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Role of Bacterial OmpA and Host Cytoskeleton in the Invasion of Human Intestinal Epithelial Cells by *Enterobacter sakazakii*

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ABSTRACT: *Enterobacter sakazakii* is an emerging pathogen in neonates and infants. Interactions of *E. sakazakii* with intestinal epithelium could be vital in the pathogenesis of enteric infections and in its systemic dissemination. The present study investigated the interaction of *E. sakazakii* with human intestinal epithelial (INT407) cells and the role of bacterial outer membrane protein A (OmpA) and host cytoskeleton in these interactions. *E. sakazakii* invaded INT407 cells with moderate efficiency. An *ompA*$^-$ mutant of *E. sakazakii* was significantly attenuated in its invasiveness, and complementation restored the invasive phenotype significantly. Drugs acting on host cell microfilaments (MF) and microtubules (MT) significantly inhibited bacterial invasion. Localization of both microfilaments (MF) and microtubules (MT) was observed in INT407 cells following *E. sakazakii* infection. The results suggest that *E. sakazakii* invasion of INT407 cells involves participation of both MF and MT and bacterial OmpA plays a critical role in invasion. **(*Pediatr Res* 62: 664–669, 2007)**

Enterobacter sakazakii is an opportunistic pathogen that causes an often fatal form of meningitis, meningoencephalitis, necrotizing enterocolitis, and sepsis in neonates and infants, with a case fatality rate of 14% (1–3). Besides the high rate of mortality, the brain infections due to *E. sakazakii* often lead to permanent impairments in mental and physical capabilities in surviving patients (4). Contaminated powdered infant formula represents the only known source of infection in neonates (3–6). A recent outbreak of neonatal infection caused by *E. sakazakii* (7) and the recall of *E. sakazakii*–contaminated infant formula preparations (8) in the Unites States have stimulated a renewed research interest on this pathogen.

Currently, very little information is available on the virulence factors of *E. sakazakii* and its pathogenic mechanisms. Some strains of *E. sakazakii* have been shown to produce an enterotoxin that is lethal in suckling mice and produce cytopathic effects in cell lines (9), however, no clear association between enterotoxin production and pathogenesis has been established. The most probable route of entry of *E. sakazakii* is the oral route since feeding of contaminated infant formula has been established as the only known link to infection in neonates. Therefore, the most likely primary colonization site of this pathogen is the intestinal tract, and stool colonization of *E. sakazakii* without overt signs of infection has been reported (7). To cause extraintestinal infections such as sepsis and meningitis, the pathogen has to break through the intestinal mucosa, gain access to the bloodstream, and survive the host defense mechanisms before gaining entry into the CNS. Fibronectin, a major glycoprotein component of the extracellular matrix of eukaryotic tissue (10) serves as an adherence target in the internalization process of many bacterial pathogens, including *Staphylococcus aureus*, *Streptococcus pyogenus*, and *Neisseria gonorrhoeae* (11–13). Our studies have identified that the outer membrane protein A (OmpA) of *E. sakazakii* binds fibronectin and facilitates the invasion of brain endothelial cells (unpublished results). This study reports the interaction of *E. sakazakii* with human intestinal epithelial cells and the role of its OmpA and host cytoskeleton in these interactions.

MATERIALS AND METHODS

This study was approved by the University of Connecticut Institutional Biosafety Committee.

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was used as host for plasmids. *E. sakazakii* and *E. coli* were grown in Luria-Bertani (LB) broth at 37°C with shaking. *E. coli* strains harboring the various plasmids were grown on LB agar and antibiotics were added when required at the following levels: ampicillin (100 μg/mL), chloramphenicol (cm; 25 μg/mL), streptomycin (100 μg/mL), and rifampin (100 μg/mL).

ompA mutation and complementation. Genomic and plasmid DNA were isolated using the AquaPure Genomic DNA isolation kit (Bio-Rad, Hercules, CA) and QIAprep spin miniprep kit (QIAGEN, Valencia, CA), respectively. Restriction endonuclease digestions, ligations, and transformations were performed as per standard protocols (14). Primers (OmpAORF-F2, 5′-GATAGTGGTGAATGAACGG-3′ and OmpAORF-R, 5′-GAGCGTAAAGAGAAGTATG-3′) designed based on *ompA* sequence of *E. sakazakii* 51329 (15) (GenBank Accession number *DQ000206*) were used to amplify the *ompA* ORF with flanking sequences from *E. sakazakii* strain ES4586. The PCR product was cloned in pGEM-T Easy (Promega, Madison, WI) yielding pG*ompA*. An internal 867-bp *Nru*I/*Bgl*II fragment of *ompA* was replaced with a 958-bp chloramphenicol acetyl transferase cassette (amplified from pACYC184 using primers CAT2F-*Bam*HI, 5′-CGC*GGATCC*GCGTATCACTTATTCAGGCGTAGC-3′ and CAT1R-*Sma*I, 5′-CATGGTA*CCCGGG*CCCTAAATACCTGTGACGGAAGAT-3′ and digested with *Bam*H1 and *Sma*I) to get pGΔ*ompA*::*cat*. The Δ*ompA*::*cat* construct was released from pGΔ*ompA*::*cat* by *Not*I digestion and ligated to *Not*I site on the suicide vector pKNG101 (16). The plasmid, pKΔ*ompA*::*cat* was transformed into *E. coli* SM10λpir and mobilized to ES4586 by conjugation. Rifampin- and cm-resistant colonies were selected for *ompA* deletion on LB plates with 5% sucrose. The sucrose-resistant colonies were screened for cm-resistance and streptomycin-sensitivity and were confirmed for mutation of the *ompA* gene by PCR, sequencing and Southern hybridization.

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Abbreviations: cm, chloramphenicol; **MF,** microfilaments; **MOI,** multiplicity of infection; **MT,** microtubules; **OMP,** outer membrane protein; **OmpA,** Outer membrane protein A

Table 1. *Bacterial strains and plasmids used in this study*

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. sakazakii		
ES4586	Enterocolitis isolate (rifampin^R)	3
ES5586	ES 4586Δ *ompA*::*cat*	*
ES6586	ES 5586 (pBBR-*ompA*)	*
E. coli		
SM10λ*pir*	*thi thr tonA lacY supE recA*::RP4-2-Te::MuKm^r	35
JM109	recA1 endA1 gyrA96 thi hsd R17 (rK–,mK+) relA1 supE44 Δ(lac-proAB) [F′ traD36 proAB lacIqZΔM15]	Promega
HB101	K-12 *supE44 ara-14 galK12 rpsL20* (Str^r)	Invitrogen
Plasmids		
pGEM-T Easy	PCR product cloning vector, Ap^r	Promega
pACYC184	General cloning vector, Cm^r	New England Biolabs
pKNG101	Sm^r mob$^+$ *sacBR*, R6K replicon	16
pG*ompA*	1593-bp PCR amplified *ompA* fragment inserted into pGEM-T Easy	*
pGΔ *ompA*	867-bp *Nru*I - *Bgl*II fragment deleted from ompA fragment of pG*ompA*	*
pGΔ *ompA*::*cat*	958-bp *cat* fragment inserted into *Nru*I - *Bgl*II site on pGΔ *ompA*	*
pKΔ *ompA*::*cat*	1684 bp *ompA*::*cat* inserted into *Not*I site of pKNG101	*
pBBR1-MCS4	Low copy broad range expression vector, Ap^R	17
pBBR-*ompA*	PCR amplified *ompA* coding sequence with promoter and terminator sequences cloned into pBBR1-MCS4 as an *XbaI* fragment	*

* This study.

For complementation, the *ompA* ORF along with the promoter and terminator sequences were amplified using primers OmpAORF-XbaF (5′-GCG*TCTAGA*GCGATAGTGGTGAATGAACGG-3′) and OmpAORF-XbaR (5′-GCG*TCTAGA*GCGGAGCGTAAAGAGAAGTATG-3′), digested with *Xba*I and cloned in the low copy expression vector pBBR1-MCS4 (17). The resultant plasmid, pBBR-*ompA* was transformed into *E. coli* SM10λpir and then mobilized to ES5586 (*ompA* $^-$) by conjugation. Expression of OmpA in the complemented strain (ES6586) was confirmed by Western blot using rabbit serum against *E. coli* OmpA (kind gift of Dr. Bruce Geller, Oregon State University, Corvallis, OR).

Outer membrane protein (OMP) extraction, SDS-PAGE, and Western blot. OMP were extracted from *E. sakazakii* strains by sodium sarcosinate treatment (18). One microgram of the OMP preparation from each strain was separated on 4% stacking–12% resolving SDS-PAGE gels at 200 V for 1 h (19) and visualized by a modified silver stain protocol (20). For Western blot, proteins from the SDS-PAGE gels were transferred to nitrocellulose membranes at 350 mA for 1 h (21). The membranes were incubated with rabbit anti-*E. coli* OmpA serum followed by peroxidase-labeled goat anti-rabbit immunoglobulin (Biomeda, Foster City, CA), and developed with α-chloronaphthol (Sigma Chemical Co.-Aldrich, St. Louis, MO).

Binding and internalization of* E. sakazakii *to human intestinal epithelial cells. Binding and internalization of E. sakazakii to intestinal epithelial cells were studied using INT407 cells [human embryonic intestine, ATCC, Manassas, VA; (22)]. Tissue culture trays (24-well) were seeded with 1.5×10^5 cells per well in Basal Medium Eagle (BME; Invitrogen, Grand Island, NY) containing 10% FCS at 37°C in a humidified and 5% CO_2 incubator for 24 h. Monolayers were rinsed with BME and inoculated with bacterial suspension in BME-10% FCS at different multiplicities of infection (MOI) ranging from 0.1 to 10,000. The tissue culture trays were centrifuged at 600 *g* for 5 min, and incubated at 37°C for 2 h. For binding assay, monolayers were rinsed five times with PBS, and lysed with 0.1% Triton X-100 for 15 min. The number of adherent cells was determined by serial dilution and plating of the suspension on tryptic soy agar. For internalization assay, the monolayers were rinsed three times in PBS and incubated for another 3 h in BME-1% FCS containing gentamicin (200 μg/mL). The number of internalized bacteria was determined as described in the binding assay. Wild type (WT), *omp*A$^-$, and complemented mutant strains were used for the study, and the percent invasion/binding was calculated using the formula (number of surviving bacteria divided by the number inoculated) × 100. The assays were run in triplicate and replicated three times.

The role of fibronectin in the invasion of INT407 cells by *E. sakazakii* was analyzed by pretreating the monolayers with rabbit anti-human fibronectin serum (Biomeda) at 37°C for 30 min, before inoculating with *E. sakazakii*. Monolayers incubated with rabbit preimmune serum served as controls.

Invasion in the presence of eukaryotic inhibitors. To study the role of eukaryotic cytoskeleton in *E. sakazakii* invasion of INT407 cells, invasion assays were performed in the presence of inhibitors of microfilaments (MF) and microtubules (MT) (Sigma Chemical Co.-Aldrich) (23). Cytochalasin D was incubated with INT407 cells for 30 min at 37°C and vinblastine and Taxol for 1 h at 37°C, before addition of *E. sakazakii*. Colchicine and nocodazole were incubated with intestinal cells for 1 h at 4°C followed by 30 min at 37°C before infection.

Immunofluorescence. The role of the eukaryotic cytoskeleton in the invasive process of *E. sakazakii* was visualized by immunofluorescence staining and confocal microscopy (24). INT407 monolayers grown on 12-mm coverslips were infected with ES4586 as described above. The coverslips were fixed in 2% paraformaldehyde at room temperature for 15 min and permeabilized with 0.5% Triton X-100 in PBS containing 1% goat serum (Sigma Chemical Co.; PBSG) for 15 min. Actin was stained with TRITC-labeled phalloidin (Sigma Chemical Co.; 0.5 μg/mL in PBSG, 1 h) and tubulin with mouse anti α-tubulin antibody (Sigma Chemical Co.; 1:1000 in PBSG, 1 h) followed by TRITC-labeled goat anti-mouse immunoglobulin (Sigma Chemical Co.; 1:64 in PBSG, 1 h). *E. sakazakii* cells were stained with rabbit anti-OmpA serum (1:1000 in PBSG, 1 h) followed by FITC–labeled goat anti-rabbit serum for 1 h. The coverslips were mounted on glass slides on Vectashield H-1000 (Vector Labs, Burlingame, CA) mounting medium, sealed with nail polish, and observed under a Leica true confocal scanner SP2 confocal microscope.

Statistical analysis. Data were expressed as mean values and SEM. For calculating the statistical significance ($p < 0.05$), two-tailed, unpaired *t* test was used.

RESULTS

Invasion efficiency. The effect of the number of bacteria in the inoculum on the invasive process of *E. sakazakii* was monitored by assessing the percentage invasiveness and the number of internalized bacteria per well against over a wide range of MOI from 0.1 to 10,000 bacteria per INT407 cell. The percentage invasiveness was found to decrease with an increase in the MOI, and a maximum invasiveness of 0.36 ± 0.06% was observed at an MOI of 0.1, which declined sharply with an increase in MOI (Fig. 1*A*). In the MOI range of 1 to 100, ES4586 exhibited a percentage invasiveness ranging between 0.26 ± 0.03 and 0.08 ± 0.002. However, the noninvasive strain *E. coli* HB101 showed a significantly ($p < 0.05$) lower invasiveness (0.008–0.004) at the same MOI range.

In contrast to the percentage invasiveness, the number of internalized bacteria per well increased with an increase in

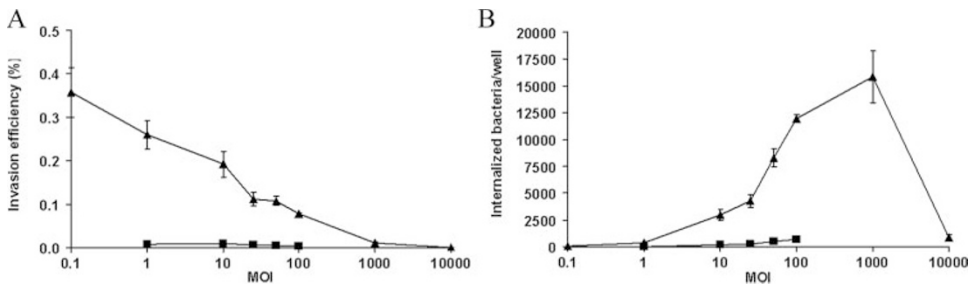

Figure 1. Effect of multiplicity of infection (MOI) on the invasive efficiency of *E. sakazakii* (▲) and *E. coli* HB101 (■) into INT407 cells expressed as (*A*) percentage invasiveness or (*B*) as the number of internalized bacteria per well (error bars indicate SE).

MOI from 0.1 to 1000, followed by a sharp decline at a MOI of 10000 (Fig. 1*B*). The maximum number of *E. sakazakii* was internalized (15,800 ± 2,418) at a MOI of 1000. The number of *E. coli* HB101 cells internalized remained significantly ($p < 0.05$) lower compared with ES4586 over a similar range of MOI. A MOI of 10,000 was found to produce rounding and detachment of a major portion of the monolayer from the wells, which could account for the sharp reduction in the number of internalized bacteria that was observed.

Effect of eukaryotic inhibitors on* E. sakazakii *invasion of Int407 cells. Role of MF. Cytochalasin D, an actin depolymerization agent was used to study the role of the actin cytoskeleton in *E. sakazakii* invasion. At the concentrations used, cytochalasin D caused rounding and retraction of epithelial cells without any detachment, and did not cause any significant changes in the viability of cells as evaluated by trypan blue staining (results not shown) compared with untreated controls. Cytochalasin D produced a dose dependent reduction in the invasiveness of *E. sakazakii* into INT407 cells (Fig. 2*A*) with approximately 93% inhibition observed at a concentration of 2 μM. *Salmonella Typhimurium* H3380 used as a positive control demonstrated 94% reduction in its invasiveness at the same concentration (Fig. 2*F*). However, the total number of bacteria associated with the epithelial cells did not vary significantly between the treated and untreated cells, indicating that the inhibitory effect is specifically at the level of invasion (results not shown).

Role of MT. The involvement of MT in *E. sakazakii* invasion was studied by conducting the invasion assays in the presence of different inhibitors of MT. Nocodazole, a MT depolymerization agent, produced a dose-dependent reduction in the invasiveness, which was not found to be statistically significant ($p > 0.05$) (Fig. 2*B*). At 20 μM, level nocodazole reduced *E. sakazakii* invasiveness by approximately 40%. However, 10 μM of each of the other two MT depolymerization agents, colchicine (Fig. 2*C*) and vinblastine (Fig. 2*D*), resulted in 40% and 72% reduction in *E. sakazakii* invasion, respectively ($p < 0.05$) compared with untreated controls. Similarly, the MT stabilizing agent, Taxol, at 20 μM level produced 44% reduction in invasiveness ($p < 0.05$; Fig. 2*E*). All of these chemicals induced retraction and flattening of the epithelial cells without any detachment indicative of MT disruption, when viewed microscopically (results not shown). Similarly, none of these treatments caused any significant changes in the viability of epithelial cells as evaluated by trypan blue staining (results not shown) compared with untreated controls. Moreover, none of the inhibitors, at the greatest concentrations tested produced any significant reduction in *S. Typhimurium* invasiveness into INT407 cells (Fig. 2*F*). DMSO was used as a solvent for the cytoskeleton inhib-

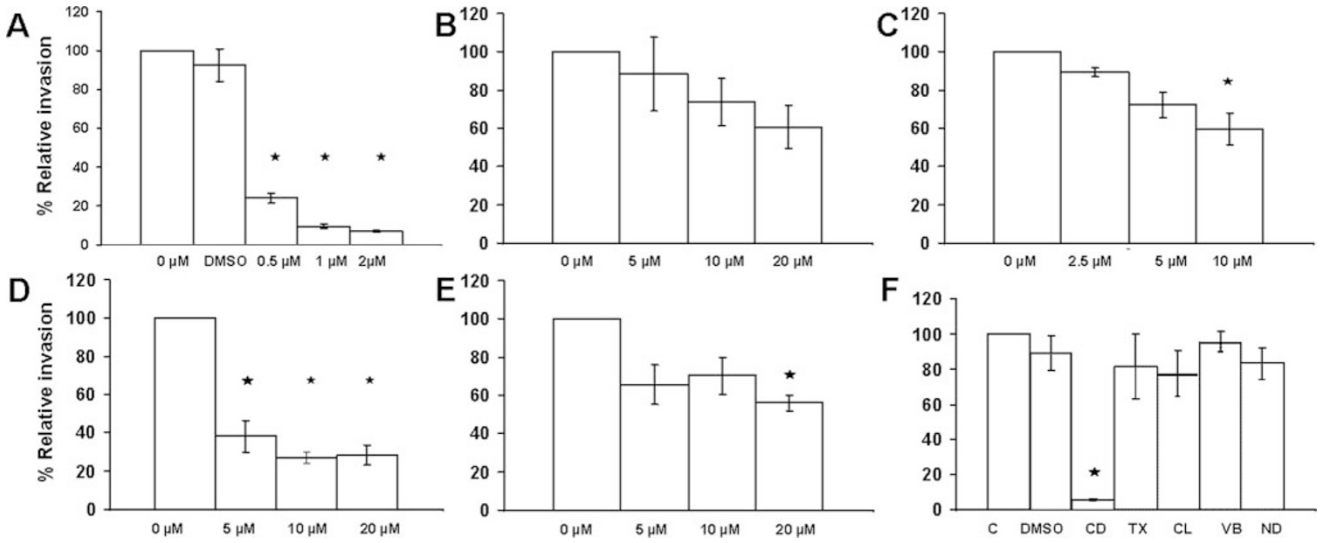

Figure 2. Effect of inhibitors of eukaryotic cytoskeleton on invasion of INT407 cells by (*A–E*) *E. sakazakii* and (*F*) *S. Typhimurium* H3380. Invasion assays using ES4586 were carried out in the presence of different concentrations of (*A*) cytochalasin D, (*B*) nocodazole, (*C*) colchicine, (*D*) vinblastine, or (*E*) Taxol. (*F*) Invasion assays using *S. Typhimurium* H3380 were carried out in the presence of no inhibitors (C), 0.2% DMSO (DMSO), 2 μM cytochalasin D (CD), 20 μM taxol (TX), 10 μM colchicine (CL), 20 μM vinblastine (VB), or 20 μM Nocodazole (ND). Error bars indicate SE. *$p < 0.05$.

itors, which at 0.2% did not exert any significant effect on the invasiveness of *E. sakazakii* or *S. Typhimurium*.

Fluorescence staining of actin and tubulin. Confocal and epifluorescence microscopy were used to visualize bacteria and MF or MT in infected epithelial cells. Uninfected monolayers showed brightly stained cortical actin at the apical surface and a dense accumulation of thick long filaments (stress fibers) at the basal surface. Uninoculated cells treated with cytochalasin D exhibited complete dissolution of the stress fibers (results not shown). Monolayers observed at 30 and 60 min postinfection revealed the presence of bacteria attached in groups or in numbers ranging from one to a few to a subset of epithelial cells. Actin condensation was observed directly beneath the adherent bacteria in epithelial cells (Fig. 3, *A* and *B*), although attached bacteria not associated with actin condensation was also observed. Intense fluorescence of actin condensation associated with attached bacterial cells was observed especially around the periphery of epithelial cells.

Similarly, *E. sakazakii* was found to interact with projections formed by bundles of MT extending from the epithelial cells (Fig. 4*A*) and *E. sakazakii* were found to co-localize with areas of MT polymerization in infected epithelial cells (Fig. 4, *B* and *C*). However, not all the bacterial cells were found to be associated with MT polymerization and areas of intense tubulin staining not associated with attached bacterial cells were observed.

Role of fibronectin in invasion by **E. sakazakii.** Blocking the INT407 monolayers by incubation with rabbit anti-human fibronectin serum (1:100) for 30 min resulted in 47% ($p < 0.05$) reduction in the invasiveness of ES4586. Under similar conditions, the invasiveness of the mutant strain declined by 28% ($p > 0.05$). Incubation with preimmune rabbit serum (1:100) was found not to exert any significant effect on the invasiveness of both mutant and WT strains. Pretreatment of monolayers with anti-fibronectin serum resulted in a significant ($p < 0.05$) reduction in the percentage of cells adhering to the monolayer for both the WT and mutant strains (30% and 33%, respectively) compared with untreated controls (results not shown).

ompA mutation and complementation. The *E. sakazakii ompA* was mutated by replacing a gene fragment corresponding to the N-terminus of the mature protein with a cm marker. PCR, Southern hybridization and nucleotide sequencing of the *ompA* region in the mutant strain revealed the internal deletion in the *ompA* gene and insertion of the cm marker at the point of deletion (results not shown). Further, SDS-PAGE and Western blot revealed lack of expression of an approximately 35 kD protein in the mutant strain, that corresponds to the OmpA in the wild type (Fig. 5). However, the expression of OmpA was restored in the complemented strain, as observed in SDS-PAGE and Western blot profiles (Fig. 5).

Role of ompA in **E. sakazakii** ***invasion of int407 cells.*** The ability of the WT, mutant and complemented strains to invade INT407 cells was compared (Fig. 6) in a gentamicin protection assay, where the different strains were inoculated at a MOI of 10. The invasiveness of the mutant, expressed as percentage relative invasiveness, was only 13% of that of the WT ($p < 0.05$). However, complementation of the mutant with *ompA* significantly restored the invasive phenotype resulting in 64% invasiveness compared with the WT. Under similar conditions, there were no significant differences ($p > 0.05$) in the total number of bacteria adhering to the monolayer between the WT, mutant, and complemented strains. This indicates that the reduced invasiveness of the mutant strain was a result of some defect in the invasive process rather than any failure of bacteria to associate with the monolayer. *E. coli* HB101, used as a negative control, was the least adhesive and invasive strain with 2% and 6% relative adhesiveness and invasiveness, respectively, compared with ES4586.

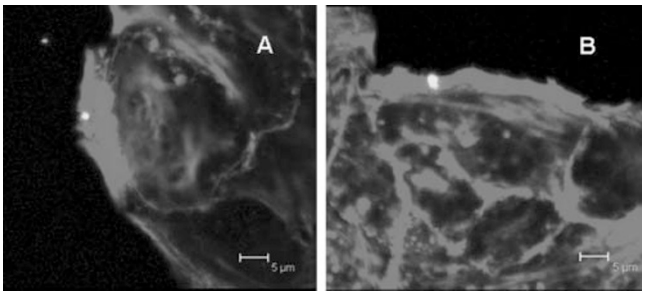

Figure 3. Representative confocal microscopic images of *E. sakazakii*–infected INT407 cells showing bacterial interactions with MF. Shown are overlays of pixels of light derived from FITC (fluorescing bacteria) and TRITC (fluorescing actin filaments) filters.

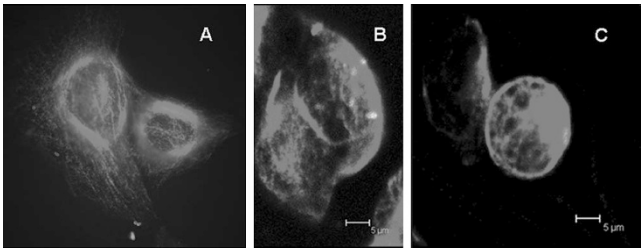

Figure 4. Representative (*A*) epifluorescence and (*B, C*) confocal microscopic images of *E. sakazakii*–infected INT407 cells showing bacterial interactions with MT. Shown are overlays of pixels of light derived from FITC (fluorescing bacteria) and TRITC (fluorescing tubulin filaments) filters.

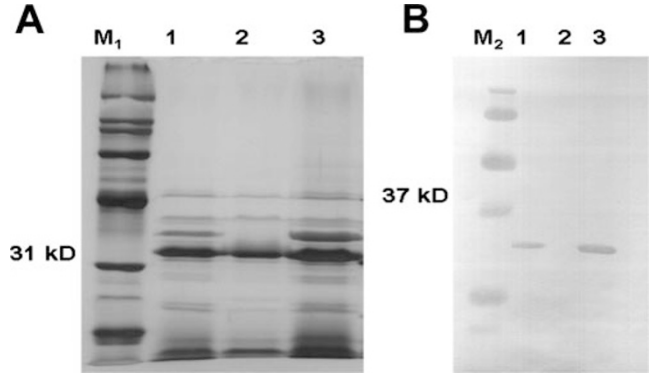

Figure 5. (*A*) SDS-PAGE and silver staining to detect OmpA expression. *Lane* M_1 , broad range molecular weight standard; *lane 1,* ES4586 (WT); *lane 2,* ES5586 (*ompA* $^-$); *lane 3,* ES6586 (complemented). (*B*) Western blot to detect OmpA expression. *Lane* M_2, prestained protein standard; *lane 1,* ES4586 (WT); *lane 2,* ES5586 (*ompA* $^-$); *lane 3,* ES6586 (complemented).

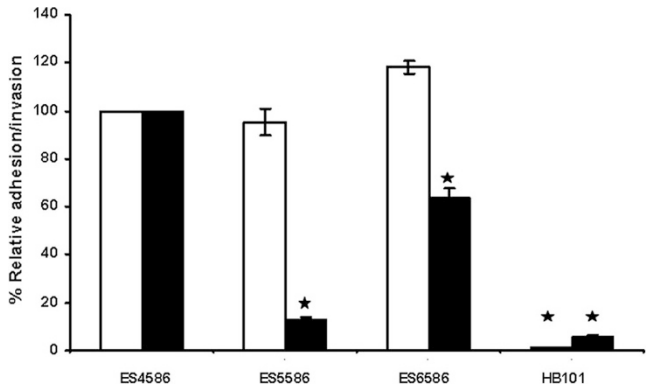

Figure 6. Percentage relative adhesion (*white bars*) and invasion (*black bars*) of ES4586 (WT), ES5586 (*ompA*$^-$), ES6586 (complemented) and *E. coli* HB101 (negative control) strains into INT407 cells. Error bars indicate SE. $^*p < 0.05$.

DISCUSSION

E. sakazakii is an opportunistic pathogen causing intestinal and extraintestinal infections in susceptible infants and neonates. The pathogenesis of these infections remains elusive and very limited information is available on the virulence factors involved. Since this pathogen is known to enter the body through the oral route, we undertook this study to closely examine the interaction between *E. sakazakii* and human intestinal epithelial cells using a cell culture model. A second objective of this study was to investigate the contribution of *E. sakazakii* OmpA and host factors such as the actin and tubulin cytoskeleton in these interactions.

In our study, *E. sakazakii* was found to invade intestinal epithelial cells, albeit with a moderate efficiency. Invasion of host epithelial cells is a critical step in the pathogenesis of many bacterial infections. Highly invasive pathogens such as *S. Typhi* were reported to invade INT407 cells with an invasive efficiency up to 60% in 60 min (25), whereas for moderately invasive pathogens such as *Campylobacter jejuni,* invasion efficiencies between 0.1 and 0.4% were reported (24). The moderate invasiveness (approximately 0.2%) of *E. sakazakii* observed is consistent with its pathogenic potential as an opportunistic pathogen. However, the invasiveness of *E. sakazakii* at a MOI of 10 (0.192 ± 0.029) was significantly greater (21-fold) than that of the noninvasive *E. coli* HB101 (0.0090 ± 0.0012). Invasion of intestinal cells could be an important step in the pathogenesis of intestinal infections and might aid in the translocation of *E. sakazakii* into the bloodstream from the intestinal tract, resulting in extra-intestinal infections such as sepsis and meningitis.

The exact role of fibronectin in the internalization process of *E. sakazakii* into intestinal epithelial cells is not clearly understood. Blocking fibronectin associated with the monolayer was found to significantly reduce both the total number of *E. sakazakii* attaching to the epithelial cells and invading them. Fibronectin-mediated binding could be a nonspecific first step where *E. sakazakii* comes in contact with the target cells, facilitating other specific interactions between bacterial ligands and host receptors. Alternatively, fibronectin could function as a bridge between a specific bacterial ligand and host cell receptor such as β1-integrin, leading to internalization in specific cell types, as observed in *S. aureus, S. pyogenes,* and *N. gonorrhoeae* (11–13).

Our findings have identified that *E. sakazakii* OmpA plays an important role in the invasion of INT407 cells. In the absence of OmpA expression, the invasiveness of *E. sakazakii* declined by 87%, whereas complementation of the mutant with *ompA* restored the invasiveness to 64% of that of the WT. However, the OmpA$^-$ mutant did not significantly differ from the WT in its ability to adhere to the monolayer. Diffused adhesion of *E. sakazakii* to human epithelial and brain microvascular cells was inhibited by mannose, suggesting the presence of mannose sensitive type I fimbriae (26). Moreover, genes encoding the putative type I and curli fimbriae have been detected in *E. sakazakii* (26,27). These and other yet unidentified surface determinants of *E. sakazakii* could also contribute to attachment to eukaryotic cells, making any potential OmpA-mediated adherence to host cells redundant.

Results of the present study clearly demonstrate that *E. sakazakii* invasion of INT407 cells involves participation of both MF and MT and bacterial OmpA plays a critical role in the invasion. Pathogens such as *Salmonella, Listeria monocytogenes*, *Shigella flexneri,* and *Yersinia* use an internalization pathway involving actin polymerization and the mechanisms behind their invasive processes are well understood (28). Pathogens such as *N. gonorrhoeae* and *Klebsiella pneumoniae* use uptake pathways that are both MF- and MT-dependent (29,30). In our study, the effect of MF inhibition on INT407 invasion by *E. sakazakii* was found to be more pronounced compared with that of MT inhibition. A similar trend has been observed in bacterial pathogens such as *K. pneumoniae* and *E. coli,* where bacterial invasion of eukaryotic cells was more markedly inhibited by drugs acting on MF than those acting on MT (30,31). This could be due to the existence of two independent pathways of internalization employing MF and MT simultaneously or due to the ability of MF to compensate the role of MT to some degree in the invasive process (30). Although the mechanisms behind MF-mediated bacterial internalization are fairly understood, the molecular basis of MT-dependant invasion is largely unknown. The effect of MT inhibitors could be an indirect effect on MF, as MT regulate the turn over of cells' focal adhesions, where the MF are cross-linked with membrane-associated adhesins (32). Alternatively, MT could be directly involved in *E. sakazakii* uptake or in intracellular trafficking or both. Inhibition of invasion by MT-stabilizing Taxol (which specifically blocks molecular motor such as dynein or kinesin-independent, MT pathway of internalization) (33) besides other MT-depolymerizers used, indicates that uptake through a direct MT-driven membrane invagination process is more likely. Further studies are required to elucidate the mechanisms behind involvement of MF and MT in *E. sakazakii* invasion. Similarly, the exact nature of interaction between OmpA and intestinal cells is not clear. *E. sakazakii* OmpA could function as a microbial ligand that up on interaction with a specific host receptor could trigger signaling events precipitating MF and/or MT rearrangements leading to bacterial internalization. OmpA of *E. sakazakii* shares a high level of sequence homology with that of *E. coli,*

both at the nucleotide and the amino acid levels (15). Specific interactions between the surface-exposed domains of *E. coli* OmpA and host cell receptors have been shown to be critical for actin rearrangements and invasion into human brain endothelial cells (34). The possibility of similar interactions between *E. sakazakii* OmpA and receptors on INT407 cells needs to be investigated to fully characterize the mechanisms behind OmpA-mediated invasion of *E. sakazakii* into intestinal epithelial cells.

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