

Outer Membrane Proteins A (OmpA) and X (OmpX) Are Essential for Basolateral Invasion of *Cronobacter sakazakii*[∇]

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***Cronobacter sakazakii* is an opportunistic pathogen that actively invades host eukaryotic cells. To identify invasion factors responsible for the intestinal translocation of *C. sakazakii*, we constructed for the first time outer membrane protein X (OmpX) and A (OmpA) deletion mutants using the lambda Red recombination system. The *ompX* and *ompA* deletion mutants showed significantly reduced invasion of human enterocyte-like epithelial Caco-2 and human intestinal epithelial INT-407 cells, and significantly fewer mutant cells were recovered from the livers and spleens of rat pups. Furthermore, compared with intact target cells, the invasion and initial association potentials of the mutants increased at a rate similar to that of the wild type in tight-junction-disrupted target cells, suggesting that OmpX and OmpA are involved in basolateral invasion by *C. sakazakii*. This is the first report of *C. sakazakii* virulence determinants that are essential for basolateral invasion and that may be critical for the virulence of *C. sakazakii*.**

Enterobacter sakazakii is an emerging pathogen associated with several outbreaks of meningitis and local necrotizing enterocolitis in premature infants (2, 28, 37). There was considerable diversity among *E. sakazakii* isolates (13, 14), and the original taxonomic name of *E. sakazakii* was reclassified as *Cronobacter* spp., which included *Cronobacter sakazakii* (13, 14). Therefore, *C. sakazakii* will be used throughout this paper. Although the incidence of *Cronobacter* infection is rare, the mortality rate is as high as 33 to 80% (11, 27, 32, 39). Even when infants survive *Cronobacter* infection, they often experience serious sequelae, including brain abscesses, developmental delay, and impairment of sight and hearing (8). Premature infants, whose immune systems are not fully developed, may be at high risk for *Cronobacter* infection (26).

Very little is known about the mechanisms of pathogenicity and the virulence determinants of the genus *Cronobacter*. Adhesion of *Cronobacter* spp. to eukaryotic cells showed two distinct patterns, i.e., a diffuse pattern and the formation of localized clusters, which was nonfimbrial (21). Pagotto et al. (29) reported that the genus *Cronobacter* produced enterotoxins and was lethal on intraperitoneal injection into suckling mice at levels as low as 10⁵ CFU per mouse. The genus *Cronobacter* interacts with and damages intestinal epithelial cells, which results in intestinal injury and villus disruption (12). In addition, the cell-bound zinc-containing metalloprotease encoded by *zpx* caused rounding of Chinese hamster ovary (CHO) cells (19), which may be important in dissemination of the pathogen into the systemic circulation. Further-

more, Townsend et al. (36) showed that *Cronobacter* can persist within rat macrophages.

As an oral pathogen causing a systemic infection, *C. sakazakii* must translocate from the intestinal lumen into the blood circulation. The genus *Cronobacter* is capable of actively invading various epithelial and endothelial cells of human and animal origin (17, 25, 31). Kim and Loessner (17) reported that the active invasion of human intestinal Caco-2 cells by *C. sakazakii* requires *de novo* bacterial protein synthesis and the host cell cytoskeleton and that the invasion efficiency of *C. sakazakii* was enhanced in the absence of cellular tight junctions. With regard to the virulence determinants related to *Cronobacter* penetration of the host cells, Mohan Nair and Venkitanarayanan (25) and Singamsetty et al. (31) reported that outer membrane protein A (OmpA) of *Cronobacter* plays an important role in the invasion of human intestinal epithelial INT-407 cells and human brain microvascular endothelial cells (HBMECs); invasion was dependent on both microfilaments and microtubules in INT-407 cells but only on microtubule condensation in HBMECs. Obviously, bacterial translocation in the intestines is multifactorial, and more detailed studies are needed to gain a better understanding of *C. sakazakii* pathogenesis.

Outer membrane protein X (OmpX) of *C. sakazakii* was identified in this study. Previously, OmpX in other bacteria was shown to be involved in the invasion of host cells (7, 18), neutralizing host defense mechanisms, and bacterial defense against the complement systems of the host (10, 38).

In this study, we report for the first time a successful application of the lambda Red recombination system to construct in-frame OmpX and/or OmpA deletion mutants in *C. sakazakii*. We further report that both outer membrane proteins (OMPs) of *C. sakazakii*, OmpX and OmpA, play critical roles in its invasion through not only the apical side, but also the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>C. sakazakii</i>		
ATCC 29544	Wild-type strain	16
ES1001	29544 harboring pKD46 (Ap ^r)	This study
ES2004	$\Delta ompA::kan$	This study
ES2005	$\Delta ompA$	This study
ES2006	pACYC184- <i>ompA</i>	This study
ES2007	$\Delta ompX::kan$	This study
ES2008	$\Delta ompX$	This study
ES2009	pACYC184- <i>ompX</i>	This study
ES2017	$\Delta ompX::kan \Delta ompA$	This study
ES2018	$\Delta ompX \Delta ompA$	This study
<i>E. coli</i>		
DH5 α	$\lambda^- \phi 80dlacZ\Delta M15 \Delta(lacZYA-argF)U169 recA1 endA1 hsdR17(r_{K^-} m_{K^-}) supE44 thi-1 gyrA relA1$	9
TOP10	F ⁻ <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC) \phi 80lacZ\Delta M15 \Delta lacX74 recA1 araD139\Delta\Delta(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG$	Invitrogen
Plasmids		
pKD13	<i>oriR6K</i> γ Ap ^r FRT Kan ^r FRT	5
pKD46	<i>oriR101 repA101(Ts) Ap^r araBADpgam-bet-exo</i>	5
pCP20	<i>oriPSC101(Ts) Ap^r Cm^r cI857λ P_Rflp</i>	5
pACYC184	Tet ^r Cm ^r p15A <i>ori</i>	5
pBAD202/D-TOPO	Kan ^r	Invitrogen
pBAD202- <i>ompA</i>	Harboring <i>C. sakazakii ompA</i> gene	This study
pBAD202- <i>ompX</i>	Harboring <i>C. sakazakii ompX</i> gene	This study
pWM1007	Km; pMW10 $\Delta lacZ W[(T1)_4-P_c-gfp-T1]$	23

^a Ap^r, ampicillin resistance; Kan^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance.

basolateral side, of the host cells. We also show that OmpX and OmpA are responsible for *C. sakazakii* translocation into the deeper organs (i.e., liver and spleen).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study are listed in Table 1. *C. sakazakii* and *Escherichia coli* were grown in tryptic soy broth (TSB) (Difco, Detroit, MI) and Luria-Bertani (LB) medium, respectively, at 37°C with constant shaking unless otherwise indicated. Strains containing the temperature-sensitive plasmid pKD46 or pCP20 were grown at 30°C. When antibiotics were required, chloramphenicol (Cm) was added at a final concentration of 25 μ g/ml and/or ampicillin (Ap), kanamycin (Km), and/or tetracycline (Tc) was added at 50 μ g/ml. After the mutants were obtained, the strains were grown at 42°C to induce deletion of the plasmids.

Construction of $\Delta ompA$, $\Delta ompX$, and $\Delta ompX \Delta ompA$ mutants using the lambda Red recombination method. The whole genome sequence of *C. sakazakii* BAA-894 was obtained from GenBank (accession number NC_009778) and used in this study (20). In-frame deletion mutants of *C. sakazakii* ATCC 29544 were generated by the lambda Red recombination method, as described by Datsenko and Wanner (5). Briefly, the kanamycin resistance (Km^r) cassette from plasmid pKD13 was amplified using the following primers: for *ompA* deletion mutant construction, *ompA-F* (5'-GTG AAG GAT TTA ACC GTG AAC TTT TCC CAA GGA AAA GCG C TG TAG GCT GGA GCT GCT TCG-3') and *ompA-R* (5'-AAA AAA CCC CGC CGT GGC GGG GTT TTT CTT AAC GTT TAA C AT TCC GGG GAT CCG TCG ACC-3'), and for *ompX* deletion mutant construction, *ompX-F* (5'-TTA AAT CTT AGG ACT TAC TTG AAG CAC ATT TGA GGT GGT T TG TAG GCT GGA GCT GCT TCG-3') and *ompX-R* (5'-AAA ATC CGC CCG TGG GCG GAT TTT TTC ATC ACC GAA GTG A AT TCC GGG GAT CCG TCG ACC-3'). (The nucleotide sequences originating from the *C. sakazakii* gene of interest are shown in italics, and those from pKD13 are underlined.) PCR was performed in a 50- μ l reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 200 μ M deoxynucleoside triphosphates (dNTPs), 2 U of DNA polymerase (τ Taq; Takara, Otsu, Japan), 0.25 μ M each primer, and 2.5 μ l of template DNA. The amplification reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal-cycling conditions consisted of a hold at 94°C for 10 min,

followed by amplification for 30 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 90 s and a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis in 1% agarose gels with 0.5 \times TAE buffer (10 \times TAE is 0.4 M Tris acetate, 0.01 M EDTA, pH 8.0). The gels were stained for 30 min in 0.5 \times TAE buffer containing 1 μ g/ml ethidium bromide and examined on a UV transilluminator.

The PCR products were transformed into the wild-type (WT) strain, *C. sakazakii* ATCC 29544, harboring the pKD46 plasmid, by electroporation (Gene Pulser; 2.5 V, 200 Ω , 25- μ F capacity; Bio-Rad, Hercules, CA) as described previously (17), and cells were selected for Km-resistant transformants, *ompA::Km* and *ompX::Km*. Finally, the kanamycin resistance cassette was removed using the pCP20 plasmid, as described by Datsenko and Wanner (5). The $\Delta ompX \Delta ompA$ double mutant was constructed using the $\Delta ompA$ mutant and the primers *ompX-F* and *ompX-R*.

Complementation study. To generate complementation strains, the complete *ompA* and *ompX* open reading frames (ORF) were amplified using the primer pairs *ompA* HindIII-F (5'-GCA TGG TGC ATT AAG CTT TTT TAG-3') and *ompA* SphI-R (5'-AGA GGG GCA TGC GGT TCT T-3') and *ompX* HindIII-F (5'-ACG GGG TAA GCT TGG CAT CCT-3') and *ompX* SphI-R (5'-AAT ACG GCG CAT GCG ATG CG-3'), respectively (nucleotide sequences in italics are artificially added restriction enzyme recognition sites). The thermal-cycling conditions consisted of a hold at 94°C for 10 min, followed by amplification for 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 5 min. The PCR products were digested with HindIII and SphI and cloned into a pACYC184 vector that had been digested with the same enzymes (3). The plasmid was then transformed into the mutant cells.

Cell culture and EGTA treatment. Human enterocyte-like epithelial Caco-2 (ATCC, Manassas, VA) and human epithelial INT-407 (ATCC) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Invitrogen), unless otherwise indicated. Trypsin-treated cells from up to six passages were seeded (approximately 5 \times 10⁴ cells per well) into 24-well cell culture plates (Sarstedt, Newton, NC) and grown at 37°C in the presence of 5% CO₂. The cells formed monolayers after 1 to 2 days and were cultured for up to 21 days, as needed. The medium was replaced every 2 days, and cell viability was determined by trypan blue staining.

For treatment with EGTA, Caco-2 monolayers were preincubated for 1 h with

DMEM containing 3 mM EGTA. The cells were washed once with phosphate-buffered saline (PBS) (pH 7.4) and used for further studies.

Invasion assay. To determine bacterial invasion of mammalian Caco-2 and INT-407 cells, a gentamicin protection assay was performed as described previously (17). Briefly, bacteria were prepared by transferring 2% inoculum from overnight cultures into fresh, prewarmed TSB, followed by incubation for 2 h at 37°C with constant shaking. *C. sakazakii* cells were collected by centrifugation at $13,000 \times g$, washed with PBS (pH 7.4), and resuspended in 1 ml of PBS for infection. Monolayer cells were infected with bacteria at a multiplicity of infection (MOI) of 100 and incubated for 1.5 h without subsequent centrifugation. After the cells were washed four times with PBS, fresh medium containing gentamicin (100 $\mu\text{g}/\text{ml}$; Sigma, St. Louis, MO) was added, and the plates were further incubated for 1.5 h, followed by five washes with PBS. Then, 1 ml of Triton X-100 (0.4% in PBS) was added, and the plates were incubated for a further 30 min before the bacteria were collected and plated on tryptic soy agar (TSA) in decimal dilutions. Bacterial invasion was expressed as the relative percentage of invasion, which was calculated using the following formula: (number of surviving bacteria/number of surviving WT) \times 100.

Adhesion assay. Bacterial adhesion to mammalian cells was performed as described previously (21). Briefly, *C. sakazakii* was prepared as described for the invasion assay and added to a mammalian cell monolayer at an MOI of 100. After 30 min of incubation, the cells were washed five times with PBS and treated with Triton X-100. Adherent *C. sakazakii* cells were counted.

Inhibition assay using purified OMPs. (i) **Overexpression of *C. sakazakii* OmpA and OmpX in *E. coli*.** To overexpress OMPs of *C. sakazakii*, the complete *ompA* gene was amplified using the primers *ompA*-TOPO-F (5'-C ACC ATG GCC TTT TTG GAT GAT AAC G-3') and *ompA*-TOPO-R (5'-AGC CTG CGG CTG AGT TAC AAC G-3'), and the complete *ompX* gene was amplified using *ompX*-TOPO-F (5'-C ACC ATG AAA AAA ATT GCA TGT CTT TCA G-3') and *ompX*-TOPO-R (5'-GAA GCG GTA ACC CAC ACC TGC-3'). The thermal-cycling conditions consisted of a hold at 94°C for 10 min, followed by amplification for 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 80 s, with a final extension at 72°C for 5 min. The PCR products were inserted into the TOPO recognition site of pBAD202/D-TOPO by a TOPO cloning reaction using *E. coli* TOP10 following the manufacturer's instructions (Invitrogen, Carlsbad, CA). The recombinant Omp proteins have His-Patch thioredoxin at the N-terminal end and a His₆ tag at the C-terminal end. Insertion of the complete gene was confirmed by sequencing using a BigDye terminator cycle-sequencing kit (Applied Biosystems) and an ABI Prism 3700 DNA analyzer (PerkinElmer, Wellesley, MA) at the National Instrumentation Center for Environmental Management (Seoul, South Korea). The activities of the recombinant Omp proteins were confirmed by complementation tests.

(ii) **Purification of *C. sakazakii* OMPs expressed in *E. coli*.** The overnight cultures of *E. coli* TOP10 harboring a plasmid expressing OmpA or OmpX were transferred to new LB medium, and 0.2% L-arabinose was added to the culture when the optical density at 600 nm (OD_{600}) reached 1.0. The induced bacterial cultures were further incubated for 2 h, recovered by centrifugation at $15,000 \times g$ for 5 min at 4°C, and suspended in Tris buffer (20 mM Tris-Cl, pH 7.4, 300 mM NaCl), which was then sonicated with an Ultrasonic Processor GE 130PB (Hielscher Systems, Teltow, Germany) with a 30-s pulse and a 30-s pause for 10 min. After centrifugation of the bacterial lysates at $16,000 \times g$ for 10 min at 4°C, the supernatants were decanted. The pellets were washed three times using Tris buffer, and OMPs were resolved using buffer A (50 mM CAPS [N-cyclohexyl-3-aminopropanesulfonic acid], pH 10.5). After centrifugation at $16,000 \times g$ for 10 min at 4°C, the supernatant was loaded onto a Ni-NTA superflow affinity column (Qiagen, Valencia, CA) and eluted using a gradient of combinations of buffer A and buffer B (50 mM CAPS, pH 10.5, 300 mM imidazole). Peak fractions were confirmed by SDS-PAGE, pooled, and dialyzed against buffer A. The protein concentration was determined by the Bradford method (1).

(iii) **Inhibition assay.** Various concentrations (5, 25, and 50 $\mu\text{g}/\text{well}$) of the Omp preparation were preincubated with Caco-2 cell monolayers for 1 h, followed by three washes with PBS. *C. sakazakii* adhesion and invasion were assayed as described above.

Preparation of crude membrane proteins of *C. sakazakii*. Outer membrane proteins were prepared as described previously (6), with modifications. Bacteria from overnight cultures were transferred to new LB medium and then grown until their OD_{600} reached 4.0. The bacterial cultures were recovered by centrifugation at $6,000 \times g$ for 10 min at 4°C, suspended in 1 ml of PBS (pH 7.4), and sonicated with an Ultrasonic Processor GE 130PB (Hielscher Systems, Teltow, Germany) with a 30-s pulse and a 30-s pause for 10 min. Cell debris was removed by centrifugation as described above, and 10 ml of the supernatant was added to 1 ml of 2% N-lauroylsarcosine (Sarkosyl; Sigma), followed by incubation for 1 h at room temperature. Crude outer membrane proteins were prepared by cen-

trifugation of the mixture at $100,000 \times g$ for 1 h. The pelleted protein was resuspended in 0.2 ml of PBS and stored at -80°C for further experiments. The protein concentration was determined by the Bradford method (1).

SDS-PAGE. Purified OMPs or crude membrane proteins of *C. sakazakii* were obtained as described above. Aliquots of 1 μg of purified OMPs or 10 μg of crude membrane proteins of *C. sakazakii* were prepared in a solution containing 0.05 M Tris-HCl (pH 8), 1.6% SDS, 25% glycerol, 5% 2-mercaptoethanol, and 0.003% bromophenol blue (Sigma), and the proteins were separated by SDS-PAGE in 5% stacking and 12% separating gels. The proteins were visualized by staining them with Coomassie brilliant blue R250 (Bio-Rad, Hercules, CA) and destaining them with a solution of 30% methanol and 10% acetic acid.

Confocal laser fluorescence microscopy. For the expression of green fluorescent protein (GFP), the plasmid pWMM1007 (23) was transformed into *C. sakazakii* strains by electroporation. The presence of the plasmid and cytosolic GFP did not affect the invasion properties of *C. sakazakii* (data not shown). For confocal laser scanning microscopy, round coverslips (diameter, 12 mm) were placed in the wells of a 24-well tissue culture plate and seeded with 10^4 Caco-2 cells, which were incubated for 24 h. After infection with *C. sakazakii* carrying pWMM1007 for 30 min and four washes with PBS, the coverslips were removed, and the cells were fixed with formaldehyde (3.7% in PBS; 15 min), washed three times with PBS, and permeabilized for 3 min with 0.1% Triton X-100. Rhodamine-labeled phalloidin (1:200 dilution of 0.1-mg/ml stock solution; Sigma) was added, and the cells were incubated for 20 min, followed by extensive washing with PBS. Fluorescence signals were recorded using an inverted confocal laser scanning microscope combined with fluorescence correlation spectroscopy (Carl Zeiss Microscopy, Jena, Germany). Different series of images from 0.4- μm x-y-z sections were captured, analyzed, and stacked using appropriate software.

In vivo animal study. Sprague-Dawley rat pups (SD rat), 2 to 3 days old, were purchased from the Institute of Laboratory Animal Resources, Seoul National University (Seoul, South Korea). Overnight cultures of *C. sakazakii* strains were collected and resuspended in PBS. Rat pups in each group ($n = 5$) were fed by oral gavage with 5×10^9 CFU of bacteria. For analysis of bacterial colonization in organs, all rats were euthanized with a mixture of ketamine and xylamine at 24 h postinfection. The spleens and livers were removed aseptically, homogenized in 1 ml of ice-cold PBS, and serially diluted. Bacterial loads were determined by plating on LB agar plates.

Statistical analysis. All experiments were performed at least in triplicate. Data from cell culture experiments were analyzed by unpaired *t* tests or one-way analysis of variance with Duncan's posttest at a 95% confidence interval using SAS software (version 9.1.3; SAS Institute, Cary, NC). Values of *P* below 0.05 were considered significant.

RESULTS

The lambda Red recombination method was successfully applied to *C. sakazakii*. To determine whether OmpA and/or OmpX expression is required for the invasion of mammalian cells by *C. sakazakii*, we used lambda Red and FLP/FLP recombination target (FRT)-mediated site-specific recombination to construct a series of deletion mutants in *C. sakazakii* ATCC 29544: ΔompA , ΔompX , and a $\Delta\text{ompX} \Delta\text{ompA}$ double deletion (Fig. 1A). The deletions in the target genes were confirmed by PCR with specific primers, which showed no amplification of the 1.4-kb *ompA* or 0.7-kb *ompX* genes in the respective mutants (Fig. 1B). In-frame deletion was also confirmed by nucleotide sequencing of the PCR product (data not shown). Furthermore, SDS-PAGE revealed lack of expression of OmpA (38-kDa) and OmpX (18-kDa) proteins, corresponding to the wild type, in the mutant strains (Fig. 1C). OmpA and OmpX were restored in complemented strains, as observed in SDS-PAGE profiles (Fig. 1C). The $\Delta\text{ompX} \Delta\text{ompA}$ double mutant was constructed using the ΔompA mutant as the parental strain and *ompX*-F and *ompX*-R primers for recombination and confirmed by PCR (data not shown).

OmpA and OmpX are required for invasion of host eukaryotic cells. The abilities of the WT, mutants, and complemented strains to invade Caco-2 and INT-407 cells were compared in

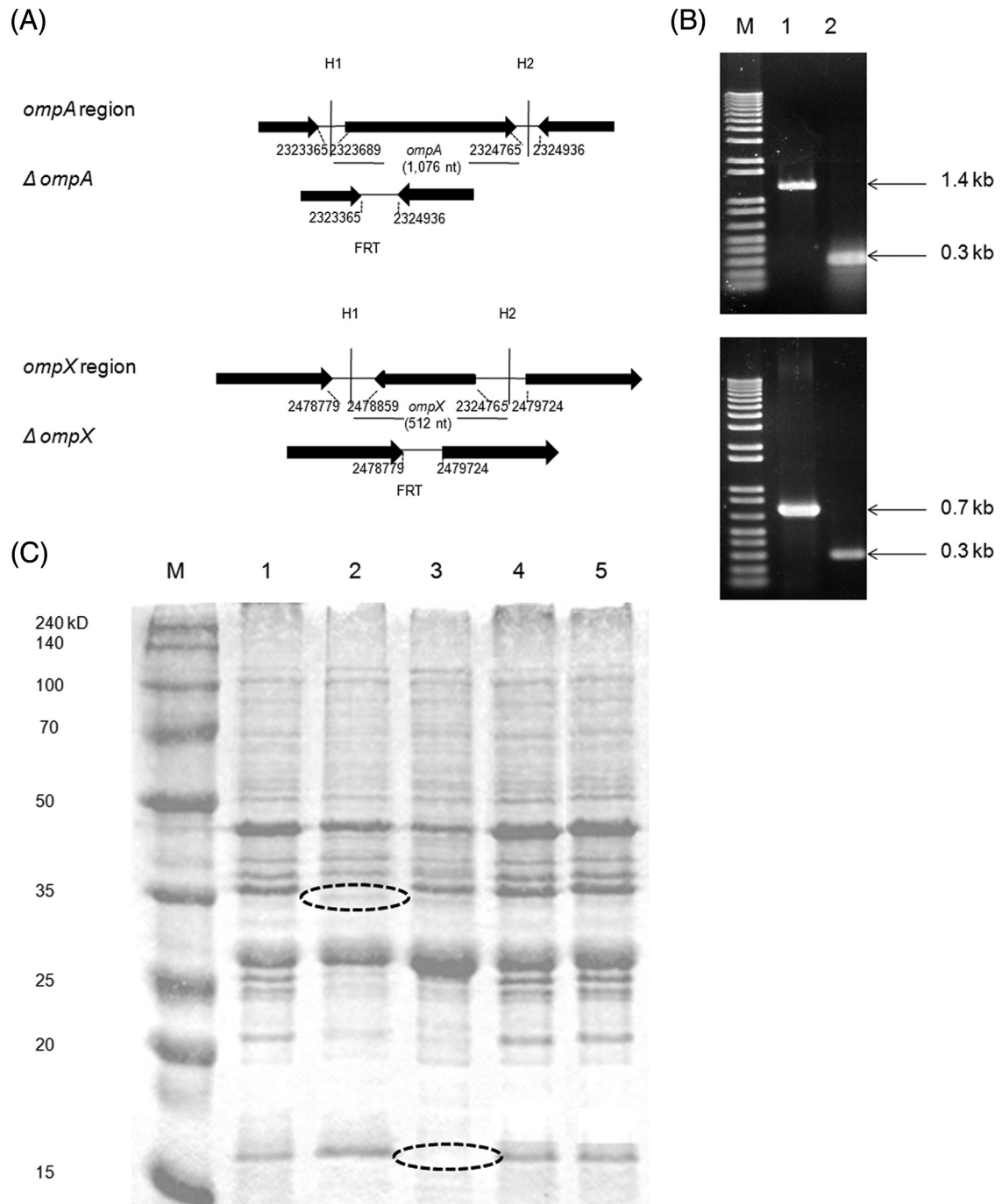


FIG. 1. Construction of $\Delta ompA$ and $\Delta ompX$ mutants with the lambda Red system. (A) Gene arrangement near *ompA* and *ompX* in *C. sakazakii* WT and the mutants. H1 and H2 indicate 40-nucleotide (nt) homology extensions. The nucleotide numbers are adopted from the *C. sakazakii* whole-genome database. (B) PCR confirmation of the mutants. Lane M, nucleotide size markers; lane 1, WT; lane 2, mutant. (C) Confirmation of mutant construction by SDS-PAGE of crude outer membrane proteins. Lane M, protein marker; lane 1, *C. sakazakii* ATCC 29544; lane 2, *ompA* deletion mutant; lane 3, *ompX* deletion mutant; lane 4, *ompA* complementation; lane 5, *ompX* complementation. The dashed ovals indicate the approximate positions of OmpA (38 kDa) and OmpX (18 kDa).

a gentamicin protection assay (Fig. 2). Compared with the WT, approximately 20% ($1.6 \times 10^4 \pm 4.9 \times 10^4$ CFU/well) of the $\Delta ompA$ mutant, 40% ($3.2 \times 10^4 \pm 1.7 \times 10^4$ CFU/well) of the $\Delta ompX$ mutant, and 17% ($1.26 \times 10^4 \pm 1.4 \times 10^4$ CFU/well) of the double mutant were able to penetrate Caco-2 cells. The

complementation strains showed restored invasion ability similar to that of the WT strain (Fig. 2A). The differences in invasion efficiency between the $\Delta ompA$ and $\Delta ompX$ mutants and between the $\Delta ompX \Delta ompA$ double mutant and the $\Delta ompX$ mutant were statistically significant, whereas there was

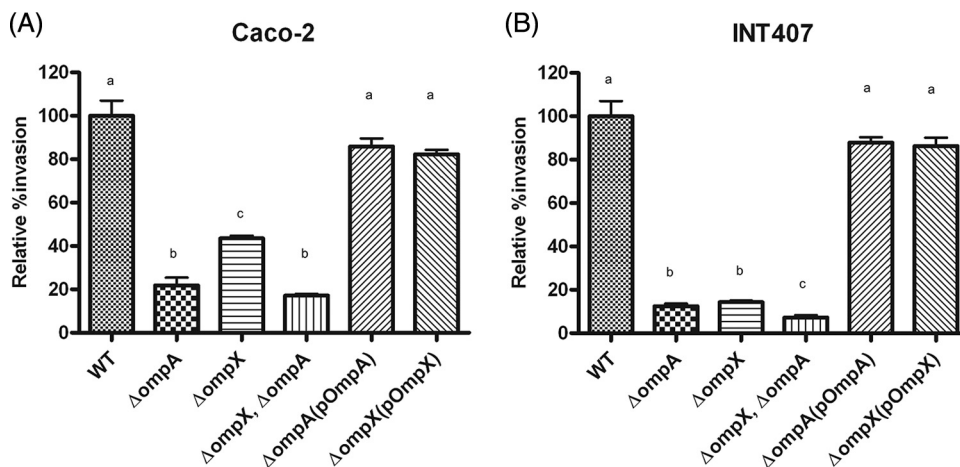


FIG. 2. Invasion by *C. sakazakii* in Caco-2 (A) and INT-407 (B) cells. Confluent monolayers of eukaryotic cells were infected with *C. sakazakii* at an MOI of 100 and incubated for 1.5 h, followed by gentamicin treatment (100 μ g/ml) for 1.5 h. The cells were then treated with 0.4% Triton X-100 to obtain the intracellular bacteria. The bars represent means plus standard deviations (SD) from three independent experiments performed in triplicate. Statistical differences are indicated by lowercase letters at P values of <0.05 .

no significant difference between the $\Delta ompX \Delta ompA$ double mutant and the $\Delta ompA$ mutant (Fig. 2A). These observations suggest that OmpA and OmpX play important roles in the invasion of Caco-2 cells but that OmpA and OmpX do not act synergistically in Caco-2 cells.

In the case of INT-407 cells, only 12.5% ($8.0 \times 10^2 \pm 0.9 \times 10^2$ CFU/well) of the $\Delta ompA$ mutant, 19.1% ($1.2 \times 10^3 \pm 1.5 \times 10^3$ CFU/well) of the $\Delta ompX$ mutant, and 8.5% ($5.4 \times 10^2 \pm 0.4 \times 10^2$ CFU/well) of the double mutant invaded the cells compared to the WT. The difference in invasion efficiency between the $\Delta ompA$ and $\Delta ompX$ mutants was not statistically significant, whereas those between either the $\Delta ompA$ or $\Delta ompX$ single mutant and the $\Delta ompX \Delta ompA$ double mutant were statistically significant (Fig. 2B). These observations indicate that OmpA and OmpX act synergistically to invade INT-407 cells. The addition of an extrachromosomal copy of the deleted gene restored the invasion potential of the mutants (Fig. 2B). Taken together, these results suggest that OmpA and OmpX are required for invasion of host cells.

OmpA and OmpX proteins can block Caco-2 cell invasion by *C. sakazakii*. To determine whether *C. sakazakii* OmpA and OmpX proteins interact directly with Caco-2 cells, we performed an inhibition assay using purified OmpA and OmpX proteins. Each protein was overexpressed in *E. coli* TOP10 by L-arabinose induction, and the OMPs were isolated using metal affinity resin (Fig. 3A). Preincubation of Caco-2 cells with purified OmpA or OmpX decreased the invasion of Caco-2 cells by *C. sakazakii* ATCC 29544 in a dose-dependent manner (Fig. 3B) ($P < 0.05$). These observations suggest that preincubated OmpA and OmpX may occupy an unknown host receptor(s) on Caco-2 cells, thereby inhibiting the invasive ability of *C. sakazakii*.

To validate the function of these recombinant OMPs as the original OMPs in *C. sakazakii* ATCC 29544, an invasion assay was conducted using $\Delta ompA$ and $\Delta ompX$ mutants harboring pBAD202-ompA and pBAD202-ompX, respectively. Complementation by pBAD202-ompA and pBAD202-ompX could re-

cover the defective phenotype of $\Delta ompA$ and $\Delta ompX$ mutants in their invasion ability (data not shown).

OmpA, but not OmpX, is required for adhesion to Caco-2 cells. Compared with the WT, only approximately 30% of the $\Delta ompA$ mutants or the $\Delta ompX \Delta ompA$ double mutants were able to adhere to Caco-2 cell monolayers in an adhesion assay (Fig. 4A) ($P < 0.005$). The *ompA* complement restored adhesion capability to a level similar to that of the parental strain, *C. sakazakii* ATCC 29544. On the other hand, deletion of *ompX* did not affect adhesion efficiency. In addition, there were no significant differences in adhesion to INT-407 cells among the WT, the $\Delta ompA$ mutant, the $\Delta ompX$ mutant, and the $\Delta ompX \Delta ompA$ double mutant (Fig. 4B).

OmpA and OmpX are essential for basolateral invasion by *C. sakazakii*. We examined whether the invasion of Caco-2 or INT-407 cells by the mutant strains was affected by tight junctions or cell age (Fig. 5). First, with 14- to 21-day-old Caco-2 cells, the invasion potential increased approximately 1.5-fold (150.1%, 35.9%, 63.1%, and 27.2% for the WT, $\Delta ompA$ and $\Delta ompX$ mutants, and $\Delta ompX \Delta ompA$ double mutant, respectively) compared with the invasion of 4- to 7-day-old Caco-2 cells (100%, 20%, 40%, and 17%, respectively) (Fig. 5A). Second, when 14- to 21-day-old Caco-2 cells were pretreated with EGTA before bacterial infection, the invasion potential of the WT increased approximately 7.3-fold (Fig. 5B), compared with the invasion of untreated 4- to 7-day-old Caco-2 cells (Fig. 5A). Similarly, 6.0-, 9.0-, and 5.8-fold increases were found for the $\Delta ompA$, $\Delta ompX$, and $\Delta ompX \Delta ompA$ mutants, respectively (Fig. 5A and B). Third, when 14- to 21-day-old INT-407 cells were used, the invasion capability of the *C. sakazakii* strains was increased by 1.2-fold (Fig. 5C) compared with invasion of 4- to 7-day-old INT-407 cells. Fourth, when EGTA-pretreated 14- to 21-day-old INT-407 cells were used, an approximately 3-fold increase in invasion potential was observed, regardless of the *C. sakazakii* strain, compared with the invasion of untreated 4- to 7-day-old INT-407 cells (Fig. 5D). These data

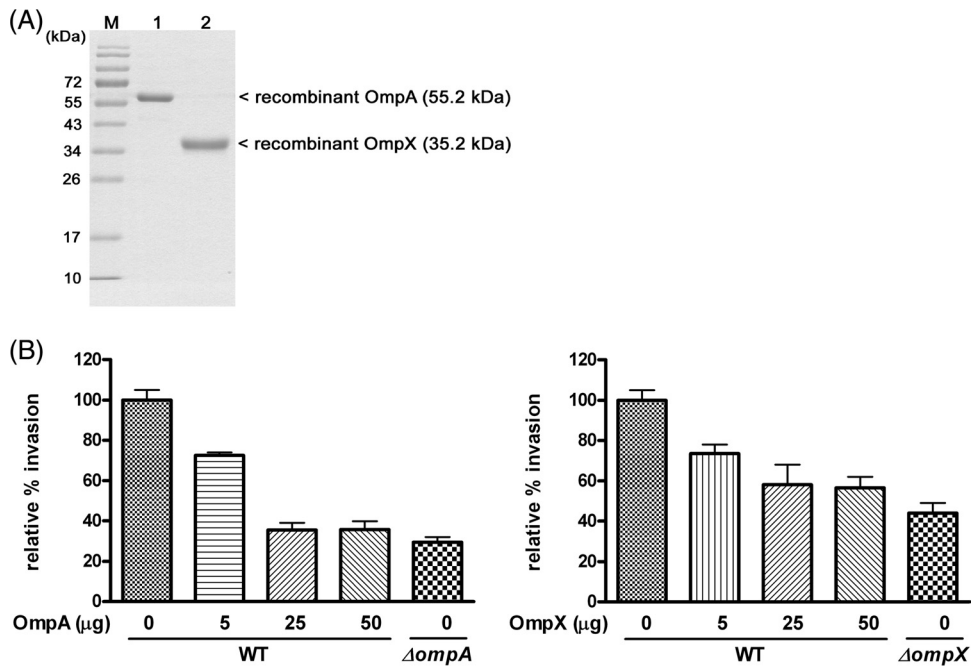


FIG. 3. Inhibition of *C. sakazakii* ATCC 29544 (WT) invasion of Caco-2 cells by pretreatment with purified proteins. (A) *C. sakazakii* recombinant OmpA and OmpX were overexpressed in *E. coli* TOP10 and isolated using metal affinity resin. The purified proteins were resolved by SDS-PAGE. Lane 1, size marker proteins; lane 2, recombinant OmpA; lane 3, recombinant OmpX. (B) Various concentrations (0.5, 25, and 50 μg) of purified outer membrane proteins were added to confluent monolayers of Caco-2 cells and incubated for 1 h at 37°C, followed by PBS washing and a gentamicin protection assay with *C. sakazakii* ATCC 29544 or the *ompA* deletion mutant. The experiments were performed at least three times in triplicate and are expressed as means plus SD. Statistical differences are indicated by lowercase letters at *P* values of <0.05.

indicate that OmpA and OmpX are required for basolateral invasion of Caco-2 cells by *C. sakazakii*.

Basolateral adhesion of *C. sakazakii* to Caco-2 cells is dependent on OmpA, but not OmpX. To examine whether OmpA or OmpX is required for basolateral adhesion to Caco-2 or INT-407 cells, we measured the initial association of *C. sakazakii* strains with Caco-2 and INT-407 cells. The association of the WT was increased slightly, by about 2-fold (218.2%), when 14- to 21-day-old Caco-2 cells were used (Fig.

6A) compared with 4- to 7-day-old Caco-2 cells. Similarly, the adhesion of the $\Delta ompA$, $\Delta ompX$, and $\Delta ompX \Delta ompA$ mutants to 14- to 21-day-old Caco-2 cells was also increased, by about 2.2-fold (80.6%), 2.6-fold (178.8%), and 2.1-fold (66.1%), respectively (Fig. 6A), compared with adhesion to 4- to 7-day-old Caco-2 cells (36.5%, 88.7%, and 31.3%, respectively). When the tight junctions of polarized Caco-2 cells were disrupted by EGTA pretreatment, the associations of the WT and $\Delta ompA$, $\Delta ompX$, and $\Delta ompX \Delta ompA$ mutants were significantly in-

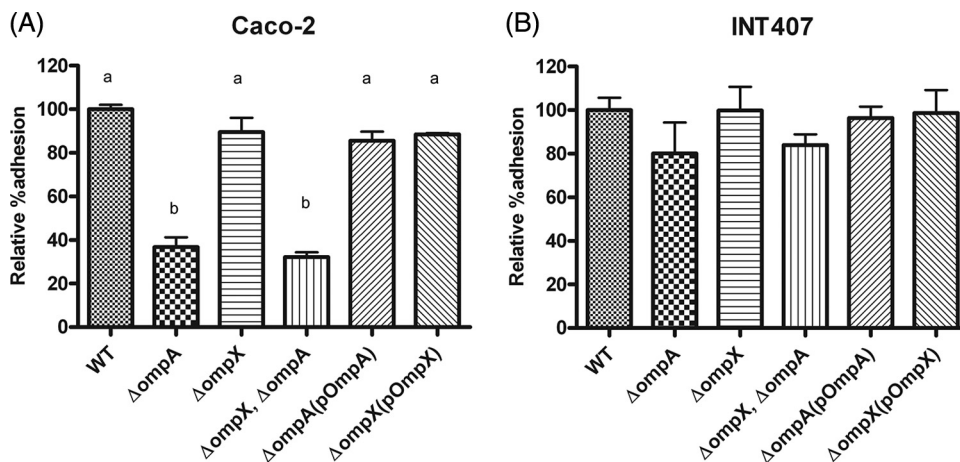


FIG. 4. Adhesion of *C. sakazakii* strains to Caco-2 (A) and INT-407 (B) cells. *C. sakazakii* was incubated with confluent monolayers of the host cells for 30 min, followed by PBS washing and Triton X-100 treatment for bacterial enumeration. The bars represent means plus SD from three independent experiments performed in triplicate.

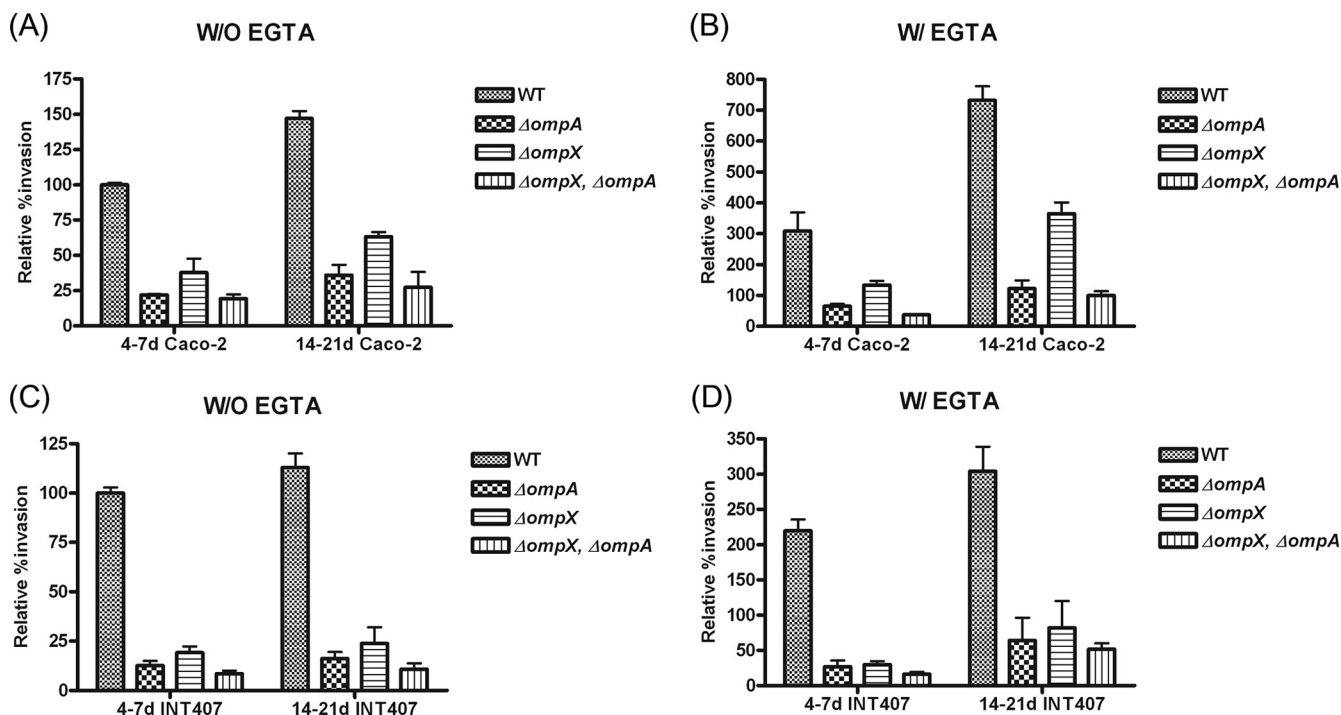


FIG. 5. Invasion of *C. sakazakii* into Caco-2 and INT-407 cells after pretreatment with EGTA. The monolayers were either 4 to 7 or 14 to 21 days old and were pretreated (W/ EGTA) or not pretreated (W/O EGTA) with 3 mM EGTA. Internalized bacteria were determined as described in the legend to Fig. 2 using Caco-2 (A and B) and INT-407 (C and D) cells. Percent invasion was determined by comparison to the non-EGTA-treated WT. Note that the scales in the y axes are different.

creased, by about 5.6-fold (565.6%), 8.6-fold (257.7%), 5-fold (503.3%), and 6-fold (184.4%), respectively, compared with adhesion to non-EGTA-treated Caco-2 cells (100%, 36.5%, 88.7%, and 31.3%, respectively) (Fig. 6B). These data indicate that OmpA, but not OmpX, is required for basolateral adhesion of *C. sakazakii* to Caco-2 cells.

For INT-407 cells, adhesion of all of the *C. sakazakii* strains was increased slightly, by 1.2-fold, when 14- to 21-day-old INT-407 cells were used (Fig. 6C) compared with 4- to 7-day-old cells. When the tight junctions of polarized INT-407 cells were disrupted, the initial association of all *C. sakazakii* strains tested showed an increase of about 1.6-fold (Fig. 6C). These data suggest that neither OmpA nor OmpX is required for the initial basolateral association of *C. sakazakii* with INT-407 cells.

To further examine the roles of OmpA and OmpX, we used confocal fluorescence microscopy to visualize the initial association of the *C. sakazakii* strains with 14- to 21-day-old Caco-2 cells pretreated with EGTA (Fig. 7). The *C. sakazakii* strains associated mainly with the basolateral region of Caco-2 cells, and this association was dependent on OmpA, but not OmpX.

OmpA and OmpX are essential for invasion into deeper tissue in rat pups. To examine whether the expression of OmpA and/or OmpX is required for *C. sakazakii* translocation in rat pups, we assessed the virulence of the WT and its isogenic $\Delta ompA$, $\Delta ompX$, and $\Delta ompX \Delta ompA$ mutant strains *in vivo*. After 24 h of incubation, the number of WT cells in the rat liver was 5.1 log CFU/g, whereas the numbers of $\Delta ompA$, $\Delta ompX$, and $\Delta ompX \Delta ompA$ mutant cells were 4.01, 4.12, and 3.80 log CFU/g, respectively (Fig. 8A). In the rat spleen, the

number of recovered WT cells was 3.85 log CFU/g, whereas the numbers of $\Delta ompA$, $\Delta ompX$, and $\Delta ompX \Delta ompA$ mutant cells were 2.12, 2.32, and 1.97 log CFU/g, respectively (Fig. 8B). *C. sakazakii* was not detected in the PBS-treated group (Fig. 8). There were no significant differences among the mutants (Fig. 8). Therefore, this experiment demonstrated that both OmpA and OmpX contribute to *C. sakazakii* virulence in rat pups.

DISCUSSION

The pathogenesis of *Cronobacter* spp., opportunistic pathogens that cause systemic infection, is virtually unknown. This may be partly because of the lack of appropriate animal models and genetic tools applicable to the genus *Cronobacter*. Nevertheless, as an oral pathogen, *C. sakazakii* is thought to be equipped with the means to cross the intestinal barrier (17). In this study, we constructed, for the first time in *C. sakazakii*, in-frame deletion mutants in the outer membrane proteins, OmpA and OmpX, using the lambda Red recombination system. *Cronobacter* OmpA was shown previously to play a role in the invasion of various mammalian host cells and is important for *Cronobacter* resistance to blood and serum killing in newborn rats (24, 25, 31). However, *C. sakazakii ompX* was first identified by a BLAST search of *Enterobacter cloacae ompX* against the *C. sakazakii* BAA-894 genome (locations 2478859 to 2479371) database (81% identity with *E. cloacae* OmpX) in this study. We report here that OmpX and OmpA are critical for the basolateral invasion of host cells by *C. sakazakii* and for its movement into deeper organs, such as the liver and spleen.

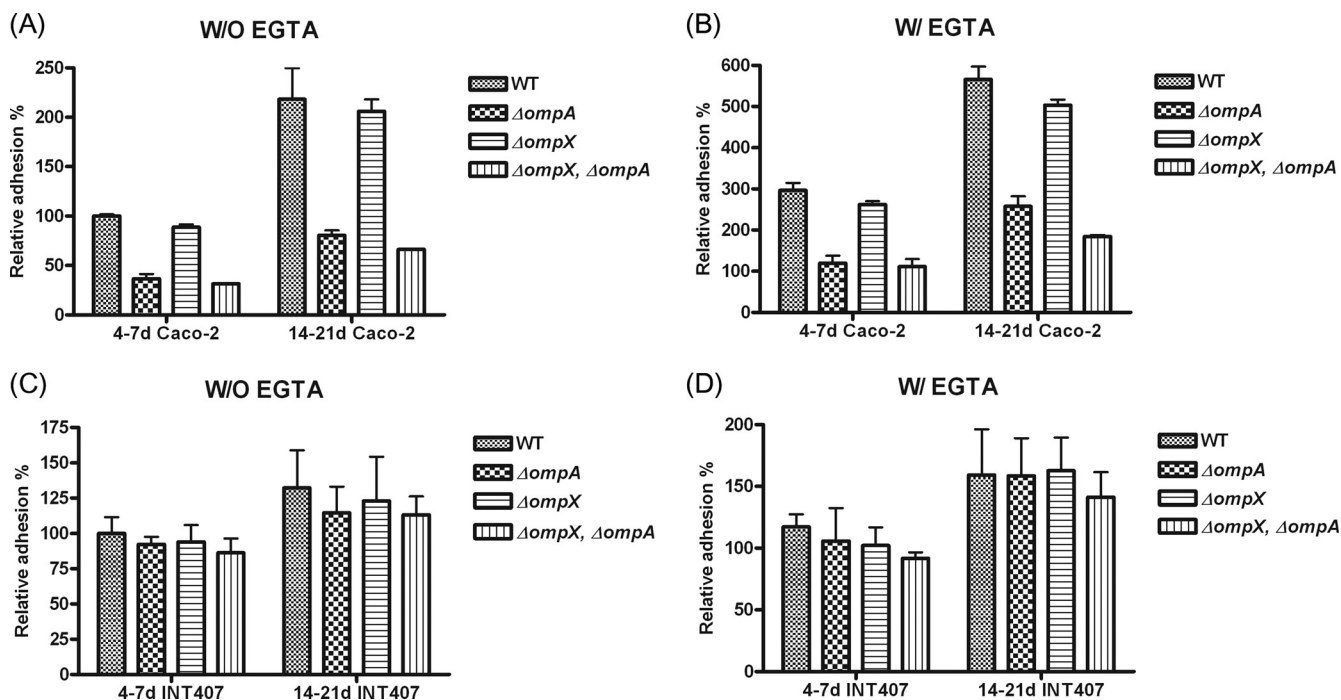


FIG. 6. Association of *C. sakazakii* with Caco-2 and INT-407 cells after pretreatment with EGTA. The monolayers were either 4 to 7 or 14 to 21 days old and were or were not pretreated with 3 mM EGTA. An adhesion assay was performed as described in Materials and Methods. The data are the means and standard deviations of three independent experiments.

Few genetic tools applicable to *Cronobacter* spp. have been reported to date. Only two different $\Delta ompA$ insertion mutants, generated by double-crossover gene replacement, have been reported (25, 31). In the present study, we applied the lambda Red recombination system to construct $\Delta ompA$ and $\Delta ompX$ deletion mutants. This site-specific recombination can generate chromosomal mutations using PCR products with as little as 30 bp of homology at the ends of the gene of interest (5). This system has been used to construct mutant strains in *E. coli* (5), *Salmonella* (4, 33), and *Klebsiella* (15). Here, we successfully constructed $\Delta ompA$, $\Delta ompX$, and $\Delta ompX \Delta ompA$ deletion mutant strains, as confirmed by PCR and the disappearance of the target protein on SDS-PAGE. Given that the whole genome sequence of *C. sakazakii* BAA-894 is now available (http://www.ncbi.nlm.nih.gov/nucleotide/156932229?ordinalpos=1&itool=EntrezSystem2.PEntrez.Sequence.Sequence_ResultsPanel.Sequence_RVDocSum), this mutation system will be useful for generating in-frame deletions in nonessential genes of *C. sakazakii*, which will facilitate the study of the pathogen.

Invasion (Fig. 2 and 5) and inhibition (blocking) (Fig. 3) studies clearly demonstrated that the OmpX and OmpA proteins are essential for the invasion of Caco-2 and INT-407 cells by *C. sakazakii*. However, these proteins may have different roles in other host cells. In Caco-2 cells, the invasion rate of the $\Delta ompX$ mutant was higher than that of either the $\Delta ompA$ or $\Delta ompX \Delta ompA$ mutant, and there was no significant difference between the $\Delta ompA$ and $\Delta ompX \Delta ompA$ mutants (Fig. 2A). This suggests that OmpA plays a more dominant role than OmpX in Caco-2 cell invasion by *C. sakazakii*. On the other hand, when INT-407 cells were used, a significant difference in the invasion rate was observed between either of the single

mutants ($\Delta ompA$ or $\Delta ompX$) and the double mutant (Fig. 2B), suggesting that the two Omp proteins act in a synergistic/additive manner.

OmpX was shown to be involved in the invasion of rabbit ileal tissue by *E. cloacae*, and *Yersinia pestis* KIM OmpX is known to be required for adhesion to and invasion of Hep-2 cells (7, 18). In addition, OmpX appears to play an important role in virulence by neutralizing host defense mechanisms and is involved in bacterial defense against the complement systems of the host (10, 38). OmpX has the same β -sheet topology (eight antiparallel amphipathic β -sheets) as the structurally related OmpA. However, their barrel structures differ with respect to the shear numbers (8 in OmpX and 10 in OmpA) and the internal hydrogen-bonding networks. At present, it is not clear whether *C. sakazakii* OmpA and OmpX share a host receptor for invasion. These data suggest that the receptors for *C. sakazakii* OmpA and OmpX may be different, as the invasion potentials of the single mutants ($\Delta ompA$ and $\Delta ompX$) were different in the different host cells (Caco-2 and INT-407).

Adhesion studies (Fig. 4 and 6) showed that OmpA was important for binding to intestinal epithelial Caco-2 cells. However, OmpA was not required for binding to INT-407 cells, consistent with the results of previous studies (25). On the other hand, OmpX did not play a significant role in binding to the eukaryotic cells examined within the incubation time used here (30 min). There are several implications of these findings. First, adhesion of *C. sakazakii* to Caco-2 cells is mediated by OmpA, but adhesion to INT-407 cells is not. Second, the initial association of *C. sakazakii* with Caco-2 and INT-407 cells is independent of OmpX. Third, the adhesion mediated by OmpA is not an absolute prerequisite for invasion within

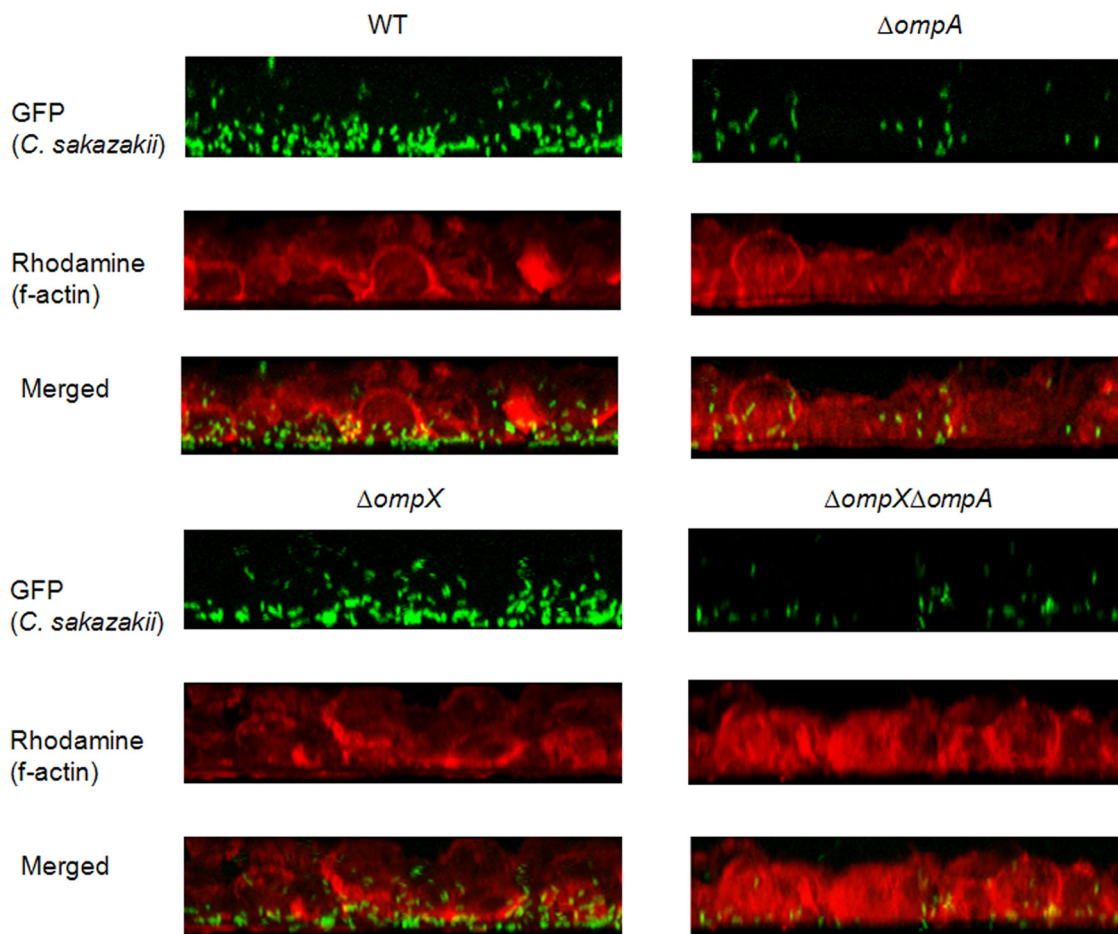


FIG. 7. Confocal fluorescence microscopy of Caco-2 cells that were EGTA treated and infected with *C. sakazakii* strains containing pWM1007 for 30 min. The f-actin molecules were stained with rhodamine-labeled phalloidin. Different series of images from 1- μ m *x-y-z* sections were obtained, analyzed, and stacked using the recommended software (LSM-FCS).

the incubation time used here, given that the association/dissociation constants between the reacting molecules were not considered. Fourth, *C. sakazakii* OmpA interacts with more than one cellular molecule.

A previous study showed that when the cellular tight junc-

tions of Caco-2 cell monolayers were removed, the invasion efficiency of *C. sakazakii* increased markedly, depending on the age of the cells (4 to 7 days versus 17 to 20 days) (17). Considering the importance of tight junctions in protection against bacterial pathogens, it was suggested that the enhanced ability

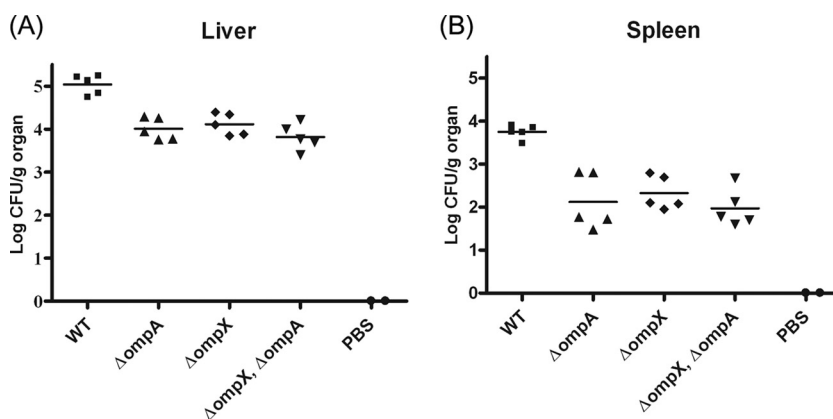


FIG. 8. *In vivo* animal study. Isogenic *C. sakazakii* strains grown aerobically were used to infect 2- or 3-day-old rat pups. A group of five rats were orally inoculated with 5×10^9 CFU of *C. sakazakii*. To analyze bacterial colonization in different organs, the rat pups were anesthetized 24 h postinfection. The spleens and livers were removed, homogenized, and then plated on LB agar plates.

of *C. sakazakii* to penetrate the host cells in the absence of this physical barrier may be important for the pathogenesis of infection by the organism in premature neonates (25). In this study, we found that the invasion potential of the mutant strains was affected by the presence of tight junctions; OmpA and OmpX mediated basolateral invasion of both Caco-2 and INT-407 cells by *C. sakazakii*. These data also suggest that the receptor for *C. sakazakii* invasin is located not only on the apical side, but also on the basolateral side, of intestinal cells.

The present study did not elucidate the precise mechanisms underlying the involvement of OmpA and OmpX in the penetration of host cells by *C. sakazakii*, and further studies are required to address this issue. Previously, it was shown that *E. coli* OmpA binds to Ecgp on the surface of HBMECs during invasion (30) and causes a temporary breakdown of the tight junctions and an increase in vascular permeability (34). It will be of interest to investigate whether *C. sakazakii* OmpA is also able to induce the breakdown of tight junctions. To our knowledge, there have been no previous reports regarding the interaction of OmpX with specific host molecules, although *Salmonella enterica* serovar Typhimurium *pagC*, which shows 81% nucleotide identity with *C. sakazakii ompX*, was reported to be involved in survival in macrophages (22).

The neonatal-rat model was used to assess bacterial translocation from the intestinal tract to deeper tissues following endotoxin administration, inducing apoptosis and intracranial infection (12, 35, 36). We also examined the translocation efficiency of $\Delta ompA$ and $\Delta ompX$ mutant strains in rat pups. The absence of *ompA* and *ompX* expression resulted in attenuated virulence in orally infected rat pups, as indicated by the decreased bacterial loads in the spleen and liver.

In conclusion, we have shown that OmpA and OmpX expression in *C. sakazakii* is required for not only apical, but also basolateral, adhesion to and invasion of mammalian cells. OmpA and OmpX show some additive effects in facilitating the invasion of host cells, suggesting that these two outer membrane proteins have different host cell receptors. Further investigations are required to determine the specific mechanisms of the interactions between *C. sakazakii* outer membrane proteins and host cell receptors.

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