profiles of *E. coli* strain RS218 and its *ompA* mutant determined by microarray analysis. By using DNA microarray analysis, we examined whether expression levels of genes differ between the wild-type RS218 and its *ompA* isogenic mutant. The most striking difference was noted with the *fim* gene cluster, which showed significantly different levels of expression in the two strains. The expression levels of *fimA*, which encodes the major subunit of type 1 fimbriae, and *fimH*, which encodes the adhesin of type 1 fimbriae, in the *ompA* mutant were approximately 40% of those in the wild-type strain (Fig. 1). The mean expression levels of *fimI*, *fimC*, *fimD*, *fimF*, and *fimG* in the *ompA* mutant were 69%, 86%, 49%, 57%, and 51%, respectively, of those in the wild-type strain (Fig. 1). These results suggested that the *ompA* deletion significantly decreased the expression of *fim* genes compared to that in the wild type.

***ompA* deletion affects the orientation of the invertible element of the *fim* gene cluster.** Type 1 fimbria expression is phase variable, which is controlled by switching the orientation of the *fim* promoter-containing invertible element (1). To investigate whether the decreased expression of *fim* genes in the *ompA* deletion mutant was the result of affecting the orientation of the invertible element (or the *fim* promoter), we performed PCR-based invertible-element orientation assays, which utilize

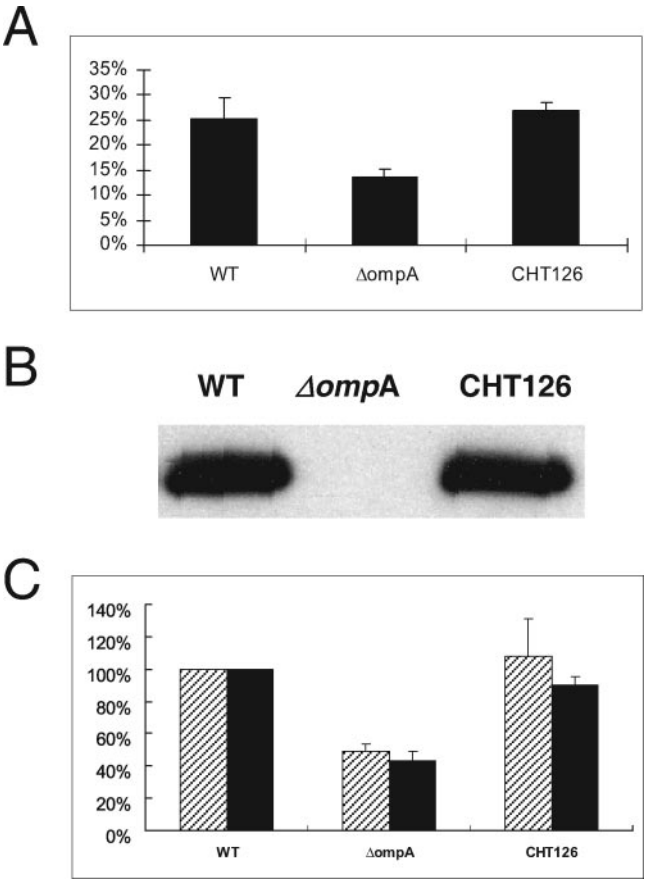

FIG. 2. (A) Invertible-element orientation assays show that *ompA* deletion preferentially directs the *fim* promoter to the phase-OFF orientation. The *y* axis indicates the percentages of the bacteria with *fim* promoter in the phase-ON orientation (mean ± SD). WT, wild type. (B) Western blot analyses of OmpA proteins from the total lysate of the wild-type RS218, the *ompA* mutant, and CHT126 with the anti-OmpA monoclonal antibody. Insertion of *ompA* into the chromosomal *lacZ* locus of the *ompA* mutant (CHT126) was able to restore the expression of OmpA to the wild-type level. (C) HBMEC association and invasion assays of the wild-type RS218, the *ompA* mutant, and CHT126. The association and invasion frequencies of CHT126 were restored to the levels of the wild type. Hatched and filled bars indicate relative association and invasion rates, respectively, compared with those of the wild-type strain, which were set at 100%. The *ompA* mutant and CHT126 exhibited 49% ± 5% and 108% ± 23% of the association frequency of the wild-type strain, respectively, while the same mutants showed 43% ± 6% and 90% ± 5% of the invasion frequency of the wild type, respectively. The invasion and association frequencies of the wild-type RS218 were 0.9% ± 0.09% and 21% ± 5% of the original inoculum, respectively. Data shown (means ± SDs) are representative of three independent experiments done in triplicate.

an asymmetric SnaBI restriction site within the invertible element to determine the orientation of the *fim* promoter (16). The invertible-element orientation assays of overnight cultures of the *ompA* mutant and the wild-type RS218 showed that the percentage of bacteria with *fim* promoters in the type 1 fimbria "phase-ON" position was significantly lower in the mutant than in the wild type (14% ± 2% in the *ompA* mutant and 25% ± 2% in the wild-type strain; $P = 0.001$) (Fig. 2A). We next estimated type 1 fimbria piliations on the surface of the bacteria by using immunofluorescence microscopy with anti-type 1

TABLE 3. Comparison of type 1 fimbria piliation and yeast aggregation among RS218 and its derivatives

Strain[a]	% of type 1 fimbria piliation (mean ± SD)[b]	Yeast aggregation titer[c]	
		Without addition of mannose	With addition of 50 mM mannose
Wild-type RS218	20 ± 5	16	—[d]
ompA mutant	7 ± 3	8	—
CHT126	22 ± 4	16	—
Locked-ON mutant	99 ± 1	32	—
CHT059	98 ± 1	32	—

[a] The strains were grown in brain heart infusion broth overnight with shaking at 200 rpm.
[b] Results are from three independent counts from different fields of the microscope; 100 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.

fimbria serum. In agreement with the results of the invertible-element orientation assays, the overnight culture of the *ompA* mutant exhibited a significantly lower percentage of type 1 fimbriated bacteria than the wild-type RS218 (7% ± 3% of the *ompA* mutant organisms versus 20% ± 5% of the wild-type RS218 organisms; $P = 0.019$) (Table 3). We also examined and compared type 1 fimbria expression by yeast aggregation assay, since 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 titer of the *ompA* mutant was twofold less than that of the parent strain (Table 3). These findings indicate that type 1 fimbria expression in the *ompA* mutant is significantly less than that in the wild-type culture.

We next complemented the *ompA* mutant by placing a new copy of the *ompA* gene with its upstream promoter region in the *lacZ* locus in the *ompA* mutant through plasmid-based allelic exchange, and the new strain was designated CHT126. OmpA expression in CHT126 was restored to the level in the wild-type RS218 (Fig. 2B). We have previously shown that the OmpA is an important bacterial determinant to facilitate *E. coli* K1 binding to and invasion of HBMEC (9, 12, 27). As shown in Fig. 2C, the decreased HBMEC association and invasion abilities of the *ompA* mutant were restored to the levels of the wild-type strain by complementation with the *ompA* gene (Fig. 2C). Of interest, the percentage of CHT126 organisms with the *fim* promoter in the phase-ON orientation (Fig. 2A), the expression of type 1 fimbriae on the surface of CHT126 (Table 3), and the yeast aggregation abilities of CHT126 (Table 3) were also restored to the levels of the wild-type RS218. These findings suggest that *ompA* deletion can preferentially switch the *fim* promoter toward the phase-OFF orientation, resulting in decreased expression of type 1 fimbriae.

Characterization of the effects of *ompA* deletion on the orientation of the *fim* promoter. To further characterize the effect of the *ompA* deletion on the *fim* promoter (or invertible-element) switching, a single colony of the *ompA* mutant or the parent strain grown on blood agar plates was resuspended in LB broth and allowed to grow under static conditions for 48 h at 37°C. Samples were taken at various time points after 12 h of incubation, and the percentages of bacteria whose *fim* promoters were in the phase-ON orientation were measured by the invertible-element orientation assay. The bacteria grown on the blood agar plates were, as expected, predominantly type 1 fimbria phase OFF (18). As shown in Fig. 3, the percentages of the phase-ON bacteria gradually increased for the parent strain and the *ompA* mutant during this experimental period (Fig. 3). However, after 16 h of incubation, the percentages of the phase-ON bacteria in the wild-type strain started to be significantly higher than those in the *ompA* mutant (27% ± 2% in the wild type versus 16% ± 3% in the *ompA* mutant at 16 h of incubation; $P = 0.006$), and the significant differences were maintained until 45 h of incubation.

The orientation of the invertible element is controlled by a site-specific DNA recombination catalyzed by two recombinases named FimB and FimE. We next compared *fimB* and *fimE* mRNA expression in the wild-type *E. coli* K1 RS218 versus its *ompA* deletion mutant by real-time qPCR analysis. Statistically significant differences between the wild type and the *ompA* mutant were observed for both genes; the expression of *fimB* and *fimE* was 1.3-fold and 2.2-fold lower in the *ompA* mutant than in the wild-type strain, respectively. The *P* values, determined by the pairwise fixed reallocation and randomization test (21), were 0.045 and 0.014 for *fimB* and *fimE*, respectively.

***ompA* deletion affects type 1 fimbria expression mainly through *fim* promoter flipping.** The above data suggest that *ompA* deletion affects type 1 fimbria piliation through preferentially driving the *fim* promoter toward a phase-OFF orientation involving both FimB and FimE. However, these results cannot exclude the possibility that the *ompA* deletion might have affected other steps of type 1 fimbria biogenesis (for example, type 1 fimbria assembly or presenting the fimbriae to the bacterial surface). To address this question, we constructed an *ompA* deletion mutant in the type 1 fimbria locked-ON background (32); this new double mutant was designated CHT059. In the locked-ON mutant, 5 bp of the 5′-end flanking region of the invertible element has been changed to abolish site-specific recombination so that the *fim* promoter is fixed in the phase-ON orientation to allow constant type 1 fimbria

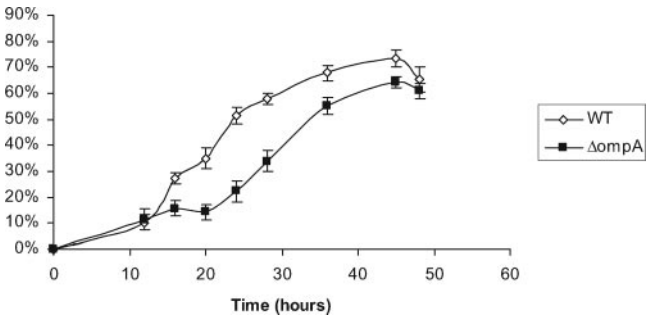

FIG. 3. Dynamics of *fim* promoter switching in the wild-type (WT) RS218 and the *ompA* mutant. The orientation of the *fim* promoter (invertible element) was determined with the invertible-element orientation assay as described in Materials and Methods. The *y* axis indicates the percentages of the bacteria with the *fim* promoter in the phase-ON orientation at different time points. Points represented the means of three separate measurements, and error bars show standard errors of the means.

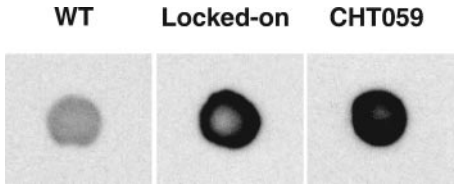

FIG. 4. Immunodot blot analysis of the wild-type (WT) RS218, the type 1 fimbria locked-ON mutant, and CHT059 (the *ompA* deletion mutant from the type 1 fimbria locked-ON mutant) with anti-type 1 fimbria polyclonal antibody.

expression (32). We assessed the percentages of type 1 fimbriated bacteria in CHT059 and the locked-ON mutant. Immunofluorescence microscopy with type 1 fimbria antiserum showed that almost all the bacteria of these two mutants were type 1 fimbriated (99% ± 1% in CHT059 and 98% ± 1% in the locked-ON mutant) (Table 3). The results with immunofluorescence microscopy suggest the percentages of type 1 fimbriated bacteria, not the total amount of the fimbriae presented on the surface of the bacterial population. To assess whether or not the total numbers of type 1 fimbriae presented on the surfaces of the two strains are similar, we performed immunodot blot assays with the type 1 fimbria antiserum. With the same numbers of bacteria, CHT059 and the locked-ON mutant showed similar densities of signals, while the wild-type RS218 showed considerably weaker signals (Fig. 4). In addition, we performed yeast aggregation assays of the two mutants to investigate the yeast aggregation abilities of their type 1 fimbriated population. The two mutants showed similar yeast agglutination titers (Table 3). Since the ability of type 1 fimbriae to aggregate yeast is through their adhesin protein, FimH, the results of the yeast aggregation assays suggest that deletion of *ompA* does not affect the proper presentation of the fimbriae on the bacterial surface in the type 1 fimbria locked-ON background. The above findings suggest that type 1 fimbria expression is similar in the type 1 fimbria locked-ON mutant and its *ompA* deletion mutant and that *ompA* deletion decreases type 1 fimbria expression mainly through the change in the *fim* promoter orientation (i.e., phase OFF).

Effect of decreased type 1 fimbria expression on the *ompA* deletion mutant's ability to bind and invade HBMEC. We have previously shown that both type 1 fimbriae and OmpA are involved in binding to and invasion of HBMEC (9, 12, 32). To investigate whether the *ompA* mutant's decreased abilities to interact with HBMEC are a result of its decreased percentage of type 1 fimbriated cells, we compared the HBMEC binding and invasion abilities of the wild-type RS218, the *ompA* mutant, the type 1 fimbria locked-ON mutant, and CHT059. The *ompA* mutant, the locked-ON mutant, and CHT059 exhibited 47% ± 7%, 650% ± 93%, and 283% ± 47% (mean ± standard deviation [SD]) of the association frequencies of the wild-type strain, respectively, while the same mutants showed 44% ± 6%, 590% ± 51%, and 271% ± 37% (mean ± SD) of the invasion frequencies of the wild-type, respectively (Fig. 5). Based on the above data, CHT059 exhibited about 44% (283%/650%) and 46% (271%/590%) of the association and invasion frequencies of the locked-ON mutant, respectively. As shown above, the locked-ON mutant and CHT059 expressed similar levels of type 1 fimbriae on their surfaces, but CHT059

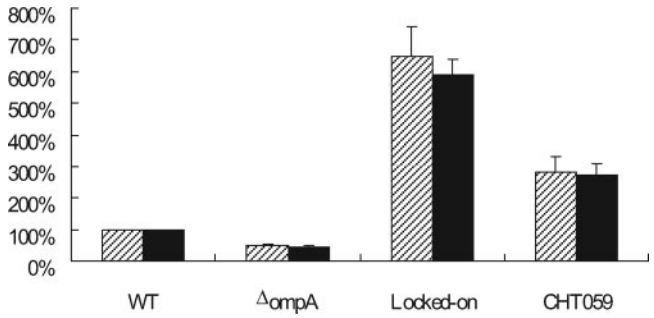

FIG. 5. HBMEC association and invasion abilities of the wild-type (WT) RS218, the *ompA* deletion mutants, the type 1 fimbria locked-ON mutant, and CHT059 (*ompA* deletion mutant in the type 1 fimbria locked-ON background). Hatched and filled bars indicate relative association and invasion rates, respectively, compared with those of the wild-type RS218, which were set at 100%. The invasion and association frequencies of the wild-type RS218 were 0.78% ± 0.07% and 17% ± 3% of the original inoculum, respectively. Data shown (means ± SDs) are representative of three independent experiments done in triplicate.

still exhibited significantly lower abilities to interact with HBMEC than the locked-ON mutant. These findings suggest that the decreased interaction of the *ompA* deletion mutant with HBMEC may not be entirely due to its decreased type 1 fimbria expression.

Contributions of OmpA and type 1 fimbriae to *E. coli* binding to and invasion of HBMEC. Since both OmpA and type 1 fimbriae were shown to be important *E. coli* K1 determinants for binding and invasion of HBMEC and since the decreased HBMEC association of the *ompA* mutant is not entirely due to its decreased expression of type 1 fimbriae, we next examined whether the contributions of these two bacterial determinants to *E. coli* K1 binding to and invasion of HBMEC are independent of each other (e.g., additive). We constructed an *ompA* deletion derivative of the type 1 fimbria locked-OFF mutant and named it CHT062. The *fim* promoter of the type 1 fimbria locked-OFF mutant was fixed in the phase-OFF orientation so that the mutant is constitutively in the type 1 fimbria phase-

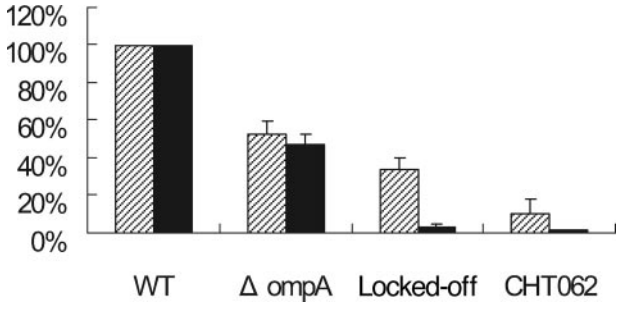

FIG. 6. HBMEC association and invasion abilities of the wild-type (WT) RS218, the *ompA* deletion mutants, the type 1 fimbria locked-OFF mutant, and CHT062 (*ompA* deletion mutant in the type 1 fimbria locked-OFF background). Hatched and filled bars indicate relative association and invasion rates, respectively, compared with those of the wild-type RS218, which were set at 100%. The invasion and association frequencies of the wild-type RS218 were 0.87% ± 0.06% and 19% ± 6% of the original inoculum, respectively. Data shown (means ± SDs) are representative of three independent experiments done in triplicate.

OFF state (32). We examined and compared the abilities of the double knockout (CHT062) and the single gene mutants (the *ompA* and locked-OFF mutants) to interact with HBMEC. The association rates (mean ± SD) of the *ompA* mutant, the locked-OFF mutant, and CHT062 were 53% ± 7%, 34% ± 6%, and 10% ± 7% of that of the wild-type RS218, respectively, while the invasion rates (mean ± SD) of these strains were 47% ± 5%, 3.4% ± 1.6%, and 1.27% ± 0.03% of that of the wild-type RS218, respectively (Fig. 6). The association and invasion abilities of the double mutant (CHT062) were significantly less than those of the single mutants (the *ompA* deletion mutant and the locked-OFF mutant). These findings suggest that OmpA and type 1 fimbriae contribute to *E. coli* K1 RS218 binding to and invasion of HBMEC at least independently of each other.

DISCUSSION

Most cases of *E. coli* meningitis develop as the result of hematogenous spread, but how circulating *E. coli* crosses the BBB remains incompletely understood. We have previously shown that successful traversal of the BBB by circulating *E. coli* requires a high degree of bacteremia, *E. coli* binding to and invasion of HBMEC, and traversal of the BBB as live bacteria (11, 12). We have identified several microbial determinants contributing to the above-mentioned steps of microbe-host interactions that are involved in the development of *E. coli* meningitis (11, 12). For example, we have shown that two *E. coli* determinants, OmpA and type 1 fimbriae, contribute to binding of *E. coli* K1 strain RS218 to HBMEC, which subsequently affects *E. coli* invasion of HBMEC (9, 12, 32), but it is unclear how OmpA and type 1 fimbriae contribute to the same phenotype, i.e., *E. coli* binding to HBMEC. In characterizing the role of OmpA in *E. coli* K1 interaction with HBMEC, we demonstrated that deletion of the *ompA* gene decreased the expression of type 1 fimbriae, thus creating a possibility that the decreased binding and invasion abilities of the *ompA* mutant may stem from its decreased expression of type 1 fimbriae.

We initially showed by *E. coli* DNA microarray analysis that the mRNA levels of the genes involved in type 1 fimbria biosynthesis were significantly lower in the *ompA* deletion mutant than in the parent *E. coli* K1 strain RS218. These findings suggest that *ompA* deletion may affect type 1 fimbria expression. Since the expression of type 1 fimbriae is phase variable, controlled by the switching of the *fim* promoter, we compared the percentages of the bacteria whose *fim* promoter is in the ON orientation between the *ompA* deletion mutant and the parent strain. The percentage of the bacteria with the *fim* promoter in the ON orientation was significantly lower in the *ompA* mutant than in the parent strain. The fact that the *fim* promoter of the *ompA* mutant is more preferentially orientated in the OFF direction compared to the parent strain is consistent with our finding of decreased *fim* expression in the *ompA* mutant. The lower type 1 fimbriation in the *ompA* mutant was also confirmed by immunofluorescence microscopy with the anti-type 1 fimbria serum and by yeast aggregation assays (Table 3).

To assess whether the surface presentation of the fimbriae was affected by the *ompA* deletion, we constructed an *ompA* deletion in the type 1 fimbria locked-ON background (strain CHT059), in which the *fim* promoter is fixed in the phase-ON orientation and the orientation of the *fim* promoter will not be affected by the deletion of *ompA*. Immunofluorescence microscopy and immunodot blot analysis as well as yeast aggregation assays revealed that the levels of type 1 fimbriae presented on the surface of strain CHT059 are similar to those of the locked-ON mutant (Table 3 and Fig. 4). We therefore conclude that the effect of *ompA* deletion on type 1 fimbria expression is mainly through the *fim* promoter switch.

It is known that the inversion of the type 1 fimbrial switch is a site-specific recombination process that is dependent upon the recombinases FimB and FimE (14). FimB is able to switch the orientation of the promoter in both directions, from ON to OFF or from OFF to ON, while FimE can switch the orientation of the promoter only from ON to OFF. The real-time PCR analysis revealed that the *fimB* and *fimE* mRNA levels of the *ompA* deletion mutant are significantly decreased in comparison with those of the parent strain. It remains to be determined whether these decreased levels of the *fimB* and *fimE* mRNAs are related to OFF orientation of the *fim* promoter in the *ompA* deletion mutant and also how *ompA* deletion affects the levels of the *fimB* and *fimE* mRNAs. Studies are in progress to determine whether *ompA* deletion affects known regulators of *fimB* and *fimE* such as leucine response regulatory protein (Lrp), integration host factor, and OmpR.

OmpA, a major outer membrane protein of *E. coli*, is important to maintain the integrity of the bacterial outer membrane structure. X-ray crystallography and nuclear magnetic resonance studies showed that the N terminus of OmpA is composed of eight transmembrane β-strands that are connected by three short periplasmic turns and four relatively large surface-exposed hydrophilic loops (15). We have demonstrated that preincubation of HBMEC with wild-type N-terminal OmpA protein, but not with OmpA with the loop deleted, decreased the *E. coli* K1 association with HBMEC in a dose-dependent manner (27). However, N-terminal OmpA alone could not fully explain the contribution of OmpA to *E. coli* K1 binding to HBMEC, because receptor-saturated concentrations of N-terminal OmpA did not decrease *E. coli* K1 binding to the level of the *ompA* deletion mutant. These findings suggest that the contribution of OmpA to *E. coli* K1 binding to HBMEC may include its binding to HBMEC as well as its effect on other binding structures of *E. coli*.

Because of the importance of type 1 fimbriae in *E. coli* K1 binding to HBMEC, we speculate that the HBMEC binding defect of the *ompA* deletion mutant might be in part through type 1 fimbriae, as the *ompA* deletion mutant exhibited decreased type 1 fimbriation compared to the parent strain. However, our data indicate that the contribution of OmpA to *E. coli* K1 binding to HBMEC may not be mainly dependent upon its effect on type 1 fimbria expression. For example, we examined whether the decreased binding and invasion abilities of the *ompA* mutant are related to its decreased type 1 fimbria expression by comparing the association and invasion abilities of strain CHT059 (the OmpA mutant derived from the locked-ON mutant) with those of the type 1 fimbria locked-ON mutant in HBMEC. Since both mutants constitutively express type 1 fimbriae, the influence of type 1 fimbria phase variation was removed, and as expected, the two strains exhibited similar levels of type 1 fimbria expression. However, the *ompA* dele-

tion mutant derived from the locked-ON background (CHT059) still exhibited significantly lower association and invasion rates than the locked-ON mutant. These findings suggest that decreased type 1 fimbria expression alone may not be fully responsible for the decreased abilities of the *ompA* deletion mutant to interact with HBMEC.

Since both OmpA and type 1 fimbriae were shown to contribute to *E. coli* K1 binding to HBMEC, we next investigated whether these two binding structures are additive in their contributions to HBMEC binding. For this purpose, we constructed the *ompA* deletion mutant from the type 1 fimbria locked-OFF background (CHT062). The association and invasion rates of CHT062 (the mutant without type 1 fimbria and OmpA expression) were significantly lower than those of the *ompA* deletion mutant and the locked-OFF mutant. Of interest, the HBMEC association and invasion defects with the locked-OFF mutant were significantly greater than those with the OmpA mutant (Fig. 6), suggesting that the contributions of type 1 fimbriae to HBMEC association and invasion are greater than those of OmpA. These findings are consistent with those of our previous studies, where the locked-OFF and *fimH* isogenic mutants were found to be defective in HBMEC association and invasion (32). It is, however, important to note that our previous studies have revealed greater defects in HBMEC invasion with the OmpA mutant (9), but these different results may stem from experimental variations, including use of different HBMEC and different OmpA mutants. These findings illustrate the importance of using the same experimental conditions for assessing and comparing the contributions of different *E. coli* structures to HBMEC association and invasion. Taken together, these findings indicate that the contributions of type 1 fimbriae and OmpA to *E. coli* K1 binding to and invasion of HBMEC are independent of each other and at least partially additive. These findings are consistent with our finding that despite similar levels of type 1 fimbria expression, the mutant derived by deletion of *ompA* from the locked-ON mutant exhibited significantly decreased association and invasion rates compared to the locked-ON mutant. We have previously shown that *E. coli* K1 association with and invasion of HBMEC involve several signaling pathways, such as phosphatidylinositol 3-kinase and RhoA (9, 10, 23, 24). We have shown that OmpA activates phosphatidylinositol 3-kinase in HBMEC, but not RhoA (9). Our preliminary data revealed that type 1 fimbriae activate RhoA in HBMEC (10a). Taken together, these findings suggest that OmpA and type 1 fimbriae contribute to *E. coli* K1 binding to and invasion of HBMEC in an additive manner, perhaps by using diverse signaling pathways.

In summary, we showed for the first time that the deletion of *ompA* decreased the expression of type 1 fimbriae by affecting the *fim* promoter orientation. The contribution of OmpA to *E. coli* K1 association with and invasion of HBMEC was, however, in part independent of type 1 fimbriae. We have previously shown that the OmpA mutant of *E. coli* K1 strain RS218 was significantly defective in penetration into the central nervous system (CNS) in vivo compared to the parent strain (34). We also showed that a high degree of bacteremia and binding to HBMEC are involved in *E. coli* K1 penetration into the CNS. Type 1 fimbriated *E. coli* has been shown to be less efficient in induction of a high degree of bacteremia (25) but has been shown to more efficient in binding to HBMEC (32). In addition, previous studies have shown that type 1 fimbriated *E. coli* was present in infant rat blood at 6 h after intraperitoneal administration of *E. coli* K1 (25). At present, the role of type 1 fimbriae in the pathogenesis of *E. coli* meningitis has not been clarified. Studies are in progress to determine whether or not the in vivo CNS penetration defect of the OmpA mutant is related to its effect on decreased expression of type 1 fimbriae.

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