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# Putative Inv Is Essential for Basolateral Invasion of Caco-2 Cells and Acts Synergistically with OmpA To Affect *In Vitro* and *In Vivo* Virulence of *Cronobacter sakazakii* ATCC 29544

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***Cronobacter sakazakii* is an opportunistic pathogen that causes neonatal meningitis and necrotizing enterocolitis. Its interaction with intestinal epithelium is important in the pathogenesis of enteric infections. In this study, we investigated the involvement of the *inv* gene in the virulence of *C. sakazakii* ATCC 29544 *in vitro* and *in vivo*. Sequence analysis of *C. sakazakii* ATCC 29544 *inv* revealed that it is different from other *C. sakazakii* isolates. In various cell culture models, an Δ*inv* deletion mutant showed significantly lowered invasion efficiency, which was restored upon genetic complementation. Studying invasion potentials using tight-junction-disrupted Caco-2 cells suggested that the *inv* gene product mediates basolateral invasion of *C. sakazakii* ATCC 29544. In addition, comparison of invasion potentials of double mutant (Δ*ompA* Δ*inv*) and single mutants (Δ*ompA* and Δ*inv*) provided evidence for an additive effect of the two putative outer membrane proteins. Finally, the importance of *inv* and the additive effect of putative Inv and OmpA were also proven in an *in vivo* rat pup model. This report is the first to demonstrate two proteins working synergistically *in vitro*, as well as *in vivo* in *C. sakazakii* pathogenesis.**

*Cronobacter sakazakii* is a Gram-negative, rod-shaped, non-spore-forming opportunistic pathogen in the family *Enterobacteriaceae* (1). *C. sakazakii* is known to transmit through reconstituted infant formula (2) and causes necrotizing enterocolitis, bacteremia, and meningitis (3–5). The mortality rate by *C. sakazakii* infection has been reported to be 33 to 80% (6–9), and the survivors often experience developmental, chronic, and neurological disorders (10). As a causative agent of neurological disorders after oral ingestion, *C. sakazakii* must be equipped with the virulence factors necessary to penetrate the intestinal epithelium, to survive the host defense mechanism, and to cross the blood-brain barrier. Production of enterotoxins (11) and the expression of genus specific cell bound zinc metalloprotease (12) were reported to play a role in *Cronobacter* pathogenesis. A putative *sod* gene (superoxide dismutase gene) (13) and *C. sakazakii* plasmid encoding *cpa* (*Cronobacter* plasminogen activator) (14) were also identified as potential virulence factors involving survival of *C. sakazakii* in *in vitro* human macrophages (U937) and contributing to serum tolerance, respectively.

Several genes are known to be crucial in the interaction of *C. sakazakii* with epithelial cells and its translocation of *C. sakazakii* through the epithelial tissue barrier. A study on *C. sakazakii* adhesion to host cells identified two distinct adherent patterns, and the authors suggested that specific adhesins may be involved in the process (15). Putative *lysR*-type transcriptional regulator (LTTR) was reported to be important in adhesion to and invasion of human intestinal cells by *C. sakazakii* (16). Outer membrane protein A (OmpA) of *C. sakazakii* was identified as playing an essential role in the adhesion and invasion of host cells (17, 18, 19) and was further revealed as important in the transcytosis of *C. sakazakii* across tight monolayers of epithelial cells on transwell cultures (20). In addition, outer membrane protein OmpX was reported as a virulence determinant (18) that is responsible for the invasion of *C. sakazakii* in Caco-2 cells. These proteins are assumed to be involved in the recognition of and binding to specific receptors of cells (21), which in turn may facilitate the successful colonization and subsequent translocation of the pathogen to blood circulation.

Previously, *inv* was reported to be involved in pathogenesis of *Yersinia* and *Salmonella* spp. (22–27). In the present study, we confirmed the presence of *inv* homolog in *C. sakazakii* ATCC BAA-894 by blast search and in *C. sakazakii* ATCC 29544 by PCR using the primers derived from *C. sakazakii* ATCC BAA-894 genome sequence. We further demonstrate that putative Inv is essential for apical and basolateral invasion of the pathogen to host epithelial cells. In addition, for the first time in *C. sakazakii*, we show that the putative *inv* gene product exhibits additive effect with OmpA in *in vitro* and *in vivo* virulence models.

## MATERIALS AND METHODS

**Bacterial strains, primers, plasmids, and media.** The bacterial strains and plasmids and the primers used in the present study are listed in Table 1 and Table 2, respectively. *C. sakazakii* ATCC 29544 and the mutants were routinely grown in half-concentrated brain heart infusion broth (BHI1/2; Difco, Franklin Lakes, NJ) at 37°C with constant shaking unless

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**TABLE 1** Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| Strains | | |
| *C. sakazakii* | | |
| ATCC 29544 | Wild-type strain | 17 |
| ES1001 | 29544 harboring pKD46(Ap$^r$) | 17 |
| ES2005 | Δ*ompA* | 17 |
| ES2010 | Δ*inv*::Kan$^r$ | This study |
| ES2011 | Δ*inv* | This study |
| ES2012 | pACYC184-*inv* | This study |
| ES2013 | Δ*ompA*::Kan$^r$ | This study |
| ES2014 | Δ*ompA* Δ*inv* | This study |
| *E. coli* | | |
| DH5α | λ$^-$ ϕ80d*lacZ*Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*($r_K^-$ $m_K^-$) *supE44 thi-1 gyrA relA1* | 17 |
| Top10 | F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) 80*lacZ*ΔM15 Δ*lacX74 recA1* Δ*araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (StrR) *endA1 nupG* | Invitrogen |
| Plasmids | | |
| pKD13 | *oriR6K*γ Ap$^r$ FRT Kan$^r$ FRT | 16 |
| pKD46 | *oriR101 repA101$^{t,s}$* Ap$^r$ *araBAD pgam-bet-exo* | 16 |
| pCP20 | *oripSC101$^{t,s}$* Ap$^r$ Cm$^r$ *c*I857λ $P_R$flp | 16 |
| pACYC184 | Tet$^r$ Cm$^r$; p15A *ori* | 16 |
| pBAD202/D-TOPO | Kan$^r$ | Invitrogen |
| pBADD202-r-*inv* | Harboring *C. sakazakii inv* gene reverse complement | This study |

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

**TABLE 2** Oligonucleotide primers used for PCR amplification and sequencing

| Primer | Sequence (5′–3′)[a] | Reference or source |
|---|---|---|
| OmpA-F | GTG CTC ATC AAT AGA CCG ACA | 17 |
| OmpA-R | ACT ACA TGC AGC AGA GAA ATT | |
| Inv_ FP | ATG CAT GAG CAA TCC ATA ATG | This study |
| Inv_RP | TTA AAG CAA ATA ATC TCT GGT | |
| Inv_F1 | ATG CTG TCT GGT CTG GCA TCG | This study |
| Inv_R1 | CCA CGT TTA ATC ACT GCT GCG | |
| Inv_F2 | GGG TCA GCG TCA CCT GGA ACG | This study |
| Inv_R2 | CGT CAT ATC CGC CAC CGC TTT | |
| Inv_F3 | CGG TGA TAA CGT GGC GTT GTT | This study |
| Inv_R3 | CAC CGG CAC CGC GGT AAA GAC | |
| Inv_Kan_F | *CAT CAT TAA GGA TAC CTG TAG CAC GGG TGT TGA TTG AGA ATT T* TGT AGG CTG GAG CTG CTT CG* | This study |
| Inv_Kan_R | *ATA CGG AGC CGA AAT CAG GGG AGT TAA TAA GAT GCC ATG A* ATT CCG GGG ATC CGT CGA CC* | This study |
| *inv* HindIII-F | AGA GAG GCA TGC GCT TTC AGA CTC** | This study |
| *inv* SphI-R | AGA GAG AAG CTT TTA AAG CAA ATA** | This study |
| Inv_pBAD_F | ATG CAT GAG CAA TCC ATA ATG | This study |
| Inv_pBAD_R | CAC CAA GCA AAT AAC TTC TGG TAT TGG T*** | This study |
| Primer_2_F inV | GCA ACA TTG GCT TAG GGG TA | This study |
| Primer_2_R inV | AGC TAT TGG CGG AAA GTT TG | This study |

[a] *, Nucleotide sequences that originated from the *C. sakazakii* ATCC 29544 *inv* gene are shown in italics, and those from pKD13 are underlined; **, artificially added restriction enzyme recognition sites are underlined; ***, an artificially added four-base sequence is underlined.

otherwise indicated. *Escherichia coli* and *C. sakazakii* strains harboring the various plasmids were grown on Luria-Bertani (LB; Difco) media at 37°C. When needed, antibiotics were added at concentrations of 25 (chloramphenicol) or 50 μg/ml (ampicillin or kanamycin).

**Identification and sequencing of *inv* gene in *C. sakazakii* ATCC 29544.** PCR for nucleotide sequencing was performed using *Pfu* DNA polymerase (Enzynomix, South Korea). In order to PCR amplify complete *inv* gene homolog in *C. sakazakii* ATCC 29544, the primers Inv_F1 and Inv_R1 were designed based on the nucleotide sequence of the genome of *C. sakazakii* ATCC-BAA 894 (NCBI accession number NC_009778.1) (Table 2). PCR was performed under the thermal cycling conditions of a hot start at 95°C for 5 min, followed by amplification for 30 cycles of 95°C for 60 s, 63°C for 60 s, and 72°C for 4 min, and with a final extension at 72°C for 10 min. After agarose gel electrophoresis and gel extraction, the PCR amplicon was sequenced using Inv_F1, Inv_R1, Inv_F2, Inv_R2, Inv_F3, and Inv_R3 (Table 2) in Macrogen, Inc. (Seoul, South Korea). Sequence information was submitted to the GenBank (NCBI accession number KC602378).

**Bioinformatics.** Protein similarity search was performed using the BLASTP algorithm (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]). TMPred (28) and HMMTOP (29) were used to predict transmembrane domains and subcellular localization of the putative Inv protein. The secondary structure of the protein was determined using the online program Phyre2, and conserve protein family and domains were identified using InterProScan program (Pfam database [http://www.ebi.ac.uk]).

**Mutant construction.** In-frame deletion mutants of *C. sakazakii* ATCC 29544 were generated by the lambda red recombination method, as described by Datsenko and Wanner (16). Briefly, to construct the *inv* deletion mutant, a kanamycin resistance (Kan$^r$) cassette from plasmid pKD13 was amplified using the primers, Inv_Kan_F (forward primer) and Inv_Kan_R (reverse primer) (Table 2). The PCRs were performed (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA) under the thermal cycling conditions of a hot start at 95°C for 5 min, followed by amplification for 30 cycles of 95°C for 60 s, 63°C for 60 s, and 72°C for 4 min, and with a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gel and examined on a UV transilluminator after staining with ethidium bromide. The resulting PCR products were electroporated (Gene Pulser, 2.5 V, 200 Ω, 25 μFD capacity; Bio-Rad, Hercules, CA) into the wild-type strain (WT), *C. sakazakii* ATCC 29544, harboring the pKD46 plasmid as described previously (18). Kan$^r$ transformants (*inv*::Kan) were selected on LB agar plates containing 50 μg of kanamycin/ml. Finally, the Kan$^r$ cassette was removed by using the pCP20 plasmid, as described previously (16). The Δ*ompA* Δ*inv* double mutant was constructed by using the Δ*ompA* mutant (17) as a parental strain and the primers Inv-FP and Inv-RP (Table 2).

**Complementation.** For construction of complement strains, complete *inv* open reading frame (ORF) plus promoter region of *C. sakazakii* ATCC 29544 *inv* (350 bp upstream from the start codon) was amplified using the forward primer *inv* HindIII-F and reverse primer, *inv* SphI-R (Table 2). The thermal cycling conditions consisted of a hot start at 95°C, for 5 min, followed by amplification for 30 cycles of 95°C for 60 s, 62°C for 60 s, and 72°C for 4 min, with a final extension at 72°C for 10 min. The PCR products were digested with HindIII and SphI and cloned into pACYC184 vector (30), followed by transformation into single (Δ*inv* or Δ*ompA*) and double (Δ*ompA* Δ*inv*) mutants.

**Cell culture.** Human intestinal epithelial Caco-2 (HTB 37 [derived from human colon adenocarcinoma]; American Type Culture Collection [ATCC], Manassas, VA), human intestinal epithelial INT-407 (ATCC CCL6 [derived from human embryonic jejunum and ileum]), and Hep-2 (ATCC CCL-23 [derived via HeLa contamination]) cells were maintained in Dulbecco modified Eagle medium (DMEM; Lonza, MD) containing 10% fetal bovine serum (FBS) (Lonza). Trypsin-treated cells were seeded (approximately $5 \times 10^4$ cells per well) into 24-well cell culture plates (Sarstedt, Newton, NC) and grown at 37°C in the presence of 5% $CO_2$. The cells formed monolayers after 3 to 4 days. The medium was replaced every 2 days, and cell viability was determined by trypan blue staining.

**EGTA treatment.** To disrupt the tight junction of Caco-2 cells, EGTA (Sigma, St. Louis, MO) treatment was used. The Caco-2 monolayer was incubated with DMEM containing 5 mM EGTA (pH 7.5) or phosphate-

buffered saline (PBS; pH 7.5) as a control for 60 min before bacterial infection (17). The cells were then washed twice with PBS (pH 7.4) and used for further studies.

**Invasion assay.** To test the ability of *C. sakazakii* to invade the cultured mammalian cells (Caco-2, INT-407, and Hep-2), invasion assay was performed as described previously (17, 18). Briefly, *C. sakazakii* was prepared by transferring overnight culture (2% inoculum) to fresh prewarmed BHI1/2 medium and was incubated at 37°C for 3 h with constant shaking (160 rpm; optical density at 600 nm [$OD_{600}$] of 0.6). Cells were collected by centrifugation at 10,000 × *g* for 5 min, and the cell pellet was suspended in 1 ml of DMEM (10% FBS). The mammalian cells grown in 24-well tissue culture plates were infected with $2 \times 10^7$ CFU of bacteria (multiplicity of infection [MOI] of 40) and incubated for 1.5 h. Monolayers were washed three times with PBS and further incubated for another 1.5 h with fresh medium containing gentamicin (100 μg/ml; Sigma) to kill the bacteria outside the cell. The cells were washed three times with PBS and treated with Triton X-100 (0.25% in PBS) for 10 min (Triton X-100 treatment [0.25%, 10 min] of bacteria has no effect on bacterial viability). Internalized *C. sakazakii* cells were enumerated by plating on BHI1/2 agar medium in duplicate. The results were expressed as an average of log CFU/well or log relative percentage of invasion compared to 4- to 7-day-old EGTA untreated Caco-2 cells from at least three independent experiments.

**Invasion assay and TEER measurement using transwell culture of Caco-2 cells.** Caco-2 cells grown in upper chamber (12-mm insert in diameter) of transwell system (0.4-μm-pore-size polycarbonate filters; Corning Life Sciences, Inc.) for 14 days were treated with either EGTA (5 mM) for 1 h, purified *Salmonella* lipopolysaccharide (LPS; 10 ng/ml; Sigma) (31), or PBS (pH 7.5) for 3 h (20). The cells were washed three times with PBS and used for an invasion assay (MOI of 100) as described above. The 0.4-μm-pore-size inserts efficiently prevented *C. sakazakii* from crossing the filter (0.08% translocation rate in the absence of Caco-2). In order to evaluate disruption of tight junction, transepithelial electrical resistance (TEER) across the transwell filter was measured before and after the treatment using a Millicell-ERS meter (Millipore, Inc., Billerica, MA) (20).

**Blocking assay.** Overexpression and purification of OmpA recombinant protein was carried out as previously described (32) with modifications. Briefly, the overnight culture of *E. coli* Top10 harboring pBAD202/D-TOPO::*ompA* (17) was transferred into fresh LB medium, and culture was induced with 2% L-arabinose when it reached an $OD_{600}$ of 0.5. The induced culture further incubated for 4 h, and the cells were recovered by centrifugation at 10,000 × *g* for 5 min at 4°C, followed by suspension in denaturation buffer B (100 mM Tris-HCl, 300 mM NaCl, 8 M urea [pH 8.0]) supplemented with 0.01% Triton X-100, 10 mM 2-mercaptoethanol, and 1% glycerol. After the cells were lysed by gentle vortexing at room temperature, lysate was centrifuged at 8,800 × *g* for 30 min at room temperature. The supernatant was loaded onto an Ni-NTA Superflow affinity column (Qiagen, Valencia, CA), and recombinant protein was eluted four times with 0.5 ml of buffer D (100 mM Tris-HCl, 300 mM NaCl, 8 M urea [pH 5.9]), followed by four elutions with 0.5 ml of buffer E (100 mM Tris-HCl, 300 mM NaCl, 8 M urea [pH 4.5]). Peak fractions were determined by SDS-PAGE, pooled, and dialyzed against PBS (pH 7.4) overnight at 4°C. Protein concentration was analyzed using NanoDrop (Biotek, South Korea) and stored at −20°C for further experiments. For blocking assay, Caco-2 monolayers were preincubated with purified OmpA protein (50 μg/well) for 1 h, followed by three washes with PBS (17). *C. sakazakii* invasion was determined as described above.

**Inhibition of *inv* translation by introduction of antisense *inv*.** To construct the plasmid transcribing reverse complement of *inv* gene, first complete *inv* gene of *C. sakazakii* ATCC 29544 was PCR amplified using the forward primer Inv_pBAD_F and the reverse primer Inv_pBAD_R (Table 2). The PCR conditions consisted of a hot start at 95°C for 5 min, followed by amplification for 30 cycles of 95°C for I min, 62°C for 1 min, and 72°C for 5 min and a final extension at 72°C for 10 min. The PCR products were inserted into the TOPO recognition site of pBAD202/D-TOPO and transformed into *E. coli* Top10 cells according to the manufacturer's instructions (Invitrogen). Insertion of *inv* in required orientation was confirmed by sequencing (Macrogen). The construct was transformed into *C. sakazakii* ATCC 29544 Δ*ompA* and Δ*inv* single mutants. The transformants were selected on LB agar plates containing 50 μg of kanamycin/ml.

To confirm the production of antisense mRNA from pBAD::r-*inv* construct, reverse transcriptase PCR was performed. Total RNA was isolated from cultures of WT, Δ*inv*, WT(pBAD::r-*inv*), and Δ*inv*(pBAD::r-*inv*) strains at mid-exponential phase ($OD_{600}$ = 0.6). After removal of genomic DNA by DNase treatment, cDNA was synthesized by using 1 μg of RNA (reverse transcription master mix; Qiagen). For amplification of the region from bp 436 to 589 of *C. sakazakii* ATCC 29544 *inv* gene or its reverse complement, PCR was performed using the forward primer Primer_2_F inV and the reverse primer Primer_2_R inV (Table 2) under the following thermocycling conditions: a hot start at 95°C for 5 min, followed by amplification for 30 cycles of 95°C for I min, 54°C for 1 min, and 72°C for 3 min, with final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gel and examined on a UV transilluminator after staining with ethidium bromide.

***In vivo* animal study.** Four-day-old pathogen-free Sprague-Dawley rat pups (DBL, South Korea) (17) were used. To prepare the bacteria, a 2% overnight culture of *C. sakazakii* was inoculated into fresh BHI1/2 medium, and late exponential-phase cells ($OD_{600}$ = 1.5) were collected. Cells were washed once with PBS and resuspended in PBS ($2 \times 10^8$ CFU/ml). Fifty-microliter portions ($10^7$ CFU) of four different 1:1 (vol/vol) mixtures (WT–Δ*ompA*::Kan$^r$, WT–Δ*inv*::Kan$^r$, Δ*ompA* Δ*inv*–Δ*ompA*::Kan$^r$, and Δ*ompA* Δ*inv*–Δ*inv*::Kan$^r$) were fed to each group of five rat pups ($n$ = 5) by oral gavage. Rat pups were euthanized at 24 h postinfection by carbon dioxide inhalation. For analysis of the bacterial colonization in organs, spleens and livers were removed aseptically and homogenized in 1 ml of ice-cold PBS, and the bacterial loads in each tissue were enumerated by plating on BHI1/2 agar and BHI1/2 agar containing 50 μg of kanamycin/ml. The competitive index (CI) was calculated by as follows: (the CFU of the reference strain − the CFU of the test strain)/(the CFU of the test strain). The results were expressed as the CI, where 1 indicates that two strains invade the tissue at equal rates *in vivo*. All experiments were performed according to Chonbuk National University Animal Care and Use Committee guidelines (approval number CBU 2013-0016).

**Statistical analysis.** All experiments were performed at least in triplicate. The data from cell culture experiments were analyzed by one-way analysis of variance with Duncan's post test with a 95% confidence interval using SAS software (v9.1.3; SAS Institute, Cary, NC). $P$ values of $<0.05$ were considered significant.

## RESULTS

**The *inv* homolog of *C. sakazakii* ATCC 29544 encodes a putative outer membrane protein.** PCR amplification of the *C. sakazakii* ATCC 29544 *inv* gene homolog using primers (Inv_FP and Inv_RP; Table 2) originating from *C. sakazakii* BAA-894 resulted in a 2.8-kp product which was different from that of *C. sakazakii* BAA-894 (3,083 bp, GenBank accession number NC_009778.1) (Fig. 1A). Sequencing of the PCR amplicon showed that the *inv* gene of *C. sakazakii* ATCC 29544 (GenBank accession number, KC602378) is 2,796 nucleotides long and that a nucleotide sequence from nucleotides 1951 to 2239 in *C. sakazakii* BAA-894 is absent in *C. sakazakii* ATCC 29544. Further sequence analysis showed that the homolog exhibits 97% identity at the nucleotide level and 92% identity at the protein level (93% of sequence coverage) with *inv* in other *C. sakazakii* strains (*ESA_00987* in *C. sakazakii* BAA-894 and *ES15_1237* in *C. sakazakii* ES15, 3,066 bp, NC_017933.1). The deduced *inv* gene product of the *C. sakazakii* ATCC 29544 is composed of 931 amino acids and is predicted to contain an N termi-

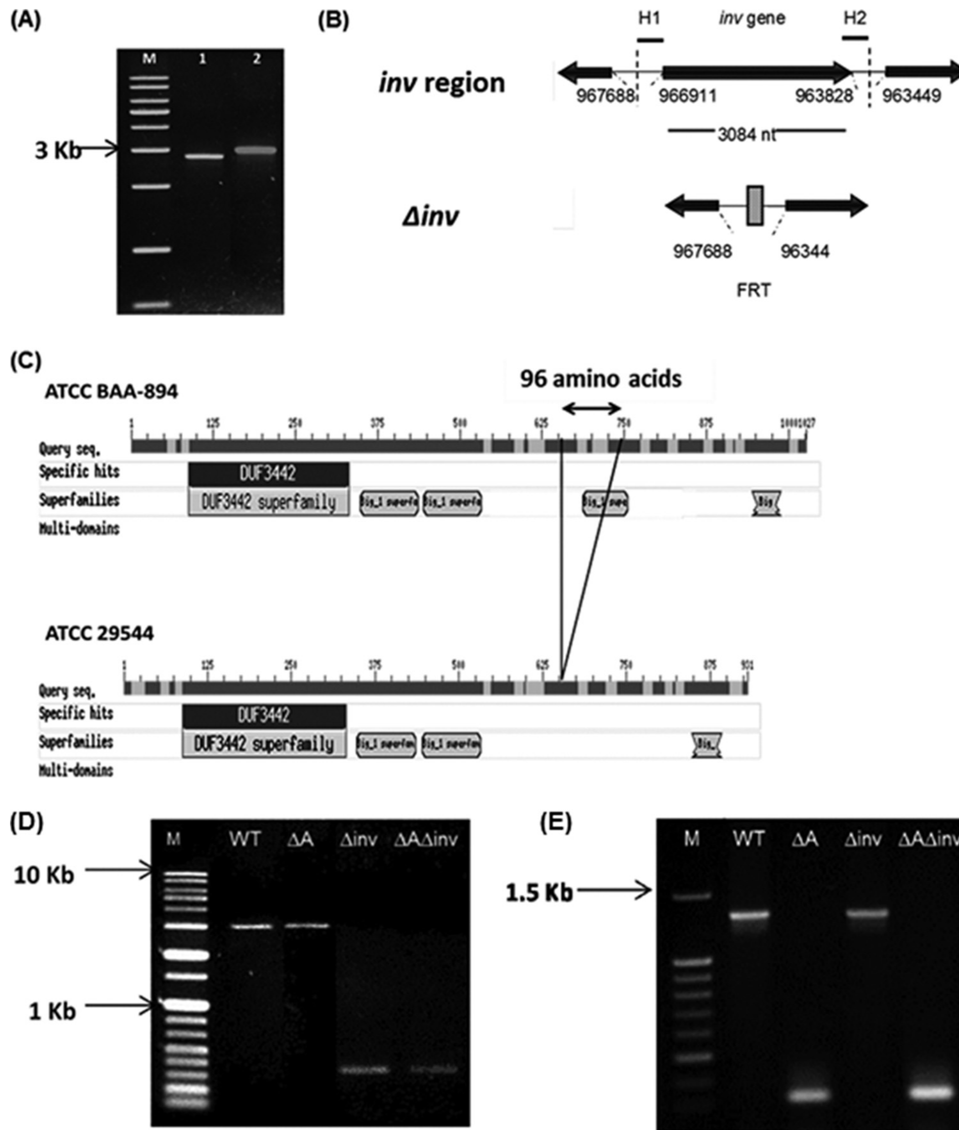


**FIG 1** Construction of in-frame deletion mutants of the *inv* gene homolog in *C. sakazakii* ATCC 29544. (A) PCR amplification of the *inv* gene ORF. Lane M, nucleotide 1-kb size markers; lane 1, *inv* of *C. sakazakii* ATCC 29544; lane 2, *inv* of *C. sakazakii* ATCC-BAA 894. (B) Gene arrangement near *inv* in *C. sakazakii* and the Δ*inv* mutant. H1 and H2 denote 40-nucleotide homology extensions located immediately downstream from the stop codon and upstream from the start codon of *inv*, respectively. The nucleotide numbers are adopted from the *C. sakazakii* ATCC-BAA 894 whole-genome database. (C) Domain analysis of putative Inv of *C. sakazakii* ATCC BAA894 and ATCC 29544 (the figure is adapted from the Pfam database [http://www.ebi.ac.uk]). Gap (96-amino-acid region in ATCC BAA 894) and domains are indicated in the figure. (D) Confirmation of *inv* deletion mutant construction. Lane 1, nucleotide size markers (1 kb); lane 2, WT; lane 3, Δ*ompA* single mutant; lane 4, Δ*inv* single mutant; lane 5, Δ*ompA* Δ*inv* double mutant. (E) Confirmation of *ompA* deletion mutant construction. Lane 1, nucleotide size markers (100 kb); lane 2, WT; lane 3, Δ*ompA* single mutant; lane 4, Δ*inv* single mutant; lane 5, Δ*ompA* Δ*inv* double mutant.

nus transmembrane domain in TMpred and InterproScan analysis (data not shown). In addition, a series of C terminus intimin and invasin domains which localize on the bacterial cell surface and allow the bacteria to adhere (32) and to penetrate mammalian cells (22) were also found (see the Discussion).

**Site-specific deletion mutants (Δ*inv*, Δ*ompA*, and Δ*ompA* Δ*inv*) were constructed and confirmed by PCR.** In order to study a possible role of the *inv* gene product in the pathogenesis of *C. sakazakii* ATCC 29544, a site-specific deletion mutant was constructed by using the lambda red recombination system (Fig. 1B). In addition, a double mutant (Δ*ompA* Δ*inv*) was generated using the previously reported Δ*ompA* strain (17) as a parental strain. PCR amplifications of the respective mutant strains using *inv*-specific primers (Table 2) which were designed 109 bp upstream (Inv_F1) and 119 bp downstream (Inv_R1) of *inv* showed 3.0-, 3.0-, 0.3-, and 0.3-kb bands for WT, Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* strains, respectively, confirming the *inv* gene deletion in Δ*inv* and Δ*ompA* Δ*inv* mutants (Fig. 1D). In addition, PCR using the *ompA*-specific primers OmpA-F and OmpA-R (17) showed 1.4-, 0.3-, 1.4-, and 0.3-kb bands for WT, Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* strains, respectively, confirming the deletion of *ompA* in Δ*ompA* and Δ*ompA* Δ*inv* strains (Fig. 1E). Growth of the mutants in different media (LB medium, TSB, and DMEM) was comparable to that of the WT (data not shown).

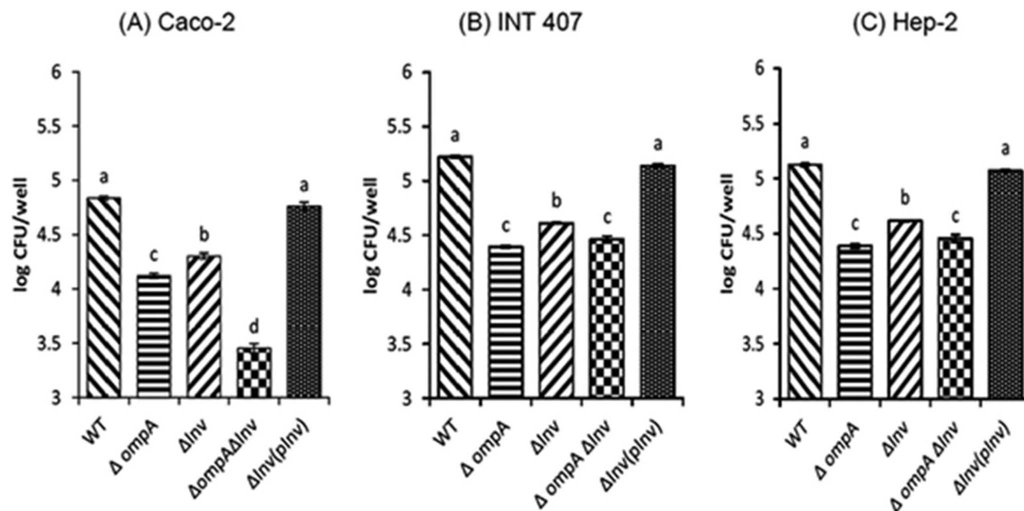


**FIG 2** *C. sakazakii* ATCC 29544 invasion of Caco-2 (A), INT-407 (B) and Hep-2 (C) cells. Confluent cell monolayers were infected with bacteria at an MOI of 40 and incubated for 1.5 h, followed by a gentamicin protection assay. The cells were treated with 0.25% Triton X-100 to release the bacteria, and intracellular bacteria were enumerated. The data represent the average log CFU per well ± the standard deviation (SD) from at least three independent experiments performed in duplicate. Values of $P$ of $<0.05$ were considered significantly different, and bars with the same lowercase letter are not significantly different from one another.

***C. sakazakii* putative Inv contributes to the invasion of host cells and works synergistically with OmpA for Caco-2 cells.** To determine the ability of *C. sakazakii* to invade various host cells (Caco-2, INT-407, and Hep-2), an invasion assay was performed (Fig. 2). We compared the invasion efficiency of each strain relative to the number of gentamicin-protected WT after 1.5 h of incubation (100%).

When Caco-2 cells were used, 4.83 ± 0.01 log CFU/well (100%) of WT was recovered after 1.5 h of incubation, while significantly lower numbers of the mutants were intracellular (4.12 ± 0.02 [17.72%] and 4.30 ± 0.02 [24.19%] log CFU/well for the Δ*ompA* and Δ*inv* strains, respectively) (Fig. 2A). The complementation strain showed fully restored invasion ability (4.76 ± 0.03 log CFU/well) for the *inv* deletion mutant.

Interestingly, when the Δ*ompA* Δ*inv* mutant was used, a significantly lower number of bacteria (3.45 ± 0.04 log CFU/ml) was recovered than when WT or single mutants (Δ*ompA* and Δ*inv*) were used. Differences in invasion efficiencies between Δ*ompA* and Δ*inv* mutants and between those of either of the two single mutants and a double mutant were statistically significant, suggesting an additive effect of the putative Inv and OmpA proteins in *C. sakazakii* invasion to Caco-2 cells.

In the case of INT-407 cells (Fig. 2B), a higher invasion rate than that in Caco-2 was observed, recovering 5.25 ± 0.01 log CFU/ml (100%) for the WT. For the single mutants, significantly lower numbers of the Δ*ompA* (4.39 ± 0.01 log CFU/well) and Δ*inv* (4.68 ± 0.01 log CFU/well) strains were intracellular, representing invasion rates that were 17.49 and 34.01%, respectively, of the WT rate. A similar pattern was observed in Hep-2 cells (Fig. 2C), where WT, Δ*ompA*, and Δ*inv* strains showed 5.19 ± 0.01 (100%), 4.38 ± 0.01 (17.26%), and 4.61 ± 0.01 (32.97%) log CFU/well, respectively. The complementation strain showed completely restored invasion ability for the *inv* deletion mutant in INT-407 and Hep-2 cells.

In contrast to Caco-2 cells, however, no additive effect was observed in the invasion of the Δ*ompA* Δ*inv* double mutant to INT-407 and Hep-2 cells showing 4.47 ± 0.01 log CFU/well (22.25%) and 4.45 ± 0.03 log CFU/well (20.81%), respectively.

**Pretreatment of Caco-2 cells with purified recombinant OmpA significantly decreases invasion efficiency of the Δ*inv* mutant.** To confirm the involvement of *inv* in invasion of *C. sakazakii* and the additive effect of putative Inv and OmpA, a blocking assay was carried out using purified recombinant OmpA (Fig. 3). Recombinant OmpA was obtained using a previously reported overexpression construct (17). After purification of His-tagged proteins, two major bands were observed in SDS-PAGE, approximately 55 and 37 kDa (Fig. 3A), that were not found in the control strain, *E. coli* with pBAD-TOPO (data not shown). Mass spectroscopy analysis showed that the 37-kDa protein was a degradation product of OmpA, which is 55 kDa (data not shown). A purified protein mixture was used for the blocking assay in the present study.

When Caco-2 cells 4 to 7 days old were pretreated with recombinant OmpA, the invasion rate of the WT was significantly reduced to 19.64%, compared to 100% for WT in Caco-2 cells without OmpA pretreatment. Furthermore, the rate of invasion for the Δ*inv* mutant was more significantly attenuated in OmpA-pretreated Caco-2 cells (5.74%) than in cells without OmpA pretreatment (24.29%).

In contrast, no difference in invasion rate of the Δ*ompA* mutant was observed between Caco-2 cells pretreated (18.77%) and not pretreated (17.72%) with purified OmpA. In addition, OmpA pretreatment of Caco-2 cells made only a marginal effect on the invasion rate of the double mutant compared to the effect seen with the Δ*inv* mutant in OmpA-pretreated Caco-2 cells.

When 14- to 17-day-old Caco-2 cells were used, significantly increased WT invasiveness (1.7-fold) was observed compared to 4- to 7-day-old Caco-2 cells. Regarding the effect of pretreatment with purified OmpA, a similar pattern of invasion efficiency was observed for the 14- to 17-day-old OmpA-pretreated Caco-2 cells challenged with WT (18.23%), Δ*ompA* (18.10%), Δ*inv* (6.11%),

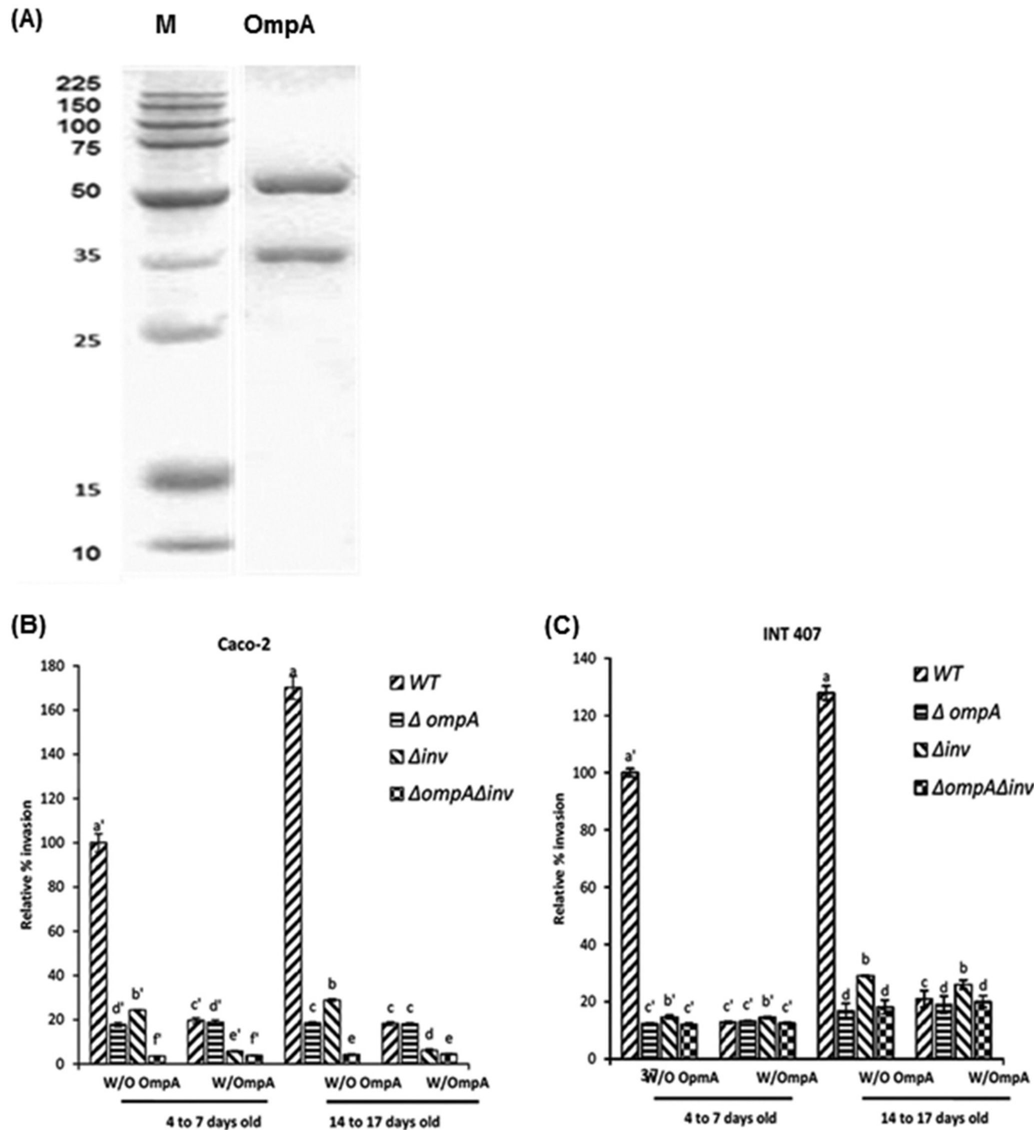


**FIG 3** Attenuation of Δ*inv* mutant invasion of Caco-2 cells pretreated with purified OmpA. (A) *C. sakazakii ompA* mutant was overexpressed in *E. coli* Top10 and purified by using metal affinity resin under denaturation conditions as described in Materials and Methods. Lane 1, protein size marker; lane 2, purified recombinant OmpA. (B and C) Invasion of *C. sakazakii* ATCC 29544 in recombinant OmpA-pretreated Caco-2 (B) and INT-407 (C) cell monolayers. Purified recombinant OmpA protein was added to Caco-2 or INT-407 cell monolayers at 5 μg/well and incubated for 1 h, followed by three washes with PBS and invasion assay with WT, Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* strains. The data represent the relative percentage invasion ± the SD from at least three independent experiments performed in duplicate compared to the invasion of WT to OmpA-untreated Caco-2 or INT-407 cells that were 4 to 7 days old. *P* values of $<0.05$ were considered significantly different, and bars with same lowercase letter are not significantly different from one another.

and Δ*ompA* Δ*inv* (4.48%) (Fig. 3C) strains compared to a 100% rate for WT in 4- to 7-day-old Caco-2 cells without OmpA pretreatment.

In INT-407 cells, when 4- to 7-day-old cells were pretreated with purified OmpA, the invasion efficiency for the WT, Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* strains were 12.78, 13.11, 14.41, and 12.50%, respectively, compared to 100% for the WT in INT-407 without OmpA pretreatment. In addition, OmpA-pretreated 14- to 17-day-old INT-407 cells did not show any significant differences in invasion pattern compared to 4- to 7-day-old cells recovering 20.91, 19.02, 25.94, and 19.97% for WT, Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* strains, respectively, compared to 100% for WT in 4- to 7-day-old INT-407 cells at without OmpA pretreatment. These results favor the presence of an additive effect involving *ompA* and the *inv* gene products in *C. sakazakii* ATCC 29544 invasions into Caco-2 cells but not in INT-407 cells.

**Introduction of the antisense *inv* gene further reduced the invasion of the Δ*ompA* mutant into Caco-2 cells.** In order to further clarify the role of the *inv* gene in the invasion of *C. sakazakii* ATCC 29544 and its relationship with *ompA*, we introduced the reverse complement of the *inv* gene (r-*inv*) into the WT in which the resulting antisense mRNA would form a duplex with the mRNA of chromosomal *inv* to inhibit functional Inv production. The construct contained the reverse complement of the complete *inv* gene (without a stop codon) inserted into the pBAD-TOPO vector cloning site (723 to 727 bp), which would make a

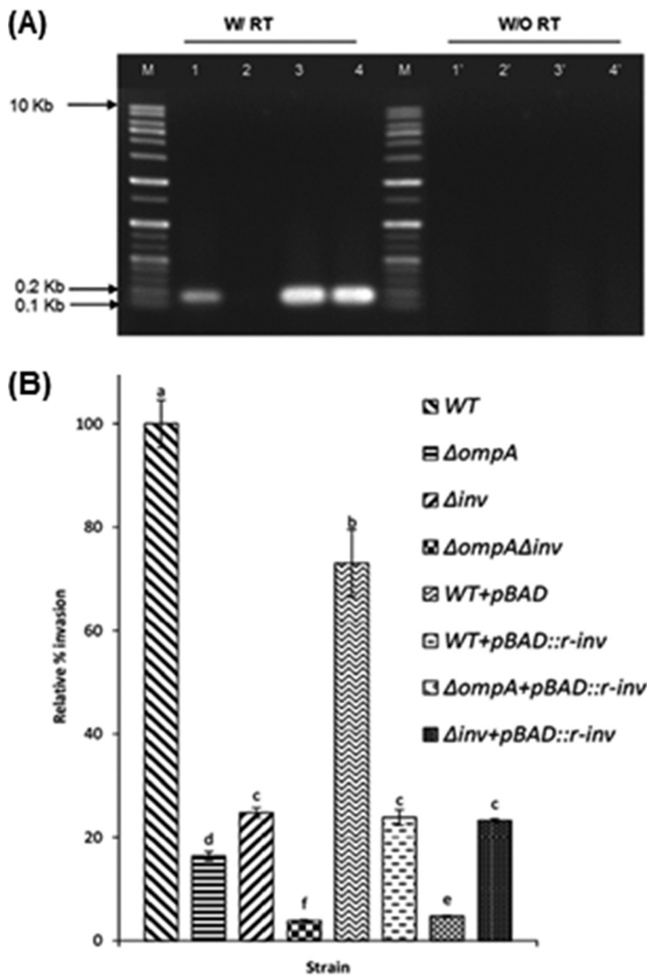


**FIG 4** Antisense expression of *C. sakazakii inv* gene. (A) PCR-amplified cDNA from reverse transcription of *inv* or *inv* reverse complement (r-*inv*) genes. Total RNA from *C. sakazakii* and its isogenic strains were treated with DNase I, and cDNA was generated by reverse transcription using random hexamer primers. The *inv* gene (region from bp 436 to 589) or *inv* reverse complement were amplified by using Primer_2_F inV and Primer_2_R inV to give 153-bp PCR products. Lane M, DNA size marker; lane 1, *C. sakazakii*; lane 2, Δ*inv* mutant; lane 3, *C. sakazakii* + pBAD::r-*inv*; lane 4, Δ*inv* + pBAD::r-*inv*. (B) Invasion of *C. sakazakii* carrying pBAD-r-*inv* into Caco-2 cell monolayers (4 to 7 days old). The data represent the relative percentages of invasion ± the SD from at least three independent experiments performed in duplicate. *P* values of <0.05 were considered significantly different, and bars with same lowercase letter are not significantly different from one another.

transcript fusion of upstream thioredoxin, r-*inv*, and the downstream his-tag sequence (see Fig. S1A in the supplemental material). The correct orientation of pBAD::r-*inv* construct was confirmed by sequencing (see Fig. S1B in the supplemental material). In addition, reverse transcriptase PCR targeting the region from bp 436 to 589 of the *inv* gene or *inv* reverse complement resulted in amplification of 0.15 kb in WT, WT(pBAD::r-*inv*), and Δ*inv*(pBAD::r-*inv*) strains but no amplification in the Δ*inv* mutant (Fig. 4A), confirming the formation of the antisense mRNA from pBAD::r-*inv* construct.

When pBAD-TOPO without r-*inv* was introduced into WT as a control, the invasion rate decreased to 75% compared to WT (100%) without the plasmid. In contrast, when pBAD::r-*inv* was introduced into WT, the invasion ability decreased to 23.86% compared to WT without pBAD-TOPO, confirming the importance of functional Inv protein in *C. sakazakii* invasion. In addition, the presence of pBAD::r-*inv* further lowered the invasiveness of the Δ*ompA* mutant, which was measured at 4.75%, compared to 100% for WT without pBAD-TOPO, whereas no significant difference was observed between WT(pBAD::r-*inv*) and Δ*inv* (23.23%) strains. These data support an additive effect of putative Inv and OmpA protein in invasion of *C. sakazakii* ATCC 29544 in Caco-2 cells.

**Additive effect of *ompA* and *inv* deletions is observed in tight-junction-disrupted Caco-2 cells.** To determine whether the opening of a tight junction and the age of the host cells have an effect on the invasion of *C. sakazakii*, we carried out invasion assays with EGTA-pretreated Caco-2 cells that were either 4 to 7 days old or 14 to 17 days old. In these experiments, we compared the invasion potential of each strain relative to that of WT (100%) in 4- to 7-day-old, EGTA-untreated Caco-2 cells (Fig. 5).

When the tight junctions of Caco-2 cells (4 to 7 days old) were disrupted by EGTA pretreatment (Fig. 5B), the invasion efficiency of WT (265.77%) increased ~2.6-fold compared to EGTA-untreated Caco-2 cells that were 4 to 7 days old (100%) (Fig. 5A). For EGTA-treated Caco-2 cells that were 14 to 17 days old, the WT invasiveness (762.13%) (Fig. 5B) increased by 7.6-fold compared to untreated Caco-2 cells that were 4 to 7 days old.

When Caco-2 cells (4 to 7 days old) were pretreated with EGTA, the invasion efficiencies of the Δ*ompA* and Δ*inv* mutants were 26.62% ± 1.03% and 28.81% ± 5.08%, respectively (Fig. 5B). Furthermore, the invasiveness of the Δ*ompA* Δ*inv* double mutant (8.54% ± 1.24%) was significantly lower compared to the Δ*ompA* and Δ*inv* single mutants (Fig. 5B). A similar pattern was found when EGTA-pretreated 14- to 17-day-old Caco-2 cells were used; we recovered 33.15% ± 4.89%, 58.98% ± 11.31%, and 9.29% ± 1.84% for the Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* mutants, respectively (Fig. 5B), compared to EGTA-untreated Caco-2 cells that were 4 to 7 days old (100%) (Fig. 5A).

For EGTA-untreated Caco-2 cells that were 14 to 17 days old, a slight but significant increase in invasiveness was observed for all four strains: 194.41% ± 1.49%, 19.10% ± 5.74%, 33.88% ± 1.94%, and 5.72% ± 3.66% for the WT, Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* strains, respectively, compared to Caco-2 cells that were 4 to 7 days old with intact tight junctions (Fig. 5A).

**Use of a transwell system confirmed the opening of tight junctions and the additive effect of *ompA* and *inv* in tight-junction-disrupted Caco-2 cells.** In order to verify the importance of the *inv* gene in the basolateral invasion of *C. sakazakii*, we carried out the invasion assay in a transwell system. First, disruption of the tight junction by EGTA treatment was confirmed by measuring the TEER values. When 14-day-old Caco-2 cells were treated with PBS, EGTA, and LPS, the TEER values were 506.5, 131.5, and 143.0 $\Omega/cm^2$, respectively, indicating disruption of the tight junction (see Fig. S2A in the supplemental material). Second, when PBS-treated 14-day-old Caco-2 cells were infected with *C. sakazakii*, the invasiveness values of the mutants were 13.38, 29.88, and 1.51% for the Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* mutants compared to the WT (100%). A similar decrease in invasion rates was observed in EGTA-pretreated Caco-2 cells, showing 25.09, 111.15, and 3.66% for the Δ*ompA*, Δ*inv*, and Δ*ompA* Δ*inv* mutants compared to the WT (509.69%). Comparable results were also observed in LPS-treated Caco-2 cells (see Fig. S2B in the supplemental material).

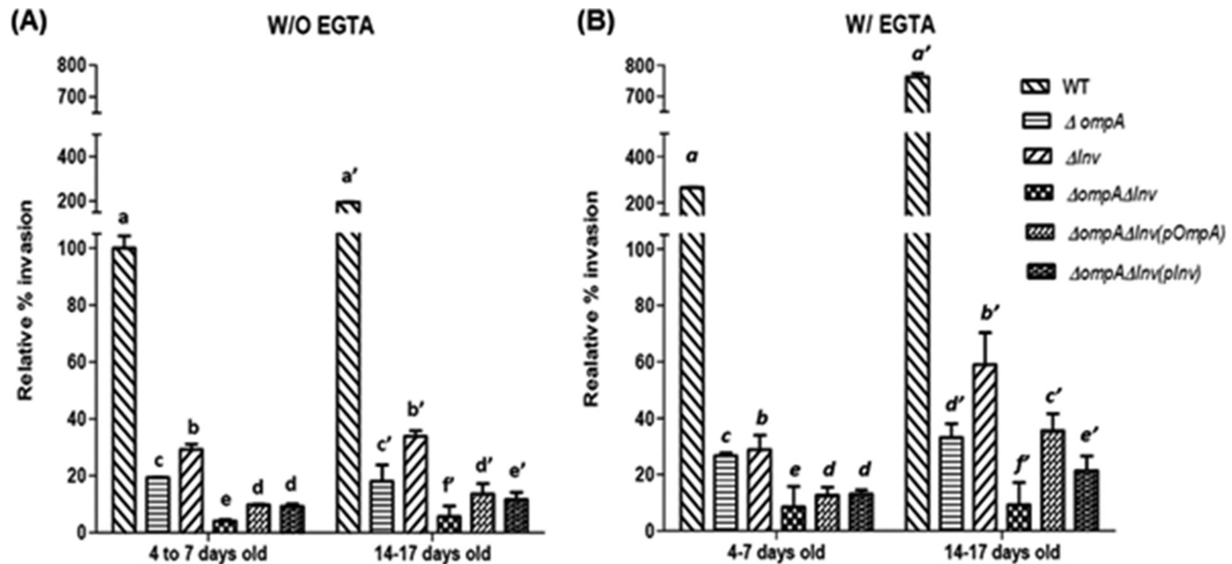


**FIG 5** Invasion of *C. sakazakii* into Caco-2 cells (4 to 7 days old or 14 to 17 days old) after pretreatment with EGTA. Cell monolayers were pretreated with 5 mM EGTA (or not pretreated) and used for an invasion assay. The data represent the relative percent invasion ± the SD from at least three independent experiments performed in duplicate compared to the invasion of WT to EGTA-untreated Caco-2 cells that were 4 to 7 days old. *P* values of <0.05 were considered significantly different, and bars with same lowercase letter are not significantly different from one another.

**Complementation of either *ompA* or *inv* partially recovered the invasion efficiency of the Δ*ompA* Δ*inv* double mutant.** When complement strains were used, partial recovery of the invasion ability was found in the double mutant (Fig. 5). The invasion efficiencies of the Δ*ompA* Δ*inv*(p*ompA*) and Δ*ompA* Δ*inv*(p*inv*) strains were 9.80% ± 0.20% and 9.29% ± 0.84%, respectively, for EGTA-untreated Caco-2 cells that were 4 to 7 days old and 13.66% ± 3.54% and 11.67% ± 2.52%, respectively, for untreated cells that were 14 to 17 days old.

In addition, when EGTA-pretreated Caco-2 cells were used, the invasion efficiencies of the Δ*ompA* Δ*inv*(p*ompA*) and Δ*ompA* Δ*inv*(p*inv*) mutant strains were 12.64% ± 2.89% and 13.08% ± 1.40%, respectively, for cells that were 4 to 7 days old and 35.56 ± 6.08 and 21.36% ± 5.20%, respectively, for cells that were 14 to 17 days old.

***inv* is essential and exhibits an additive effect with *ompA* for the translocation of *C. sakazakii* to deeper organs in a rat pup model.** We applied a CI assay to examine the ability of *C. sakazakii* and its isogenic strains to translocate into deeper organs such as the liver and spleen. We first compared the translocation efficiencies of Δ*inv* or Δ*ompA* mutants relative to the WT using WT–Δ*inv*::Kan$^r$ or WT–Δ*ompA*::Kan$^r$ strain combinations (1:1 CFU ratios) to infect a single host. At 24 h postinfection, the calculated average CI values for recovered *C. sakazakii* administered in a WT–Δ*inv*::Kan$^r$ (or WT–Δ*ompA*::Kan$^r$) strain combination inoculum from the liver (Fig. 6A) and spleen (Fig. 6B) were 4.65 (5.8) and 4.64 (6.1), respectively. Next, we tested the potential additive effect of *inv* and *ompA* mutants *in vivo* using Δ*ompA* Δ*inv*–*ompA*::Kan$^r$ and Δ*ompA* Δ*inv*–Δ*inv*::Kan$^r$ strain combinations. The average CI values in the liver (Fig. 6A) were 0.18 and 0.19, respectively, and those in the spleen (Fig. 6B) were 0.17 and 0.35, respectively.

## DISCUSSION

In an effort to identify genes related to *C. sakazakii* pathogenesis, *inv* was recognized as an outer-membrane-localized potential virulence factor in *in silico* genomic sequence analysis. It contains a number of bacterial Ig-like domains belonging to the Big_1 superfamily. Bacterial Ig-like domains are found in bacterial surface proteins such as intimin/invasin, which have been reported to be involved in bacterial pathogenesis (15). Previously, Invs of *Yersinia* or *Salmonella* spp. exhibiting Ig domains similar to those of the *C. sakazakii inv* gene product have been reported to be involved in the penetration of various host epithelial cells (Hep-2 and M cells) (22–27). Putative Inv of *C. sakazakii* BAA-894 exhibits its homologies to those of *Yersinia* (50% similarity) and *Salmonella* (68% similarity) spp. at the protein level. This correspondence implies the possibility of a role for the putative *inv* gene product in *C. sakazakii* pathogenesis.

Based on the *inv* sequence of *C. sakazakii* BAA-894 (GenBank accession number NC_009778.1), we amplified and sequenced the *inv* homolog (GenBank accession number KC602378) in *C. sakazakii* ATCC 29544. Interestingly, when the DNA sequences are compared, the *inv* of *C. sakazakii* ATCC 29544 is significantly different from those of *C. sakazakii* BAA-894, ES15 (GenBank accession number NC_017933.1), and SP291 (GenBank accession number NC_020260.1). The differences were due to the deletion of a nucleotide sequence from nucleotides 1951 to 2239 that is present in *C. sakazakii* BAA-894, which encodes 96 amino acids (651- to 746-amino-acid region). This missing region contains an Ig-like domain (3 found in ATCC 29544 and 4 found in other sequenced *C. sakazakii* strains, according to the pfam database) (Fig. 1C). However, other previously reported outer membrane proteins, such as OmpA and OmpX, exhibit no difference among *C. sakazakii* isolates (BAA-894, ES15, SP291, and ATCC 29544), showing 100% identity at the amino acid level.

Phenotypic variation, including virulence properties among the different *C. sakazakii* isolates, has been well described in previous studies (12, 20, 33, 34, 35), and BAA-894 and ATCC 29544 also differ in their growth (data not shown). However, strain heterogeneity has not been elucidated at the molecular level in *C.*

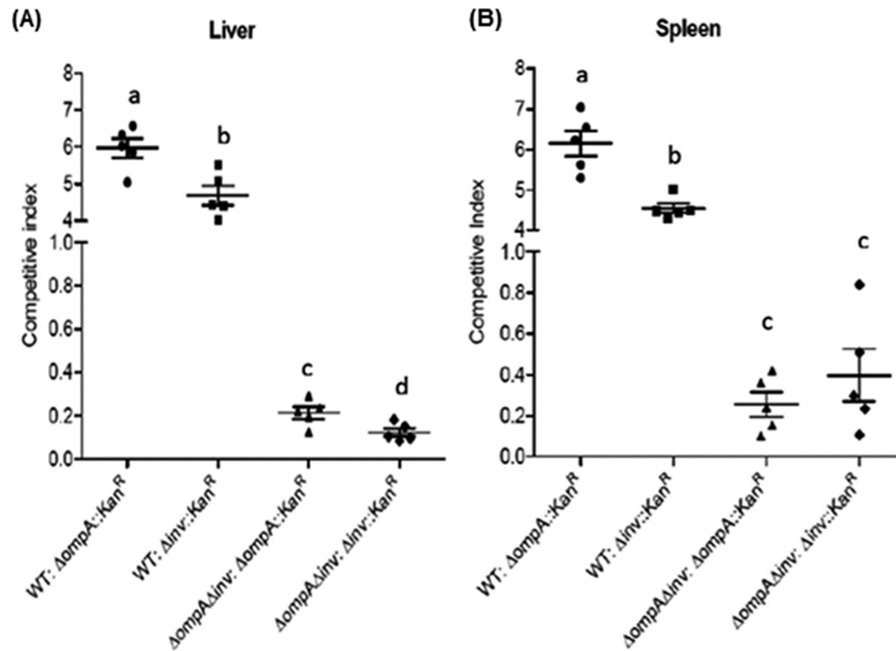


**FIG 6** *In vivo* animal study. *C sakazakii* WT and mutants (Δ*ompA*::Kan$^r$, Δ*inv*::Kan$^r$, and Δ*ompA* Δ*inv*) were grown to exponential phase and mixed in a 1:1 ratio (reference strain to test strain) to give four different mixed cultures that were fed ($10^7$ CFU) to each group of five ($n = 5$) rat pups (4 days old) by oral gavage. The pups were then sacrificed at 24 h postinfection. The livers and spleens were removed, homogenized, and plated on BHI1/2 agar and BHI1/2 agar containing 50 μg of kanamycin/ml. The CI score per organ [(the CFU of the reference strain – the CFU of the test strain)/(the CFU of the test strain)] was plotted with the mean CI ± the SD, where a CI of 1.0 indicates no difference in virulence between the reference and test strains. *P* values of <0.05 were considered significantly different, and bars with same lowercase letter are not significantly different from one another.

*sakazakii*. Currently, it is not clear whether the differences in *C. sakazakii inv* genes that exist among the isolates result in any functional differences. Previously, studies on *inv* of *Y. pseudotuberculosis* and *Y. enterocolitica* showed that, despite their high sequence homology (71% identity at the DNA level and 77% identity at the protein level), *E. coli* harboring *Y. enterocolitica inv* (92-kDa protein) was noninvasive to MDCK cells, whereas *inv* of *Y. pseudotuberculosis* (103-kDa protein) conferred the invasive property to *E. coli* (27).Thus, it would be interesting to determine whether the absence of the extra Ig domain found in *C. sakazakii* ATCC 29544 might be able to explain the observed phenotypic variation, such as the invasive property of various *C. sakazakii* isolates.

When the Δ*inv* mutant was tested for invasion, a significant reduction was observed in three epithelial cell lines (Caco-2, INT-407, and Hep-2) compared to the WT strain (Fig. 2). The blocking assay (Fig. 3B and C) and antisense approach (Fig. 4B, see below) also clearly showed reduced invasion by *C. sakazakii* due to an absence of functional *inv* gene product. Furthermore, complete restoration of invasion phenotype by *inv* complementation strain in all three cell lines confirms the importance of the *inv* gene in *C. sakazakii* invasion. Inv is the third putative outer membrane protein, after OmpA and OmpX (17), to be identified in *C. sakazakii*.

Repeated trials to construct an *inv*-overexpressing *E. coli* strain using several expression vector systems with tightly controlled promoters have failed, suggesting a possible toxicity of the putative *inv* gene product in host *E. coli*. The toxicity of certain overexpressed transmembrane proteins is well described. For example, *E. coli unc*, which encodes the membrane-spanning subunits a (36) and b and c (37) of F-ATPase were shown to be extremely toxic, causing total growth inhibition of host *E. coli* [BL21(DE3)] upon IPTG (isopropyl-β-D-thiogalactopyranoside) induction (36, 37). The toxicity of overexpressed membrane proteins was suggested to be due to Sec translocan saturation (38), increased protonophoric activity, unbalanced synthesis of membrane proteins and lipids, and misinsertion of the protein into the membrane (36, 37, 38). In this regard, it is worth mentioning that predicted three-dimensional structure of putative Inv (using Phyre 2.0) mimics the beta-barrel membrane protein structure that is found in proteins of the outer membranes of Gram-negative bacteria and often functions as aqueous pores that allow the passage of small molecules (39). Interestingly, *C. sakazakii* outer membrane proteins, OmpA and OmpX, when successfully overexpressed in *E. coli* (17), turned out to be toxic to the host once they were induced (data not shown). Currently, the toxic mechanism of *C. sakazakii* Inv in *E. coli* is not clear, but the capacity to act as a porin/ion channel might be possible.

In order to assess the effect of *inv* gene expression on invasion, an antisense mRNA technique was used (Fig. 4). The expression of antisense mRNA from a multicopy plasmid has been successfully used in previous studies (40, 41) that were based on the silencing of the gene expression by preventing the synthesis of the protein (42, 43). We introduced here a reverse complement of the complete *inv* gene (r-*inv*) into the pBAD-TOPO vector, resulting in the production of a fused transcript that was flanked by thioredoxin- and His tag-coding sequences. We also confirmed the synthesis of antisense mRNA using reverse transcriptase PCR in an Δ*inv* mutant transformed with pBAD-TOPO::r-*inv* (Fig. 4A), and the expression of r-*inv* in the WT strain resulted in a significant reduction in invasion efficiency and further supports the importance of functional Inv protein for the invasion of *C. sakazakii* into Caco-2 cells.

Tight junctions act as a physical barrier preventing the invasion

of pathogens through the basolateral side of host cells (17, 18, 35). Previously, Kim et al. (17, 18) had reported a critical role for *ompA* and *ompX* in mediating the basolateral invasion of *C. sakazakii*. In the present study and consistent with previous data (18), the disruption of tight junctions enhanced the penetration of *C. sakazakii* WT into Caco-2 cells (with 2.6- and 7.6-fold increases in EGTA-treated Caco-2 cells that were 4 to 7 days old and 14 to 17 days old, respectively, compared to EGTA-untreated Caco-2 cells that were 4 to 7 days old) (Fig. 5). In contrast, the invasion potentials of the Δ*inv* mutant (29% in EGTA-untreated Caco-2 cells that were 4 to 7 days old) did not increase as significantly as those of the WT, showing 29 and 58% invasion potentials in EGTA-treated Caco-2 cells that were 4 to 7 days old and 14 to 17 days old, respectively. If the putative *inv* gene product mediates *C. sakazakii* invasion through the apical side only in Caco-2 cells, the invasion potential of the Δ*inv* mutant should have increased as significantly as or close to that of the WT. Comparable data were obtained when 14-day-old Caco-2 cells were pretreated with EGTA or LPS and used for invasion assay in a transwell system (see Fig. S2B in the supplemental material). Therefore, this information suggests that the putative *inv* gene product is essential for the basolateral invasion of *C. sakazakii* in Caco-2 cells.

Previously, it was reported that the *C. sakazakii* invasion rate increased over the age of the Caco-2 cells used (17, 18). In the present study, we showed that the Inv-dependent invasion potential also increased as the Caco-2 cells advanced in age. When the invasion potentials of the WT were compared between EGTA-untreated Caco-2 cells that were 4 to 7 days old and those that were 14 to 17 days old, a 1.9-fold increase ($6.8 \times 10^4$ CFU/well and $1.3 \times 10^5$ CFU/well, respectively) was observed, whereas a significantly lower fold increase (1.1) was found with the Δ*inv* mutant (Fig. 5A). A similar pattern was observed in EGTA-treated Caco-2 cells (2.9- and 2.1-fold increases, respectively) (Fig. 5B). These data indicate that the *inv*-dependent invasion potential increased as the age of the Caco-2 cells increased.

We examined the effect of the *inv* deletion on the invasion phenotype in the Δ*ompA* background by using a Δ*ompA* Δ*inv* double mutant (Fig. 2). Interestingly, a severely attenuated invasion rate was detected for the Δ*ompA* Δ*inv* double mutant relative to that of either single mutant (Δ*ompA* or Δ*inv*), indicating an additive effect in Caco-2 invasion by *C. sakazakii* in the absence of both *inv* and *ompA* (Fig. 2A). This phenomenon was further supported by the results of the blocking assay using the Δ*inv* mutant with recombinant OmpA-pretreated Caco-2 cells (Fig. 3) and antisense expression of the *inv* gene in the Δ*ompA* mutant (Fig. 4). In addition, the additive effect of OmpA and Inv was also observed in tight-junction-disrupted Caco-2 cells (Fig. 5), as well as in the *in vivo* rat pup model (Fig. 6) (see below). The present study is the first to demonstrate that two outer membrane proteins work additively in *C. sakazakii*'s invasion of Caco-2 cells and in an *in vivo* animal model.

The additive effect of the two invasin molecules, suggested in comparing the invasion potentials of single and double mutants, may imply that OmpA and Inv exhibit independent modes of action. However, the complementation study hints at the presence of different routes to penetrate Caco-2 cells by OmpA and Inv. When *inv* or *ompA* complementation in a respective single mutant background was studied, complete recovery of the invasion potential was observed (Fig. 2) (17). On the other hand, when it was performed in a double mutant, a significantly lower level of complementation was observed (Fig. 5). These data indicate a possible invasion mechanism in which OmpA and Inv are dependent upon each other.

The importance of the putative *inv* gene product and its additive effect with OmpA was also shown in an *in vivo* murine model (Fig. 6). CI values for WT versus the Δ*inv*::Kan$^r$ strain were 4.65 and 4.64 in the liver (Fig. 6A) and spleen (Fig. 6B), respectively, while those for double mutant versus the single mutant were 0.12 (0.21 for Δ*ompA*::Kan$^r$) and 0.39 (0.25 for Δ*ompA*::Kan$^r$) in the liver (Fig. 6A) and spleen (Fig. 6B), respectively. The presence of the kanamycin gene did not compromise the virulence of the Δ*ompA* and Δ*inv* mutants, as determined by an *in vitro* invasion assay (see Fig. S3 in the supplemental material), and we also confirmed the maintenance of the kanamycin gene during infection.

Previously, it was reported that *C. sakazakii* OmpA and OmpX showed an additive effect in invasion using an *in vitro* cell culture model—INT-407 cells—but not in Caco-2 cells (17). On the other hand, the same study described no additive effect for those factors in an *in vivo* rat pup model (17). In the present study, an additive effect was found in the *in vitro* Caco-2 cell model (but not in INT-407 cells) and in an *in vivo* animal model. At present, there is no clear explanation as to why invasion-related proteins behave differently in different cell lines, which obviously would be an interesting subject for further study.

In conclusion, we showed here that putative Inv is essential for invasion of *C. sakazakii* ATCC 29544 to various mammalian epithelial cells (Caco-2, INT-407, and Hep-2) and for penetration through the basolateral side of Caco-2 cells and to deeper organs in a rat pup model. In addition, an additive effect of *ompA* and *inv* was observed in Caco-2 cells and in an animal model. We have also suggested a possible dual mode of invasion involving OmpA and Inv. This report is the first to relate the findings for two proteins working additively *in vitro* as well as *in vivo* in *C. sakazakii* pathogenesis.

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## REFERENCES

1. **Iversen CM, Waddington SL, Forsythe S.** 2004. Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* species. J. Clin. Microbiol. **42:**5368–5370. http://dx.doi.org/10.1128/JCM.42.11.5368-5370.2004.
2. **World Health Organization.** 2004. *Enterobacter sakazakii* and other microorganisms in powdered infant formula: meeting report. MRA series 6. World Health Organization, Geneva, Switzerland.
3. **Caubilla-Barron J, Hurrell ES, Townsend P, Cheetham Loc-Carrillo CO, Fayet MF, Prere Forsythe SJ.** 2007. Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. J. Clin. Microbiol. **45:**3979–3985. http://dx.doi.org/10.1128/JCM.01075-07.
4. **Nazarowec-White M, Farber JM.** 1997. *Enterobacter sakazakii*: a review. Int. J. Food Microbiol. **34:**103–113. http://dx.doi.org/10.1016/S0168-1605(96)01172-5.
5. **Van Acker J, de Smet F, Muyldermans G, Bougatef A, Naessens A, Lauwers S.** 2001. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. J. Clin. Microbiol. **39:**293–297. http://dx.doi.org/10.1128/JCM.39.1.293-297.2001.
6. **Hunter CJ, Petrosyan M, Ford HR, Prasadarao NV.** 2008. *Enterobacter*

*sakazakii*: an emerging pathogen in infants and neonates. Surg. Infect. **9**:533–539. http://dx.doi.org/10.1089/sur.2008.006.

7. **Mullane NR, Iversen C, Healy B, Walsh C, Whyte P, Wall PG, Quinn T, Fanning S.** 2007. *Enterobacter sakazakii* an emerging bacterial pathogen with implications for infant health. Minerva Pediatr. **59**:137–148.
8. **Skovgaard N.** 2007. New trends in emerging pathogens. Int. J. Food Microbiol. **120**:217–224. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.07.046.
9. **Willis J, Robinson JE.** 1988. *Enterobacter sakazakii* meningitis in neonates. Pediatr. Infect. Dis. J. **7**:196–199. http://dx.doi.org/10.1097/00006454-198803000-00012.
10. **Gurtler JB, Kornacki JL, Beuchat LR.** 2005. *Enterobacter sakazakii*: a coliform of increased concern to infant health. Int. J. Food Microbiol. **104**:1–34. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.02.013.
11. **Pagotto FJ, Nazarowec-White M, Bidawid S, Farber JM.** 2003. *Enterobacter sakazakii*: infectivity and enterotoxin production in vitro and *in vivo*. J. Food Prot. **66**:370–375.
12. **Kothary MH, McCardell BA, Frazar CD, Deer D, Tall BD.** 2007. Characterization of the zinc-containing metalloprotease encoded by *zpx* and development of a species-specific detection method for *Enterobacter sakazakii*. Appl. Environ. Microbiol. **73**:4142–4151. http://dx.doi.org/10.1128/AEM.02729-06.
13. **Townsend SM, Hurrell E, Gonzalez-Gomez I, Lowe J, Frye JG, Forsythe S, Badger JL.** 2007. *Enterobacter sakazakii* invades brain capillary endothelial cells, persists in human macrophages influencing cytokine secretion and induces severe brain pathology in the neonatal rat. Microbiology **153**:3538–3547. http://dx.doi.org/10.1099/mic.0.2007/009316-0.
14. **Franco AA, Kothary MH, Gopinath G, Jarvis KG, Grim CJ, Hu L, Datta AR, McCardell BA, Tall BD.** 2011. Cpa, outer membrane protease of *Cronobacter sakazakii* activates plasminogen and mediates resistance to serum bactericidal activity. Infect. Immun. **79**:1578–1587. http://dx.doi.org/10.1128/IAI.01165-10.
15. **Marchler-Bauer A, Zheng C, Chitsaz F, Derbyshire MK, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Lu S, Marchler GH, Song JS, Thanki N, Yamashita RA, Zhang D, Bryant SH.** 2013. CDD: conserved domains and protein three-dimensional structure. Nucleic Acids Res. **41**:D384–D352.
16. **Choi Y, Kim KP, Kim K, Choi J, Shin H, Kang DH, Ryu S.** 2012. Possible roles of LysR-type transcriptional regulator (LTTR) homolog as a global regulator in *Cronobacter sakazakii* ATCC 29544. Int. J. Med. Microbiol. **302**:270–275. http://dx.doi.org/10.1016/j.ijmm.2012.06.001.
17. **Kim K, Kim KP, Choi J, Lim JA, Lee J, Hwang S, Ryu S.** 2010. Outer membrane protein A (OmpA) and X (OmpX) are essential for basolateral invasion of *Cronobacter sakazakii*. Appl. Environ. Microbiol. **76**:5188–5198. http://dx.doi.org/10.1128/AEM.02498-09.
18. **Kim KP, Loessner MJ.** 2008. *Enterobacter sakazakii* invasion in human intestinal Caco-2 cells requires the host cell cytoskeleton and is enhanced by disruption of tight junction. Infect. Immun. **76**:562–570. http://dx.doi.org/10.1128/IAI.00937-07.
19. **Mohan Nair MK, Venkitanarayanan K.** 2007. Role of bacterial OmpA and host cytoskeleton in the invasion of human intestinal epithelial cells by *Enterobacter sakazakii*. Pediatr. Res. **62**:664–669. http://dx.doi.org/10.1203/PDR.0b013e3181587864.
20. **Giri CP, Shima K, Tall BD, Sathyamoorthy V, Hanisch B, Kim KS, Kopecko DJ.** 2012. *Cronobacter* spp. (previously *Enterobacter sakazakii*) invade and translocate across both cultured human intestinal epithelial cells and human brain microvascular endothelial cells. Microb. Pathol. **52**:140–147. http://dx.doi.org/10.1016/j.micpath.2011.10.003.
21. **Leong JM, Fournier RS, Isberg RR.** 1990. Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. EMBO J. **9**:1979–1989.
22. **Isberg RR, Voorhis DL, Falkow S.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50**:769–778.
23. **Amini K, Salehi TZ, Nikbakht G, Ranjbar R, Amini J, Ashrafganjooei SB.** 2010. Molecular detection of *invA* and *spv* virulence genes in *Salmonella enteritidis* isolated from human and animals in Iran. Afr. J. Microbiol. Res. **4**:2202–2210.
24. **Galan JE, Iii RC.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. U. S. A. Microbiology **86**:6383–6387. http://dx.doi.org/10.1073/pnas.86.16.6383.
25. **Isberg RR, Bernes P, Wong KW.** 2003. Uptake of *Yersinia pseudotuberculosis* into cultured cells and integrin receptor signaling, p 37–51. *In* O'Connor CD, Smith DGE (ed), Microbial subversion of host cells. Cambridge University Press, Cambridge, United Kingdom.
26. **Isberg RR, Leong JM.** 1990. Multiple $\beta_1$ chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell **60**:861–871. http://dx.doi.org/10.1016/0092-8674(90)90099-Z.
27. **Pepe JC, Miller VL.** 1990. The *Yersinia enterocolitica* inv gene product is an outer membrane protein that shares epitopes with *Yersinia pseudotuberculosis* invasion. J. Bacteriol. **172**:3780–3789.
28. **Hofmann K, Stoffel W.** 1993. TMbase: a database of membrane spanning proteins segments. Biol. Chem. Hoppe-Seylers **374**:166.
29. **Tusnády GE, Simon I.** 2001. The HMMTOP transmembrane topology prediction server. Bioinformatics **17**:849–850. http://dx.doi.org/10.1093/bioinformatics/17.9.849.
30. **Chang AC, Cohen SN.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
31. **Yeung CY, Chiau JSC, Chan WT, Jiang CB, Cheng ML, Liu HL, Lee HC.** 2013. *In vitro* prevention of *Salmonella* lipopolysaccharide-induced damages in epithelial barrier function by various *Lactobacillus* strains. Gastroenterol. Res. Pract. **2013**:973209.
32. **Jerse AE, Yu J, Tall BD, Kaper JB.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. U. S. A. **87**:7839–7843.
33. **Mange JP, Stephan R, Borel N, Wild P, Kim KS, Pospischil A, Lehner A.** 2006. Adhesive properties of *Enterobacter sakazakii* to human epithelial and brain microvascular endothelial cells. BMC Microbiol. **6**:58–67. http://dx.doi.org/10.1186/1471-2180-6-58.
34. **Mittal R, Wang Y, Hunter CJ, Gonzalez-Gomez I, Prasadarao NV.** 2009. Brain damage in newborn rat model of meningitis by *Enterobacter sakazakii*: a role for outer membrane protein A. Lab. Invest. **89**:263–277. http://dx.doi.org/10.1038/labinvest.2008.164.
35. **Townsend S, Hurrell E, Forsythe S.** 2008. Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak. BMC Microbiol. **8**:64. http://dx.doi.org/10.1186/1471-2180-8-64.
36. **Arechaga I, Miroux B, Runswick MJ, Walker JE.** 2003. Over-expression of *Escherichia coli* F1Fo[ens]ATPase subunit a is inhibited by instability of the *uncB* gene transcript. FEBS Lett. **547**:97–100. http://dx.doi.org/10.1016/S0014-5793(03)00677-X.
37. **Miroux B, Walker JE.** 1996. Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J. Mol. Biol. **260**:289–298. http://dx.doi.org/10.1006/jmbi.1996.0399.
38. **Narayanan A, Ridilla M, Yernool DA.** 2011. Restrained expression, a method to overproduce toxic membrane proteins by exploiting operator-repressor interactions. Protein Sci. **20**:51–61. http://dx.doi.org/10.1002/pro.535.
39. **Sugawaras E, Nikaido H.** 1994. OmpA protein of *Escherichia coli* outer membrane occurs in open and closed channel forms. J. Biochem. Chem. **269**:17981–17987.
40. **Chen G, Patten CL, Schellhorn HE.** 2003. Controlled expression of an *rpoS* antisense RNA can inhibit RpoS function in *Escherichia coli*. Antimicrob. Agents Chemother. **47**:3485–3493. http://dx.doi.org/10.1128/AAC.47.11.3485-3493.2003.
41. **Yinduo JI, Marra A, Rosenberg M, Woodnutt G.** 1999. Regulated antisense RNA eliminates alpha-toxin virulence in *Staphylococcus aureus* infection. J. Bacteriol. **181**:6585–6590.
42. **Louise CVR, Hans USP, Kim KM.** 2007. Hitting bacteria at the heart of the central dogma: sequence-specific inhibition. Microb. Cell Fact. **6**:24. http://dx.doi.org/10.1186/1475-2859-6-24.
43. **Zhao RC, McIvor RS, Griffin JD, Verfaillie CM.** 1997. Gene therapy for chronic myelogenous leukemia (CML): a retroviral vector that renders hematopoietic progenitors methotrexate-resistant and CML progenitors functionally normal and nontumorigenic in vivo. Blood **90**:4687–4698.