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Possible roles of LysR-type transcriptional regulator (LTTR) homolog as a global regulator in *Cronobacter sakazakii* ATCC 29544

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ABSTRACT

Cronobacter sakazakii is a Gram-negative opportunistic pathogen that causes necrotizing enterocolitis, sepsis, and meningitis in premature infants. The genetic basis of *C. sakazakii* virulence is poorly understood. In this study, the putative LysR-type transcriptional regulator (LTTR) gene (*ESA_01081* homolog) was characterized as a possible regulator for *C. sakazakii* pathogenesis. An in-frame deletion mutant of the *ESA_01081* homolog and its cognate complementation strain were constructed and characterized for pathogenesis (adhesion/invasion potentials to human intestinal cells and in vivo rat pup challenge assay), biofilm formation, resistance to oxidative stress and induction of IL-8 secretion. LTTR-deficient *C. sakazakii* ATCC 29544 exhibits significantly attenuated phenotypes in all the properties tested, except adhesion. Our data strongly suggest that the putative gpESA_01081 homolog, plays an important regulatory role in diverse biological processes including the virulence of *C. sakazakii*. This is the first report of a functional global regulator in *C. sakazakii*.

Introduction

Cronobacter spp. (formerly known as *Enterobacter sakazakii*) is considered to be an emerging pathogen that causes bacteremia, meningitis, and local necrotizing enterocolitis in premature infants (Bar-Oz et al., 2001; Lai, 2001; Muytjens et al., 1983; Stoll et al., 2004). It can form biofilms on various materials and resist environmental stresses (Dancer et al., 2009; Hartmann et al., 2010; Johler et al., 2010). In addition, it can adhere to/invade host eukaryotic cells (Kim and Loessner, 2008), persist in macrophage (Townsend et al., 2007) and cause brain damage in a rat model (Mittal et al., 2009; Townsend et al., 2007). High mortality rates ranging from 40 to 80% have been reported (Forsythe, 2005; Mullane et al., 2007).

Although several genes have been identified to be involved in the virulence of *Cronobacter sakazakii*, we are still far from understanding its pathogenesis. The cell-bound zinc-containing metalloprotease encoded by *zpx*, caused rounding of Chinese hamster ovary (CHO) cells (Kothary et al., 2007), which might be important in dissemination of the pathogen into the systemic circulation. Outer membrane protein A (OmpA) plays a significant role in adhesion/invasion to Caco-2 and INT-407 cells (Kim et al., 2010; Mohan Nair and Venkitanarayanan, 2007; Singamsetty et al., 2008) and brain damage such as brain abscess formation and gliosis (Mittal et al., 2009). Kim et al. (2010) also reported that another outer membrane protein, OmpX, is important in basolateral invasion of *C. sakazakii* ATCC 29544. Together with the release of the complete genome sequence information (Kucerova et al., 2010), recent successful application of lambda red recombination to construct site-specific in-frame deletion mutants (Kim et al., 2010) will significantly facilitate more detailed studies on this bacterium.

In an attempt to search for invasion-related virulence factors in *C. sakazakii* ATCC 29544, we identified a LysR-type transcriptional regulator (LTTR) homologue and it was further characterized for its possible function as a global regulator. LTTRs are part of a large protein family and display a well-conserved structure with an N-terminal HTH (helix-turn-helix) DNA-binding motif and a C-terminal substrate-binding domain (Maddocks and Oyston, 2008). Orthologues of LTTRs are present in numerous species of bacteria and are known to regulate a range of regulons involved in metabolism, cell division, quorum sensing, virulence, motility, nitrogen fixation, oxidative stress responses, toxin production,

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attachment and secretion (MacLean et al., 2008; Maddocks and Oyston, 2008). This is the first report of identification of a functional global regulator in *C. sakazakii*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains used in this study are listed in Table 1. *C. sakazakii* ATCC 29544 (American Type Culture Collection, MA) and the isogenic strains were grown in Luria–Bertani (LB) broth (Difco) at 37 °C with aeration unless indicated otherwise. Strains containing the temperature-sensitive plasmids, pKD46 or pCP20, were grown on LB media at 30 °C. The antibiotic concentrations used were: 50 μg/ml ampicillin (Ap); 50 μg/ml kanamycin (Kan); 50 μg/ml tetracycline (Tc); 25 μg/ml chloramphenicol (Cm).

Cell culture

Human enterocyte-like epithelial Caco-2 (ATCC, Manassas, VA) and human epithelial INT-407 (ATCC) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Invitrogen). Trypsin-treated cells were seeded (approximately 5×10^4 cells per well) into 24-well tissue culture plates (TPP, Switzerland) and grown at 37 °C in the presence of 5% CO_2. Medium was replaced every 2 days. Cell viability was determined by trypan blue straining.

Target gene sequencing and bioinformatics

In order to acquire the complete DNA sequence of the LTTR homolog in *C. sakazakii* ATCC 29544, primers were designed based on the *C. sakazakii* ATCC BAA-894 database (LTTR-conF; 5′-GCA AAT GCT GTT TGG CGA ACA G-3′, LTTR-conR; 5′-GAT TGT AGC AGA GTT GCC GCT A-3′). PCR products were separated in 1% agarose gels, and purified using the Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI). DNA sequence was obtained and used for bioinformatic analysis.

The blastp algorithm was used for similarity searches through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). TMPred (Hofmann and Stoffel, 1993) and PSORTb (version 3.0) (Yu et al., 2010) programs were used to predict transmembrane domains and subcellular localization of the protein, respectively. Conserved protein family and domains were identified using the InterProScan program (Pfam database, http://www.ebi.ac.kr).

Construction of ESA_01081 homolog deletion mutant

An *ESA_01081* homolog deletion mutant strain was generated using the lambda red recombination method as described by Datsenko and Wanner (2000). Briefly, the homologous sequences around the target gene were used for construction of an insertion fragment containing a selectable marker. Then, homologous recombination was conducted by lambda recombinase from helper plasmid, pKD46. For induction of the recombinase from plasmid, L-arabinose (100 mM) was used (Kim et al., 2010). The kanamycin resistance (Kan^r) cassette from plasmid pKD13 was amplified by using the primers LTTR-lamb-F (5′-TAA TAA GTG GTA AGC ACG GTT GCG TTC GCA CGA GGT CAT CTG TAG GCT GGA GCT GCT TCG-3′) and LTTR-lamb-R (5′-AAA CTG ATG ATG AGC GCG CCG TGT GAC GCG CTC CGC GAA A AT TCC GGG GAT CCG TCG ACC-3′) as described by Datsenko and Wanner (2000). The PCR product was transformed into the ES1001 strain, which harbors plasmid pKD46, by electroporation, which was then selected in 50 μg/ml of kanamycin. Finally, the antibiotic cassette was removed by using pCP20 plasmid as described previously (Kim et al., 2010) and ESA2005, a null deletion mutant for *ESA_01081* homolog, was constructed.

Complementation study

For complementation, the *ESA_01081* homolog open reading frame (ORF) was amplified using primers LTTR-SalI (5′-CGT CAG GAT *GTC GAC* CAG AAT G-3′) and LTTR-SphI (5′-TGA TGA TGA *GCA TGC* CGT GTG A-3′) by PCR using 59 °C of annealing temperature and 1 min extension time. The PCR product was digested with SalI and SphI, and cloned in plasmid pACYC184, which was then transformed to DH5α by heat shock. After determining the sequence, the complementation vector, pESA_01081 was transformed into the deletion mutant by electroporation.

Invasion assay

In order to determine the bacterial invasion potential to mammalian cells, a gentamicin protection assay was performed (Kim et al., 2010) using Caco-2 and INT-407. Briefly, bacteria were prepared by transferring a 2% inoculum from overnight culture into fresh, pre-warmed LB, incubating for 2 h, collecting cells by centrifugation, washing with phosphate-buffered saline (PBS, pH 7.4)

Table 1
Bacteria strains and plasmids used in this study.

Strains or plasmid	Relevant characteristic[a]	Reference or source
C. sakazakii		
ATCC 29544	Wild-type strain	ATCC
ES1001	ATCC 29544 harboring pKD46 (Ap^r)	This study
ES2004	ATCC 29544 derivative *ΔESA_01081* homolog::*kan*	This study
ES2005	ATCC 29544 derivative *ΔESA_01081* homolog	This study
ES2006	ES2005 with pESA_01081	This study
E. coli		
DH5α	*supE44 hsdR17 recA1 gyrA96 thi-1 relA1*	Hanahan (1983)
EC100D™	*pir*$^+$	Epicentre
Plasmids		
pKD13	*oriR6Kγ* Ap^r FRT Kan^r FRT	Datsenko and Wanner (2000)
pKD46	*oriR101 repA101ts* Ap^r *araBADpgam-bet-exo*	Datsenko and Wanner (2000)
pCP20	*oripSC101*^{ts} Ap^r Cm^r cI857λ $P_R$flp	Datsenko and Wanner (2000)
pACYC184	*Tet*^{r} *Cm*^{r}; *p15A ori*	Crowe et al. (1984)
pESA_01081	pACYC184::*ESA_01081* homolog	This study

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

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and re-suspending in PBS. Monolayer cells were infected with bacteria at a multiplicity of infection (MOI) of 100 (5×10^7 CFU/ml) and incubated for 1.5 h, without subsequent centrifugation. After washing four times with PBS, fresh media was added containing gentamicin (100 μg/ml; Sigma), and plates were incubated further for 1.5 h, followed by washing five times with PBS. Then, 1 ml of Triton X-100 (0.4% in PBS) was added, and plates were incubated for another 30 min before collecting the bacteria and plating onto LB agar in decimal dilutions.

Adhesion assay

C. sakazakii prepared as for the invasion assay (5×10^7 CFU/ml), was added to the mammalian cell (Caco-2 and INT-407) monolayer at a MOI of 100, and incubated for 30 min. After washing 5 times with PBS, Triton X-100 treatment and enumeration of adhesive *C. sakazakii* were performed.

Biofilm assay

The experiment was performed as described previously (Peeters et al., 2008) with modifications. *C. sakazakii* was inoculated into 3 ml LB broth and incubated at 37 °C with aeration until the cell density reached 2.5×10^8 CFU/ml. The culture was diluted 1:100 in LB broth, and 500-μl portions were loaded in triplicate into a 24-well tissue culture plate (Nunc, Denmark), followed by further incubation at 37 °C for 48 h without shaking. For fixation of the biofilms, 100 μl of 99% methanol was added (15 min), after which supernatants were removed and the plates were air-dried. Then, 500 μl of crystal violet (CV) solution was added and after 20 min, the excess CV was removed and washed with PBS. Finally, bound CV was released by adding 250 μl of 95% ethanol (Merck). The absorbance was measured at 570 nm using a SUNRISE-BASIC TECAN microplate reader (Tecan, Austria).

H_2O_2 sensitivity assay

The overnight (16 h) culture of *C. sakazakii* was sub-cultured in LB medium for 4 h, which was then adjusted to a cell density of 10^7 CFU/ml. Diluted samples (each 10 μl) were dropped on the surface of LB agar containing 500 μM of H_2O_2 for comparing the sensitivity pattern and incubated for 8 h for CFU counting. In addition, diluted samples (each 100 μl) were plated on LB agar containing 500 μM of H_2O_2 and the sensitivity was assessed by comparing CFU after 8 h incubation.

Interleukin-8 secretion assay

Approximately 1×10^5 Caco-2 cells were infected with *C. sakazakii* at a MOI of 100 and incubated for 2 h at 37 °C in the presence of 5% CO_2. The culture medium was harvested and centrifuged at 15,000 × *g* for 10 min to pellet the residual bacteria. The supernatant was collected and the secreted interleukin-8 (IL-8) levels were determined using a BD OptEIA human IL-8 enzyme-linked immunosorbent assay II kit (BD Biosciences) according to the manufacturer's instructions.

In vivo virulence assay

In vivo virulence was tested in rat pups by competitive index analysis (Kim et al., 2010; Mohan Nair and Venkitanarayanan, 2007). Three-day-old Sprague-Dawley rat pups were purchased from the Institute of Laboratory Animal Resources, Seoul National University (Seoul, Korea). Overnight cultures of *C. sakazakii* strains were collected and re-suspended in PBS. Five rat pups in each group were infected through oral gavage with a mixed inoculum of 10^9 CFU of each WT and mutant strain (ES2004 or ES2006). For analysis of bacterial colonization in organs, all rats were euthanized with a mixture of ketamine and xylamine at 24 h post infection. 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 with appropriate antibiotics. The results are presented as competitive index (CI) values, which were calculated by dividing the number of mutant strain CFU by the number of WT strain CFU.

Results and discussion

Identification and bioinformatic analysis of LTTR in C. sakazakii ATCC 29544

During the screening of an invasion-deficient *C. sakazakii* ATCC 29544 mutant library, we obtained the nucleotide sequence of an LTTR homolog (GenBank Access Number, JQ080074). Deduced protein sequence analysis showed 100% identity with putative gpESA_01081 in *C. sakazakii* ATCC BAA-894. It contains a bacterial regulatory HTH motif at the N-terminus and a LysR substrate-binding domain. It shares high similarities (78–88% identities) with LysR type transcriptional regulators (LTTRs) (named YeiE in some bacteria) of *Enterobacter cloacae*, *Citrobacter*, *Klebsiella*, *Salmonella* Typhimurium, *Shigella*, *Erwinia* and *Pantoea*.

LTTR in C. sakazakii ATCC 29544 plays an important role in invasion but not in adhesion

We constructed an *ESA_01081* homolog deletion mutant (ES2005) using the lambda red recombination system (Fig. 1A). The deletion in the targeted gene was confirmed by PCR with specific primers, which showed no amplification of ~1.2 kb *ESA_01081* homolog gene in the mutant (Fig. 1B). We examined the growth

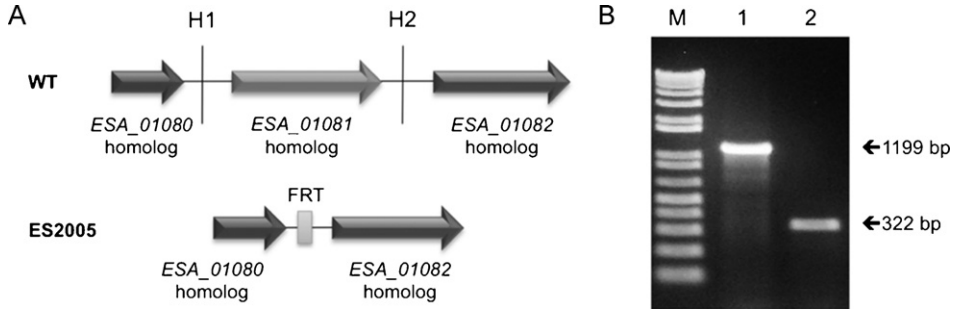

Fig. 1. Construction of the *ESA_01081* homolog mutant by lambda red system. (A) Gene arrangement near *ESA_01081* homolog in *C. sakazakii* WT as adapted from *C. sakazakii* ATCC BAA-894 database and deletion mutant (ES2005). H1 and H2 indicate 40-nt homology extensions and FRT indicates FLP recognition target site. The nucleotide numbers are adopted from *C. sakazakii* whole genome database. (B) PCR confirmation of the mutant: Lane M, the nucleotide size marker; Lane 1, WT; Lane 2, ES2005.

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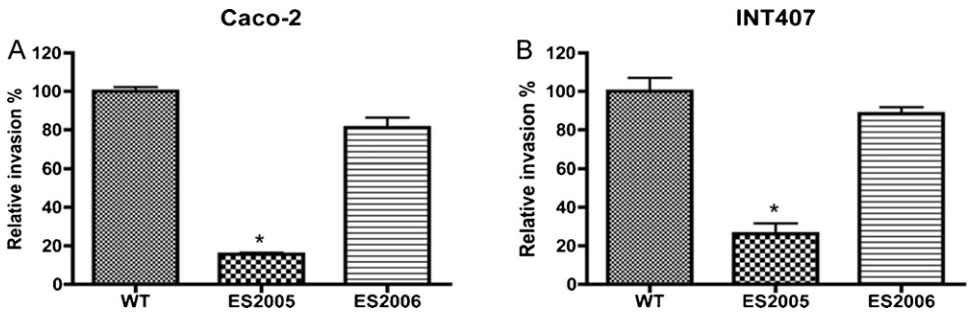

Fig. 2. Invasion of *C. sakazakii* strains in Caco-2 (A) and INT-407 (B). Confluent monolayers of eukaryotic cells were infected with *C. sakazakii* strains (WT, ES2005, and ES2006) at an MOI of 100 and incubated for 1.5 h, followed by gentamicin treatment (100 μg/ml) for 1.5 h. The cells were then treated with 0.4% Triton X-100 to obtain the intracellular bacteria. Relative percent invasion was calculated by [(CFU number of the mutants protected from gentamicin/CFU number of wild type protected from gentamicin) × 100]. The error bars represent means ± SD from three independent experiments performed in triplicate. * $p < 0.05$.

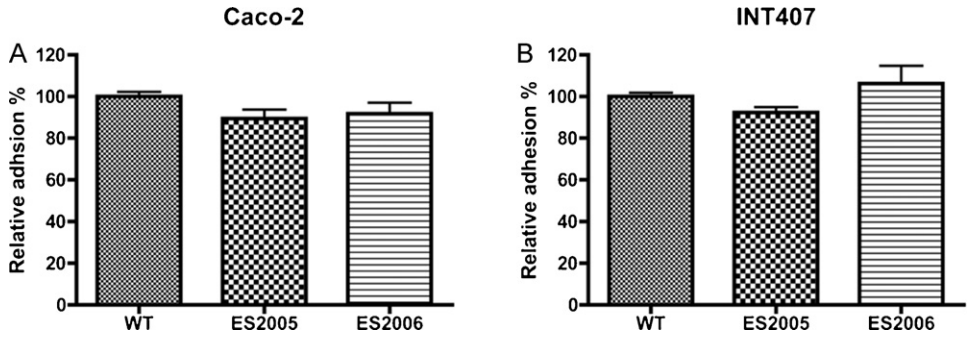

Fig. 3. Adhesion of *C. sakazakii* strains onto Caco-2 (A) and INT-407 (B). *C. sakazakii* strains (WT, ES2005, and ES2006) were incubated with confluent monolayers of the host cells for 30 min, followed by PBS washing and Triton X-100 treatment for bacterial enumeration. Relative percent adhesion was calculated by [(CFU number of the mutants adhered/CFU number of wild type adhered) × 100]. The error bars represent means ± SD from three independent experiments performed in triplicate.

level of the mutant and confirmed no growth defect (data not shown).

Invasion potentials of the WT, deletion mutant and complemented strain into Caco-2 and INT-407 cells were compared (Fig. 2). Approximately 15.6% and 26.3% of ES2005 was able to penetrate Caco-2 and INT-407 cells, respectively, compared to 100% of WT, while the complemented strain (ES2006) restored invasion ability similar to WT ($p < 0.05$) (Fig. 2A and B). In the case of the adhesion assay, no statistical significance was found between WT and the mutant strain (Fig. 3A and B). These results suggest that the putative LysR-type protein in *C. sakazakii* ATCC 29544 plays a role in regulating genes involved in host cell invasion, but not in adhesion. Taken together with a previous report on *Cronobacter* invasion (Kim et al., 2010), we speculate that there is a stepwise process in *C. sakazakii* ATCC 29544 infection.

LTTR in C. sakazakii ATCC 29544 affects biofilm formation and resistance to oxidative stress

Previously, Kovacikova et al. (2005) and Bernier et al. (2008) reported the possible involvement of a LysR type regulator in biofilm formation, and OxyR was reported to up-regulate various genes upon H_2O_2 treatment in different bacteria (Storz and Tartaglia, 1992). Biofilms are interface-associated consortia of microorganisms (Hartmann et al., 2010), and survival of *C. sakazakii* in biofilms under different environmental conditions has been investigated (Beuchat et al., 2009; Hurrell et al., 2009; Kim et al., 2008). In this study, the ES2005 showed reduced biofilm formation (46.5% compared to 100% WT, $p < 0.05$) (Fig. 4) and was more sensitive to hydrogen peroxide (46% compared to WT, $p < 0.05$) (Fig. 5), compared to WT.

Controlling of bacterial biofilm formation is important for food safety, as biofilms resist anti-bacterial treatments such as sanitizing chemical agents (Van Houdt and Michiels, 2010). Ability of biofilm formation is different for different strains and is highly dependent on the medium and surface materials used (Kim et al., 2008). A recent study showed that several genes were involved in *C. sakazakii* biofilm formation (Johler et al., 2010). We found that LTTR also plays an important role in *C. sakazakii* biofilm formation. Elucidation of the mechanism by which LTTR is involved would deepen our understanding of the genes involved in biofilm production and may lead to practical applications to prevent biofilm formation.

The LTTR family was shown also to play an important role in oxidative stress response in many pathogenic organisms. OxyR, an LTTR, up-regulates various genes upon H_2O_2 treatment in different bacteria (Storz and Tartaglia, 1992; Wang et al., 2008). During infection, intracellular pathogens have to either avoid the damage by oxygen radicals or resist their effects using thioredoxin and glutathione/glutaredoxin systems which include enzymes

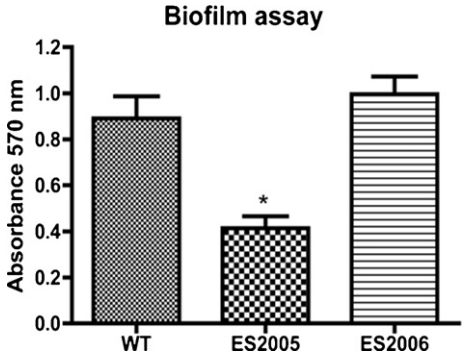

Fig. 4. Biofilm formation. The crystal violet biofilm assay was performed in triplicate and repeated three times. Biofilm formation is indicated by absorbance of 570 nm. Error bars indicate standard deviations. *$p < 0.05$.

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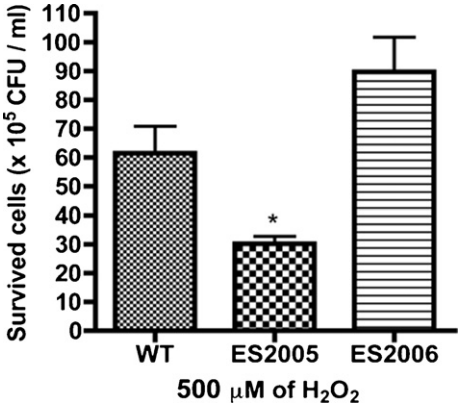

Fig. 5. Sensitivities to hydrogen peroxide of *C. sakazakii* strains. Sub-cultures of each strain were serially diluted and dropped/plated on LB agar containing 500 μM of H_2O_2. Assay was performed in triplicate and repeated three times. Error bars indicate standard deviations. $^*p < 0.05$.

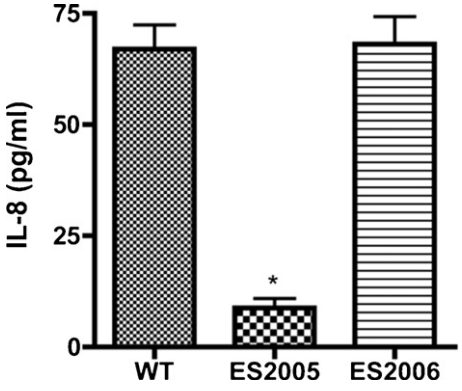

Fig. 6. IL-8 secretion of Caco-2 cells stimulated with *C. sakazakii* strains. Confluent monolayers of eukaryotic cells were infected with *C. sakazakii* at an MOI of 100 and incubated for 2 h. Culture media were separated by centrifugation. The supernatant was collected and the secreted interleukin-8 (IL-8) levels were determined by ELISA. The assay was performed in triplicate and repeated three times. Error bars indicate standard deviations. $^*p < 0.05$.

which shuttle redox potential from NADPH to cytosolic substrates, thereby providing a repair system for oxidized sulfhydryl groups in cytosolic proteins (Bjur et al., 2006; Lahiri et al., 2008). The enhanced sensitivity against hydrogen peroxide of the LTTR mutant (Fig. 5) suggests a role for LTTR in resistance to oxidative stress during the late times of infection. How LTTR is involved in oxidative stress response of *C. sakazakii* needs to be further studied.

LTTR in C. sakazakii ATCC 29544 also affects induction of IL-8 secretion

C. sakazakii is associated with local acute intestinal inflammatory responses, and infection with *C. sakazakii* induces a pro-inflammatory response in newborn rats. Recent studies showed that neutrophils and macrophages played an important role in the clearance of *C. sakazakii* during the initial stages of infection (Emami et al., 2011b). Depletion of neutrophils (polymorphonuclear leukocytes; PMNs) and macrophages led to increased recruitment of dendritic cells (DC) to the intestine of *C. sakazakii*-infected mice (Emami et al., 2011b). During infection, interaction between *C. sakazakii* and DC induced greater levels of cytokines (IL-10 and TGF-β) secreted by either epithelial cells and/or DC resulting in tight junction disruption (Emami et al., 2011a). In addition, IL-8 is a pro-inflammatory cytokine and a mediator of localized inflammatory responses, serving as a chemical signal that attracts neutrophils to the site of inflammation (Harada et al., 1994; Singer and Sansonetti, 2004). In this study, Caco-2 cells secreted low levels of IL-8 when challenged by ES2005 (10.75 ± 4.2 pg/ml) compared to WT (66.98 ± 7.2 pg/ml) (Fig. 6), while in ES2006, it was fully recovered. In the complementation strain, all the defective phenotypes were completely recovered.

C. sakazakii ATCC 29544 lacking LTTR showed attenuated virulence in rat pups

To assess the effects of LTTR on virulence in vitro, we used a newborn rat model. The neonatal rat model was used to assess bacterial translocation from the intestinal tract to deeper tissues following endotoxin administration, including apoptosis and intracranial infection (Hunter et al., 2008; Townsend et al., 2007). The lack of LTTR in *C. sakazakii* ATCC 29544 caused significant defects in phenotypes related to virulence including its invasion into epithelial cells, its ability for biofilm formation, resistance to oxidative stress, and ability to induce IL-8 secretion. Thus, we next examined whether the LTTR is required for virulence of *C. sakazakii* in vivo. To evaluate virulence of the LTTR mutant, cohorts of five 3-day-old rat pups were inoculated orally with a mixed inoculum of WT and mutant strains. The Kan-cassette-inserted mutant (ES2004) strain displayed reduced colonization compared to WT in both liver and spleen. Moreover, the introduction of pESA_01081 (ES2006) recovered the decreased colonization of the LTTR mutant strain in both liver and spleen (Fig. 7). These results suggest that putative gpESA_01081 is an important regulator of virulence in *C. sakazakii*.

In summary, characterization of the putative gpESA_01081 homolog suggests that the LysR-type transcriptional regulator

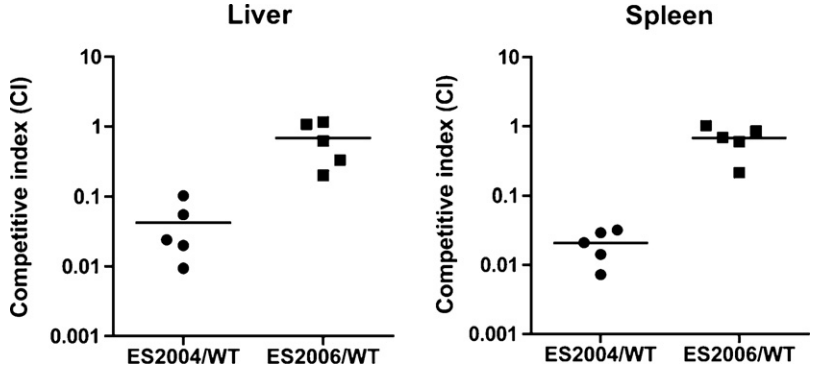

Fig. 7. Virulence phenotype of *C. sakazakii* strains in vivo. Groups of 3-day-old rat pups were infected with a mixed inoculum of WT and *ESA_01081* homolog mutant chromosomally marked with Kan marker (ES2004) or mixed inoculum of WT and complementation mutant harboring complementation plasmid, pESA_01081 (ES2006), and the competitive index (CI) values were determined in the liver and spleen. Each dot point represents one animal, and horizontal bars indicate mean value of CI.

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homologue is a global regulator affecting diverse properties including several factors involved in virulence of *C. sakazakii* ATCC 29544. Further studies are now required to elucidate more detailed molecular mechanisms, by which the putative gpESA_01081 homolog conducts its regulation.

Acknowledgments

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