creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

A Thesis for the Degree of Master of Science

Effects of murein lipoprotein (Lpp) on virulence of *Cronobacter sakazakii* ATCC 29544

Cronobacter sakazakii ATCC 29544 균의 병원성 인자 murein lipoprotein (Lpp)의 효과

August 2018

Graduate School of Agriculture and Life Science
Seoul National University
Department of Agricultural Biotechnology

Jinshil Kim

석사학위논문

Effects of murein lipoprotein (Lpp) on virulence of *Cronobacter sakazakii* ATCC 29544

Cronobacter sakazakii ATCC 29544 균의 병원성 인자 murein lipoprotein (Lpp)의 효과

지도교수 유 상 열

이 논문을 석사학위논문으로 제출함
2018 년 8 월

서울대학교 대학원
농생명공학부
김진실

김진실의 석사학위논문을 인준함
2018 년 8 월

위원장 서 진 호 (인)
부위원장 유 상 열 (인)
위원 이 기 원 (인)

ABSTRACT

Jinshil Kim

Department of Agriculture Biotechnology

The Graduate school

Seoul National University

Cronobacter sakazakii is a foodborne pathogen that causes necrotizing enterocolitis and sepsis with a high mortality particularly in neonates. Since very little is known about the mechanisms of its pathogenicity. Identifying virulence determinants of the pathogen is of high priority. Screening of outer membrane proteins in *C. sakazakii* was conducted to identify new virulence factors and murein lipoprotein, encoded by *lpp* gene, was studied here. To examine the role of *lpp* in *C. sakazakii* virulence, *lpp* mutant was constructed using Lambda-Red recombination and its phenotypes were characterized. Cell membrane permeability assays showed that the membrane integrity is altered by the *lpp* mutation. In addition, the *lpp* mutant exhibited hyper-motility on 0.3% soft agar and higher expression level of flagellar biosynthesis genes. Consistent with this, *lpp* mutant cells were hyper-flagellated

observed by transmission electron microscopy. Importantly, the absence of *lpp* in *C. sakazakii* decreased invasion (by at least 3 folds) and adhesion ability (by at least 4 folds) toward Caco-2 human intestinal epithelial cells, respectively, as well as reduced survival within RAW 264.7 murine macrophages compared with wild type. The invasiveness of the mutant was not increased as that of the wild type in tight junction-disrupted Caco-2 cells generated by EGTA treatment, indicating that *lpp* is involved in the basolateral invasion of *C. sakazakii*. Furthermore, the extracellular provision of purified recombinant Lpp decreased the adhesion ability of *C. sakazakii* toward the Caco-2 cells in a dose-dependent manner, suggesting that Lpp may function as a new adhesin binding to unknown receptor present on the surface of the host cells. These results suggest that *lpp* has crucial roles in the virulence of *C. sakazakii*.

Keywords: *Cronobacter sakazakii*, murein lipoprotein (Lpp), virulence, Caco-2, adhesin

Student Number: 2016-21720

CONTENTS

ABSTRACT.....i

CONTENTS.....iii

List of Figures.....vi

List of Tables.....vii

1. INTRODUCTION.....1

2. MATERIALS AND METHODS.....4

2.1. Bacterial strains, plasmids, and growth conditions.....4

2.2. Complementation study.....4

2.3. Growth kinetics.....4

2.4. Outer membrane permeability assays.....5

2.5. Motility assay.....6

2.6. Transmission electron microscopy (TEM) analysis.....7

2.7. RNA purification and transcript analysis.....7

2.8. Cell culture and EGTA treatment.....8

2.9. Survival assay.....9

2.10. Invasion assay.....9

2.11. Western blot analysis.....10
2.12. Adhesion assay.....11
2.13. Overexpression and Purification of *C. sakazakii* Lpp in *E. coli*.....12
2.14. Inhibition assay.....13
2.15. Statistical analysis.....14
3. RESULTS.....20
3.1. Construction and growth characteristics of the *lpp* mutant in *C. sakazakii*.....20
3.2. Cell membrane integrity of the *lpp* mutant.....23
3.3. Lpp mutant is hyper-motile due to higher expression of flagella.....26
3.4. Lpp affects intracellular survival.....29
3.5. Lpp is affected invasive ability of *C. sakazakii* into human epithelial cells.....32
3.6. Lpp is essential for basolateral invasion by *C. sakazakii*.....35
3.7. Recombinant Lpp inhibits adherence of *C. sakazakii* to Caco-2 cells..... 37
4. DISCUSSION.....43

REFFERENCES.....47

국문초록.....53

List of Figures

Fig. 1. *lpp* homolog in *C. sakazakii* ATCC 29544.....21

Fig. 2. Growth kinetics of *C. sakazakii*.....22

Fig. 3. Effect of the *lpp* gene on the regulation of motility in *C. sakazakii*.....27F

ig. 4. Intracellular survival of *C. sakazakii*.....30

Fig. 5. The effect of *lpp* mutation on the host cell invasion and cellular levels of OmpA and OmpX.....33

Fig. 6. Invasion of *C. sakazakii* into Caco−2 cells after pretreatment with EGTA.....36

Fig. 7. The effect of *lpp* mutation on the host cell adhesion and inhibition of adhesion by purified protein.....39

Fig. S1. Integrity of the membrane in the *lpp* mutant of *C. sakazakii*.....41

Fig. S2. Cytotoxicity of recombinant Lpp (rLpp) protein.....42

List of Tables

Table 1. Bacterial strains and plasmids used in this study.....15

Table 2. Oligonucleotides used in this study.....18

Table 3. Integrity of the membrane in the *lpp* mutant of *C. sakazakii*.....25

1. INTRODUCTION

Cronobacter sakazakii is a motile, nonspore-forming, facultative anaerobic Gram-negative microorganism (Iversen et al., 2008; Iversen et al., 2004). This bacterium is an emerging foodborne opportunistic pathogen that causes necrotizing enterocolitis, meningitis, and sepsis in infants, particularly those born prematurely or of low birth weight (<2500 g) (Bar-Oz et al., 2001; D. Drudy, 2006; Jøker et al., 1965). Although the incidence rate is quite low, the mortality rate by *C. sakazakii* infection is from 40 to 80% (Hunter et al., 2008; Mullane et al., 2007; Willis and Robinson, 1988). Several studies have been reported about virulence factor of *C. sakazakii*, however, little is known about the mechanism of its pathogenicity. Previous study reported that outer menbreane protein A (OmpA), OmpX, and Inv from *C. sakazakii* are essential for the invasion/adhesion into host cells, Caco-2 and INT 407 cells, and play crucial roles in the basolateral invasion of host cells (Dilini Chandrapala, 2014; Kim et al., 2010). The plasmid-encoded MCP is also important for the pathogenesis of *C. sakazakii*, involved in virulences, motility, and biofilm formation (Choi et al., 2015). Recent study revealed that Labp encoded by CSK29544_02616 is

new virulence factor to control lipopolysaccharides (LPS) in *C. sakazakii* (Kim et al., 2018).

The various lipoproteins exist in Gram−negative bacteria, and play important biological roles. Particularly, murein (or Braun) lipoprotein (Lpp), which was discovered approximately 40 years ago, is one of the major outer membrane proteins of family Enterobacteriaceae (Klaus Hantke, 1973). The Lpp is the most abundant small (~8−kDa) lipoprotein in *Escherichia coli* at ~750,000 copies per cell (Cowles et al., 2011). The Lpp exists in two separate forms, a 'bound−form' and a 'free−form'. One−third of Lpp, the bound−form, connects the outer membrane to the peptidoglycan (PG). However, the free−form Lpp, which is not attached to PG, is surface exposed, but its functions are unknown (Cowles et al., 2011; Wilson and Bernstein, 2016). In the wild type, the ratio of free to bound−form Lpp is approximate 1.8:1 ratio respectively (Egan, 2018).

Previously, the Lpp has been reported to involve in pathogenesis of various bacteria, including *E. coli*, *Salmonella enterica* serovar Typhimurium, *Klebsiella pneumoniae*, and *Yersinia pestis* (Diao et al., 2017; Fadl, Sha, Klimpel, Olano, Galindo, et al., 2005; Fadl, Sha, Klimpel, Olano, Niesel, et al., 2005; H Zhang, 1997; Hsieh et al.,

2013; Sha et al., 2008; Sha et al., 2004). For instance, *lpp* mutant exhibits increased outer membrane permeability, leakage of periplasmic, and increased outer membrane vesicle release in *E. coli* (Daniel W. Yem, 1978; Hideho Suzuki and Hirota, 1978). Recent study reported that the bound-form Lpp contributes to resistance to complement-mediated bacterial lysis by influencing group 2 capsule assembly in a Uropathogenic *E. coli* (Diao et al., 2017). The lack of Lpp in *S.* Typhimurium, which have two highly homologous genes; *lppA* and *lppB*, showed attenuated *in vivo*, reduced their motility, invasive ability, and cytokine/chemokine production compared to the wild type *in vitro* (Fadl, Sha, Klimpel, Olano, Galindo, et al., 2005; Fadl, Sha, Klimpel, Olano, Niesel, et al., 2005; Sha et al., 2004). Likewise, *K.* pneumonia Lpp contribute to virulence, involved serum resistance, antiphagocytosis, and proinflammatory cytokine stimulation (Hsieh et al., 2013).

Nevertheless, the function of Lpp in the virulence of *C. sakazakii* has not yet been determined. To examine the effect of *lpp* homolog in *C. sakazakii*, in the present study, I investigated the role of *lpp* in outer membreane integrity, motility, intracellular survival in murine macrophage, and invasive/adhesive ability in intestinal epithelial cell.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, the *C. sakazakii* and *E. coli* strains were grown in Luria-Bertani (LB) medium at 37° C with constant shaking.

2.2. Complementation study

To complement the *lpp* mutation, the coding region on the *lpp* (237 bp) and its potential promoter region (585 bp upstream of the start codon of the *lpp*) from the genomic DNA of *C. sakazakii* were amplified with primer sets, *lpp*-pBR322-F-Sca I (5′-GGT AAA TCC GCG AAA TCC GTT-3′) and *lpp*-pBR322-R-Sca I (5′-CAC ACG GTA CTA TTA CTT ACG G-3′) modified 5′ phosphorylation. The fragment were ligated into the blunt-ended Sca I-digested pB322 vector. The recombinant plasmid was transformed into *lpp* mutant by electroporation.

2.3. Growth kinetics

C. sakazakii wild type (JS001), *⊿lpp* (JS002) and *⊿lpp* harboring pLPP (JS003) were prepared by transferring 1% inoculum from overnight cultures into fresh 50 ml LB medium. The cultures were incubated with shaking at 220 rpm at 37° C for 10 h. Every 1 h, the optical density (OD) was measured at 600 nm.

2.4. Outer membrane permeability assays

The outer membrane integrity of the *C. sakazakii* wild type, *⊿lpp* was examined by using the release of β-lactamase(Hsieh et al., 2013) and sensitivity to detergents (Triton X-100 and SDS) and a hydrophobic antibiotic (rifampin)(Sha et al., 2004), as described previously. Briefly, the overnight cultures were centrifuged (16,000 x g, 1 min), and the supernatant was kept on ice. The pellet was resuspended in the original culture volume of fresh LB medium, and sonicated on ice. After centrifugation, the clear cell extracts and the original culture supernatant were used to measure the enzymatic activity. To a 100 ㎕ sample, I added 100 ㎕ of chromogenic cephalosporin substrate (CENTA; Calbiochem) at a final concentration of 25 ㎍/ml and 800 ㎕ LB medium into 96-well polystyrene microplates. The samples were measured the

absorbance at 405 nm after incubation for 3 h. The percentage of lactamase release in the culture supernatant was calculated based on total enzymatic activity in the wild type and compared with that of the *⊿lpp*. A sample of 900 ㎕ of LB medium with 100 ㎕ CENTA was used to control.

To demonstrate sensitivity the effect of detergents (Triton X-100, SDS) and antibiotic (Rifampin), the bacterial cells were prepared by transferring 1% inoculum from overnight cultures into fresh LB medium, followed by incubation for 1.5 h at 37° C to an OD of 1.0 and diluted 50-fold, and then various concentrations of Triton X-100 (2 to 14%), SDS (0.01 to 0.1%), and rifampin (0 to 3 ㎍/ml) were added. The cultures were incubated at 37° C for 3 h with shaking (220 rpm), and the OD_{600} was measured. A 50% reduction in the OD_{600} in three independent experiments indicated the sensitivity of the culture to the treatment.

2.5. Motility assay

C. sakazakii wild type (JS001), *⊿lpp* (JS002) and *⊿lpp* harboring pLPP (JS003) were prepared by transferring 1% inoculum from overnight cultures into fresh LB medium, followed by incubation for 3 h at 37° C with constant shaking. Bacteria were diluted to adjust

the OD_{600} to 1.5. Two microliters of suspension was injected into soft-agar motility plates (LB medium containing 0.3% agar). The plates were incubated at 37° C for 7 h and photographed by using a GelDoc EZ imager (Bio-Rad).

2.6. Transmission electron microscopy (TEM) analysis

Flagellar morphology was visualized by negative staining, followed by TEM. After being negatively stained with 2% uranyl acetate for 10 sec on carbon-Formvar copper grids, flagella were examined with an energy-filtering transmission microscope (E-TEM; Libra 120, Germany) at voltage of 120 kV.

2.7. RNA purification and transcript analysis

To extract RNA from *C. sakazakii* strains, bacteria were grown for 3 h at 37° C as in the motility assay. Total RNAs were isolated using an RNeasy minikit (Qiagen). After RNase free DNase (Ambion) treatment of the isolated total RNAs, cDNA was synthesized using Omnitranscript Reverse Transcription reagent (Qiagen) and random hexamers (Invitrogen). The synthesized cDNA was mixed with 2 × iQ SYBR Green Supermix (Bio-Rad),

and real-time PCR was performed using the CFX 3.1 (Bio-Rad). The mRNA level of each gene was divided by the mRNA level of the 16S rRNA. The mRNA expression values in *⊿lpp* (JS002) and *⊿lpp* harboring pLPP (JS003) were further normalized to the transcription levels in the wild type (JS001). All qRT-PCR primer sets used in this study are listed in the TABLE 2.

2.8. Cell culture and EGTA treatment

Murine macrophage RAW 264.7 and Human intestinal epithelial Caco-2 cell lines were obtained from American Type Culture Collection (Manassas, VA). Macrophage were maintained in Dulbecco's modified eagle medium (DMEM; ATCC) with 10% fetal bovine serum (FBS; Invitrogen). Caco-2 cells were maintained in Eagle's minimum essential medium (EMEM with L-glutamine; ATCC) containing 20% fetal bovine serum. Both types of cells were incubated at 37° C under 5% CO_2. The cell viability was determined with trypan blue staining.

To disrupt the tight junction of Caco-2 cells, EGTA treatment was conducted as described previously(Kim et al., 2010). Briefly, the Caco-2 monolayer was preincubated for 1 h with EMEM containing 5 mM EGTA (pH 7.4) or phosphate buffered saline (PBS; pH 7.4)

as a control before bacterial infection. The cell were then washed once with PBS and used for invasion assay.

2.9. Survival assay

A long-term survival assay was conducted as described previously(Townsend et al., 2007), with modifications. Briefly, a monolayer of 5 x 10^5 murine macrophage RAW 264.7 cells was prepared in a 24-well tissue culture plate. *C. sakazakii* strains were prepared as described above, and then added to the macrophage at an MOI of 100. After a 45 min incubation, the wells were incubated for 45 min with the fresh pre-warmed medium supplemented with 100 ㎍/ml of gentamicin to kill extracellular bacteria. The cells were replenished with medium supplemented with 10 ㎍/ml of gentamicin for assess of bacterial persistence at various time points (3, 8, 24,48, 72 and 96 h). Subsequently, the wells were washed three times with PBS, lysed in 1% Triton X-100 for 10 min, and then serially diluted in PBS. A dilution of the suspension was plated on LB agar medium with or without tetracycline to enumerate the CFU.

2.10. Invasion assay

To test the ability of *C. sakazakii* to invade the cultured Caco-2 cells, invasion assay was conducted as described previously (Kim et al., 2010), with modifications. Briefly, prior to bacterial infection, a monolayer of 2 x 10^5 Caco-2 cells was prepared in a 24-well tissue culture plate. Bacteria were prepared by transferring a 1% inoculum from an overnight culture into fresh LB and incubated at the for 3 h at 37° C (OD_{600} = 1.5). The bacterial cells were collected by centrifugation at 20,000 x g for 2 min at 4° C, washed with PBS. The cell pellets were suspended in 1 ml EMEM with 20% FBS, and then added onto the cell monolayer at a multiplicity of infection (MOI) of 100. After a 1.5 h incubation, the wells were washed three times with prewarmed PBS and then incubated for 1.5 h with the fresh prewarmed medium supplemented with 100 ㎍/ml of gentamicin to kill extracellular bacteria. Subsequently, the wells were washed three times with PBS, lysed in 1% Triton X-100 for 10 min, and then serially diluted in PBS. A dilution of the suspension was plated on LB agar medium with tetracycline to enumerate the CFU.

2.11. Western blot analysis

A Western blot analysis using anti-OmpA/OmpX was performed as described previously(Kim et al., 2015). Protein samples from the cell lysates, equivalent to 10 ㎍ of total protein, were separated by SDS-12% polyacrylamide gel electrophoresis. The resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat dry milk in 1x Tris-buffered saline-Tween 20 (TBST) buffer. The membrane was probed with anti-OmpA/OmpX polyclonal antibody and anti-DnaK antibody (Enzo Life Science) as primary antibodies. Anti-mouse IgG conjugated with peroxidase (Santa Cruz Biotechnology) was used as the secondary antibody in all Western blots. The chemiluminescent signals were developed with a West-zol plus Western blot detection system (Intron Biotecnology, South Korea) and imaged using the ChemiDoc MP imaging system (Bio-Rad) and ImageLab software (version 5.2.1, Bio-Rad).

2.12. Adhesion assay

To assess bacterial adhesion of mammalian Caco-2 cells, a adhesion assay was performed as described previously(Choi et al., 2015). Briefly, Caco-2 cells were treated with 0.8 ㎕/ml of cytochalasin D (CD; sigma) for 30 min to inhibit the internalization

of bacteria. The internalization of *C. sakazakii* wild type into Caco-2 cells was inhibited by approximately 70% in the presence of 0.8 ㎕/ml of CD. Before bacterial infection, the wells were washed with PBS, and fresh EMEM was added. Subsequently, *C. sakazakii* strains were prepared in a manner similar to that for the invasion assay, and then added to the Caco-2 cell monolayer at an MOI of 100, and incubated for 45 min. The plates were washed three times with PBS, lysed in 1% Triton X-100 for 10 min, and then serially diluted in PBS. A dilution of the suspension was plated on LB agar medium with tetracycline to enumerate the CFU of adhesive bacteria.

2.13. Overexpression and Purification of *C. sakazakii* Lpp in *E. coli*

To overexpress Lpp of *C. sakazakii*, a His6-Lpp recombinant protein was cloned and expressed in *E. coli*. Briefly, the coding region on the *lpp* gene (237 bp) without signal peptide from the genomic DNA of *C. sakazakii* was amplified by PCR by using the primer sets *lpp*-pET28-F-Nde I (5′ -AAA CAT ATG TGC TCC AGC AAC GCT AAA ATC -3′) and *lpp*-pET28-F-Sal I (5′ -AAA GTC GAC TTA CTT ACG GTA TTT AGT AGC-3′). The

PCR product was cloned into pET28-a T7 promoter-based vector. The recombinant plasmid was transformed into *E. coli* BL21(DE3).

To purified recombinant Lpp protein, the *E. coli* BL21 (DE3) harboring a plasmid expressing Lpp was were prepared by transferring 1% inoculum from overnight cultures into fresh LB medium, and 133 μM was added to the culture when the OD_{600} reached 1.0. The induced bacterial culture was further incubated for 3 h, collected by centrifugation at 6,000 x g for 15 min at 4° C. The pellets was washed using Tris buffer (20 mM Tris-Cl, 300 mM NaCl, pH 8.0) after removing supernatant, and resolved using Tris buffer, which was then sonicated with an Ultrasonic Processor GE 130PB (Hiescher Systems, Teltow, Germany). After centrifugation of the bacterial lysates at max speed for 30 min at 4° C. The supernatant was loaded onto an Ni-NTA superflow affinity column (Qiagen, Valencia, CA) and eluted using a Elution buffer (Tris buffer, pH 8.0, 300 mM imidazole). Peak fraction was confirmed by SDS-PAGE, and protein was concentrated by Amicon tube and then changed buffer to Tris buffer. The protein concentration was determined by the Brad-ford method.

2.14. Inhibition assay

As described above, Caco-2 cells were treated with CD in the same manner as the adhesion assay, and *C. sakazakii* strains were prepared in a manner similar to that for the invasion assay, and then were added with various concentrations (5, 10, 50, 100 ㎍/well) of the recombinant Lpp proteins onto Caco-2 cell monolayers for 45 min. The wells were washed three times with PBS and lysed with 1% Triton X-100. Suspensions were plated LB agar for determination of the total CFU count in each well.

2.15. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.01 software. All results were analyzed by Student's unpaired t test. The data are presented as means and standard errors of the mean. A P value of < 0.05 was considered to be statistically significant

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or characteristics [a]	Reference or source
Bacterial strains		
C. sakazakii		
ATCC 29544	Wild-type strain	(Kim et al., 2010)
ES1001	29544 harboring pKD46	(Kim et al., 2010)
JS001	29544 harboring pBR322	This study
⊿lpp	*⊿lpp*	Laboratory collection
JS002	*⊿lpp* harboring pBR322	This study
JS003	*⊿lpp* harboring pLPP	This study
E. coli		

DH5 α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA relA1*; plasmid replication	(HANAHAN, 1983)
BL21(DE3)	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen

Plasmids

pKD13	*oriR6K* Amp^r FRT Kan^r FRT	(Datsenko and Wanner, 2000)
pKD46	*oriR101 repA101*(Ts) Amp^r *ara BADpgam-bet-exo*	(Datsenko and Wanner, 2000)
pCP20	*ori*pSC101(Ts) Amp^r Cm^r c I 857 λ $P_R$flp	(Datsenko and Wanner, 2000)
pBR322	Cloning vector; Tet^r, Amp^r	(Sutcliffe, 1978)

pET28a(+)	His_6 tag fusion expression vector; Kan^r	Novagen
pLPP	pBR322 with *lpp* gene coding region and its putative promoter	This study
pMLPP	pET28a(+) with mature *lpp*	This study

[a] FRT, FLP recombinant target.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide name	Genotype and/or characteristics	Reference or source
Cloning		
lpp-pBR322-F-Sca I	GGT AAA TCC GCG AAA TCC GTT	This study
lpp-pBR322-R-Sca I	CAC ACG GTA CTA TTA CTT ACG G	This study
pBR322-seq-F	GTT TGG TAT GGC TTC ATT CAG CT	This study
pBR322-seq-R	CCG AAG AAC GTT TTC CAA TGA TG	This study
lpp-pET28-F-Nde I	AAA CAT ATG TGC TCC AGC AAC GCT AAA ATC	This study
lpp-pET28-F-Sal I	AAA GTC GAC TTA CTT ACG GTA TTT AGT AGC	This study
pET28-seq-F	TAA TAC GAC TCA CTA TAG GG	This study
pET28-seq-R	TAG TTA TTG CTC AGC GGT GG	This study
qRT-PCR		
Control-RT-F	ACG CGA AGA ACC TTA CCT GG	This study

Control-RT-R	CCG GCA GTC TCC TTT GAG TT	This study
flhA-RT-F	GCC GGC TCG TTA TCG TAT TCT	This study
flhA-RT-R	GGA TTT GCG CCT GCT CTT TC	This study
fliA-RT-F	AGG CAA TAG GCC AAC TGG AG	This study
fliA-RT-R	TTC GAT GCT ATC GCC ATG CT	This study
fliC-RT-F	AAA GGT ATG ACC CAG GCT GC	This study
fliC-RT-R	TGC CAG ACG CTG AGT GAT TT	This study
fliG-RT-F	GCT GAG CAT CAA CAC CAA CG	This study
fliG-RT-R	TAA TCT GCG GGT GTT CGT CG	This study
flgK-RT-F	GGG CAG TCT AAC AGC ACC AT	This study
flgK-RT-R	TGG TGG TGC CAG AGA CCA TA	This study

3. RESULTS

3.1. Construction and growth characteristics of the *lpp* mutant in *C. sakazakii*

Lpp has previously been shown to contribute to the pathogenesis of many bacteria(Hsieh et al., 2013; Sha et al., 2008; Sha et al., 2004). However, effects of Lpp on the pathogenesis of *C. sakazakii* ATCC 29544 have not been demonstrated. The Lpp of *C. sakazakii* ATCC 29544 showed high protein sequence similarity to the Lpp of *E. coli* K12 (97%), *K. pneumonia* KpN06 (96%), *S.* Typimurium 14028 LppA (84%) and LppB (99%) (Fig. 1). The high level of sequence similarity between the Lpp of *C. sakazakii* and other Gram-negative bacteria demonstrate a potential role for the Lpp. So, to understand the roles of *lpp* in *C. sakazakii* ATCC 29544 pathogenesis, I generated an unmarked mutant lacking the entire *lpp* using the Lambda-Red recombination technique.

When I compared the growth of the strains in LB medium at 37℃ with 220 rpm, the *lpp* mutant grew slightly slower than the wild type (Fig. 2). The complemented strain restored the reduced growth of the *lpp* mutant (Fig. 2).

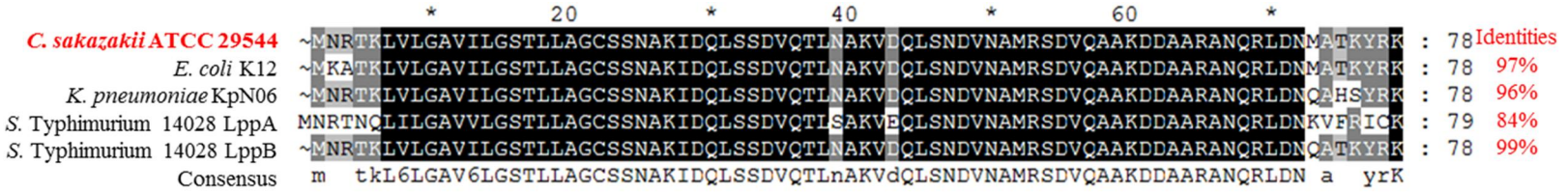


Fig. 1. *lpp* homolog in *C. sakazakii* ATCC 29544. Multiple-sequence alignment of amino acid sequences of *C. sakazakii* ATCC 29544 *lpp* against *E. coli* K12 *lpp*, *K. pneumoniae* KpN06 *lpp*, *S.* Typhimurium 14028 *lppA* and *lppB* by Clustal Omega (version 1.2.1) and Genedoc. The uppercase letters in the consensus sequence indicate conserved amino acids appearing in all the aligned sequences, and the lowercase letters indicate conserved amino acid appearing at least two sequences. Identical sequences (*black boxes*) and similar sequences (*dark gray boxes*, >70% conserved; *light gray boxes*, >40% conserved) are indicated. Every 10 amino acids are indicated by asterisks.

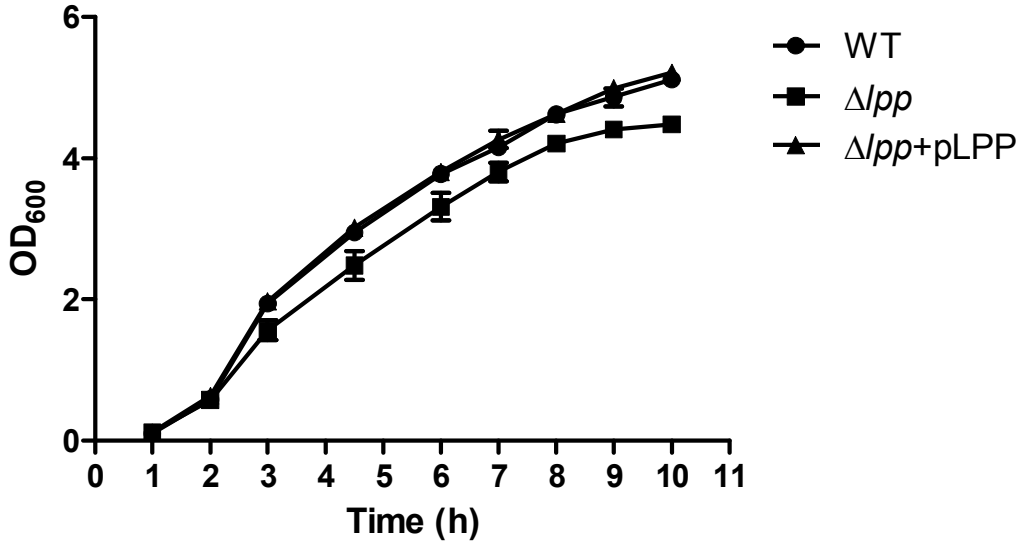


Fig. 2. Growth kinetics of *C. sakazakii*. *C. sakazakii* wild type (JS001), *⊿lpp* (JS002) and *⊿lpp* harboring pLPP (JS003) were cultured in LB broth at 37℃. After incubation, growth was measured spectrophotometrically at 600 nm every hour. Error bars represent means and standard errors of the mean (SEM) from three independent experiments.

3.2. Cell membrane integrity of the *lpp* mutant

Studies in *E. coli* showed that Lpp is involved in maintaining the structural integrity of the cell envelope (Daniel W. Yem, 1978; Hideho Suzuki and Hirota, 1978). However, the integrity of the cell envelope was not changed in the *S.* Typhimurium *lpp* mutant(Sha et al., 2004). To determine whether the *lpp* mutant in *C. sakazakii* exhibited altered outer membrane integrity, the *C. sakazakii* strains were tested different assays, the release of β-lactamase, the sensitivity to detergents (Triton X-100, SDS), and the permeability to rifampin. The β-lactamase assay indicated that wild type and the *lpp* mutant exhibited similar releases of β-lactamase into the medium (Table 3). When the mutant was treated with Triton X-100 (up to a concentration of 14%), no obvious difference was noted between the sensitivity of the *lpp* mutant and the sensitivity of wild type (Table 3 and Fig. S1A). However, the *lpp* mutant showed 50% reduction in the OD_{600} at a concentration of SDS of 0.025%, whereas the wild type was showed at this concentration of SDS (Table 3 and Fig. S1B). Also, the permeability to rifampin indicated that the *lpp* mutant decreased OD_{600} to 50% at a concentration of 1 μg/ml of rifampin, while the wild type decreased at a concentration of 2 μg/ml (Table 3 and Fig. S1C). These data suggested that *lpp*

mutant in *C. sakazakii* was a little but significantly affected cell membrane integrity.

TABLE 3. Integrity of the membrane in the *lpp* mutant of *C. sakazakii*

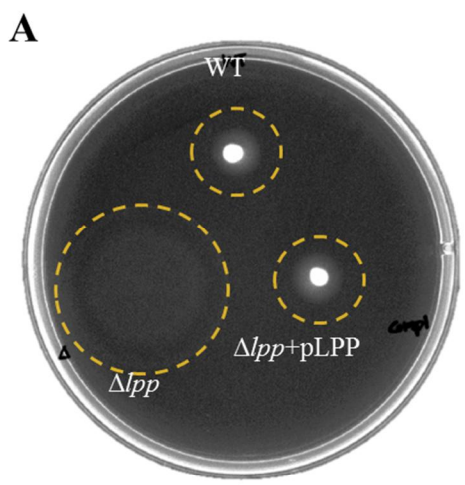
Strain	β-Lac[a] (% release)	Resistance to:		
		Triton X-100[b] (%)	SDS[b] (%)	Rif[b] (μg)
WT	47.41±1.19	>10	0.05	2
⊿lpp	49.02±0.85	>10	0.025	1

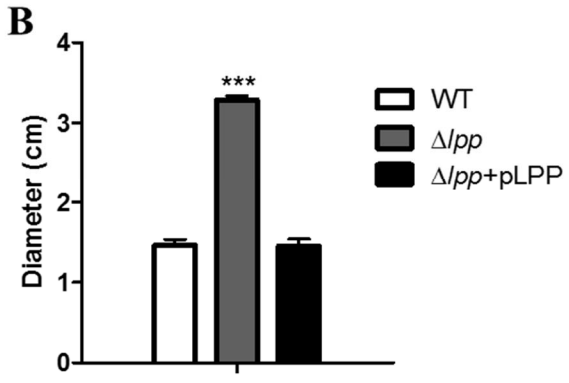
[a] β-lactamase activity present in the supernatant was indicated as percentage of the total activity (average values from triplicate experiments ± SD).

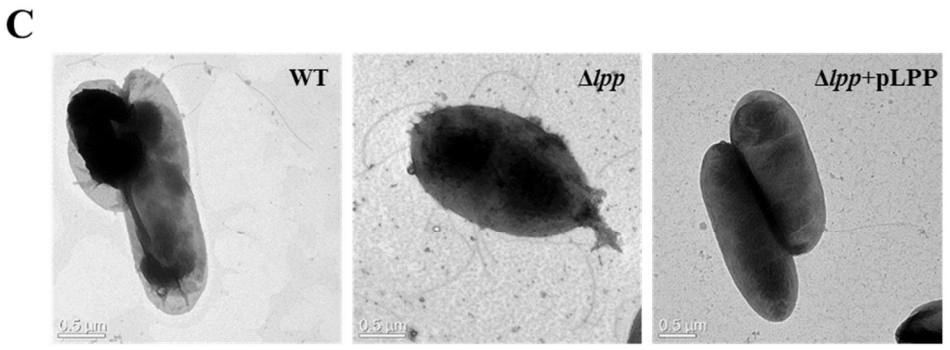
[b] Triton X-100 (% vol/vol), SDS (% weight/vol) and Rifampin (μg/ml) concentration leading to a 50% decrease in cell turbidity measured after 3 h of bacterial growth. (average values from triplicate experiments ± 10%).

3.3. Lpp mutant is hyper-motile due to higher expression of flagella

Because the motility is an important virulence factor, I performed motility assay on 0.3% soft-agar plate. Interestingly, the motility was increased by *lpp* mutation (Fig. 3A, B). The wild type showed low motility (14.7 mm in diameter), but the *lpp* mutant was hypermotile (32.8 mm). Also, the complementation strain decreased the motility (14.6 mm) (Fig. 3B). In addition, the *lpp* mutant was hyper-flagellated observed by transmission electron microscopy compared wild type (Fig. 3C), indicated that *lpp* may regulated flagellar synthesis. So, to evaluate the expression level of several flagellar biosynthesis-related genes in *C. sakazakii* strains, I performed qRT-PCR. Compared with the wild type, the levels of transcripts of *flhA* and *fliG* genes increased 4.5 fold and 7.5 fold following the *lpp* mutation (Fig. 3D). On the other hand, the transcriptional level of *flhCD*, *fliA*, *motAB*, *cheAW*, and *fliC* was not significantly different between the wild type and the *lpp* mutant (Fig. 3D). The complemented strain decreased the motility and transcription level of flagellar biosynthesis-related genes as wild type.


A
WT
Δlpp+pLPP
Δlpp


B
Diameter (cm)
***
WT
Δlpp
Δlpp+pLPP


C
WT
Δlpp
Δlpp+pLPP

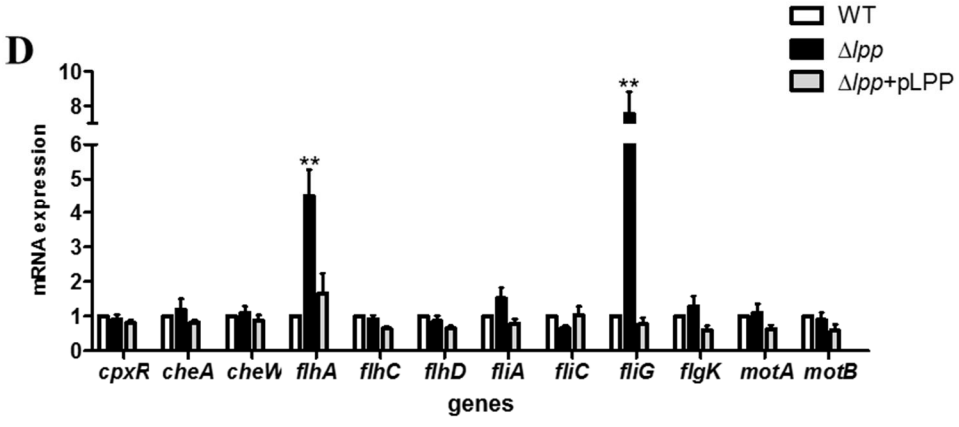

D
WT
Δlpp
Δlpp+pLPP
mRNA expression
cpxR cheA cheW flhA flhC flhD fliA fliC fliG flgK motA motB
genes

Fig. 3. Effect of the *lpp* gene on the regulation of motility in *C. sakazakii*. (A) The areas of motilities of the strains grown for 7 h on LB plates with 0.3% (w/v) soft agar. (B) The diameters of motility areas are representative from three independent biological replicates. (C) *C. sakazakii* wild type (JS001), *⊿lpp* (JS002) and *⊿lpp* harboring pLPP (JS003) were negatively stained with 2% (w/v) uranyl acetate and then observed by using a transmission electron microscope. (D) The mRNA levels of *flhA, fliA, fliC, fliG,* and *flgK* from *C. sakazakii* wild type (JS001), *⊿lpp* (JS002), and *⊿lpp* harboring pLPP (JS003) were determined by qRT-PCR. The mRNA level of each gene in the wild type was set as 1. Error bars represent the means and SEM from three independent experiments. The asterisks indicate a significant difference (***, $P < 0.001$; **, $P < 0.05$).

3.4. Lpp affects intracellular survival

To examine the impact of *lpp* mutation on the survival of *C. sakazakii* within cultured macrophages, a RAW 264.7 macrophage cell line was infected with the wild type and *lpp* mutant for long-term (96 h). Although the initial uptake of *lpp* mutant by macrophages was 10 fold lower than that of the wild type, the intracellular survival of *lpp* mutant was significantly decreased in RAW 264.7. After 24 h, the number of *lpp* mutant decreased to 3.5-log-unit in comparison to the number of wild type, which were 0.6-log-unit. At 72 h post-infection, clearance of the mutant (5.7-log-unit decrease in survival) was significantly faster than for the wild type (1.8-log-units) on average (Fig. 4A). In addition, the *lpp* mutant was cleared completely after 96 h post-infection, whereas wild-type strain (approximately 4 log CFU/ml) was still able to persist within macrophages at same time point (Fig. 4A). The complemented strain restored the ability to survive within macrophages (Fig. 4B). These data suggest that *lpp* is necessary for intracellular survival of *C. sakazakii* in host macrophages.

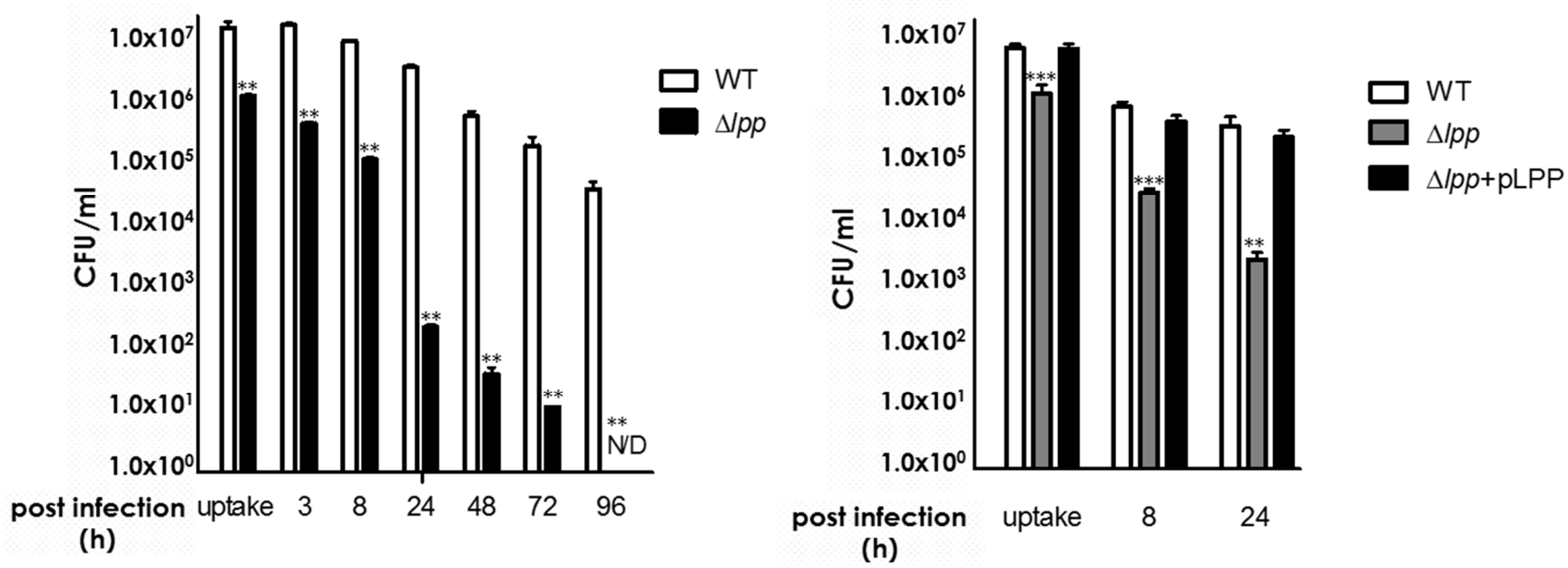


Fig. 4. Intracellular survival of *C. sakazakii*. (A) RAW 264.7 macrophage-like cells were infected with wild type and *⊿lpp*, and the intracellular CFU were enumerated at uptake (1.5 h), 3, 8, 24, 48, 72, and 96 h postinfection. (B) RAW 264.7 macrophage-like cells were infected with wild type (JS001), *⊿lpp* (JS002) and *⊿lpp* harboring pLPP (JS003). The intracellular CFU were enumerated at uptake (1.5 h), 8, and 24 h postinfection. (C) CD-pretreated Caco-2

epithelial cells were infected with *C. sakazakii* strains. The numbers of intracellular bacteria were determined 45 min after infection without the use of gentamicin. Error bars represent the means and SEM from three independent experiments. The asterisks indicate a significant difference (***, $P < 0.001$; **, $P < 0.05$).

3.5. Lpp is affected invasive ability of *C. sakazakii* into human epithelial cells

Previously, *lpp* mutant decreased the invasive ability compared to the wild type in *S.* Typhimurium(Sha et al., 2004). The invasion assay was evaluated to determine invasive ability in *lpp* mutant of *C. sakazakii*. As shown in Fig. 5A, the invasive ability of the *lpp* mutant was decreased by 3 fold from that of wild type. However, the invasive ability of the mutant was significantly restored after complementation (Fig. 5A). It has been reported that outer membrane protein OmpA and OmpX were invasin of *C. sakazakii* into Caco-2 cells. To investigate whether the OmpA and OmpX affect invasive ability in *lpp* mutant of *C. sakazakii*, the Western blot analysis was performed using anti-OmpA and anti-OmpX polyclonal antibodies. The result of the Western blot analysis showed that the expression of OmpA and OmpX was not significantly different (Fig. 5B).

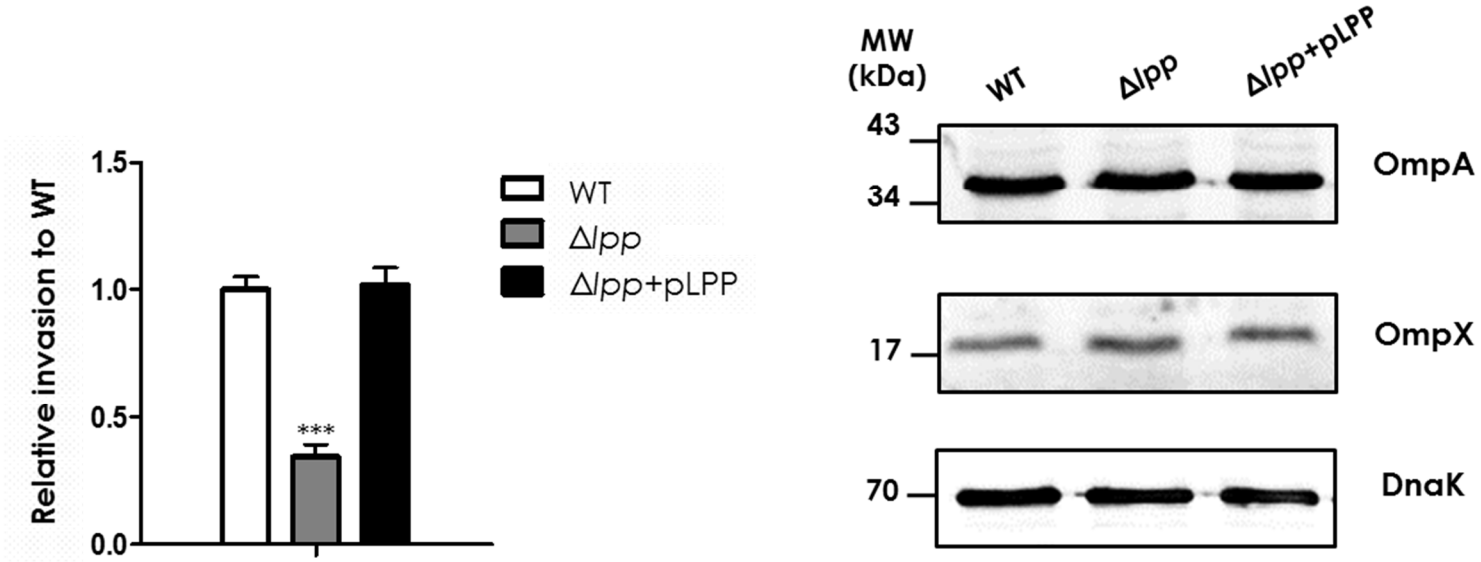


Fig. 5. The effect of *lpp* mutation on the host cell invasion and cellular levels of OmpA and OmpX. (A) Caco-2 epithelial cells were with wild type and *⊿lpp*, and *⊿lpp* harboring pLPP. The numbers of intracellular bacteria were determined 1.5 h after infection using the gentamicin protection assay. Error bars represent the means and SEM from three independent experiments. The asterisks indicate a significant difference (***, $P < 0.001$). (B) Cellular levels of OmpA

and OmpX were assessed by immunoblotting in exponentially grown cells. DnaK was used as a loading control. The position of protein size markers (in kDa; Bio-rad) are shown on the left of the gel. The blots are representative of three independent experiments.

3.6. Lpp is essential for basolateral invasion by *C. sakazakii*

To investigate whether the *lpp* gene affects basolateral invasion, I disrupted tight junctions by pretreating EGTA to Caco-2 cells. When Caco-2 cells were pretreated with EGTA before bacterial infection, the invasion efficiency of the wild type increased about 2.3 fold compared with the untreated wild type (Fig. 6). In addition, the invasion efficiency of the *lpp* mutant increased about 1.3 fold compared with the untreated *lpp* mutant (Fig. 6). These data indicate that Lpp are required for basolateral invasion of Caco-2 cells by *C. sakazakii*.

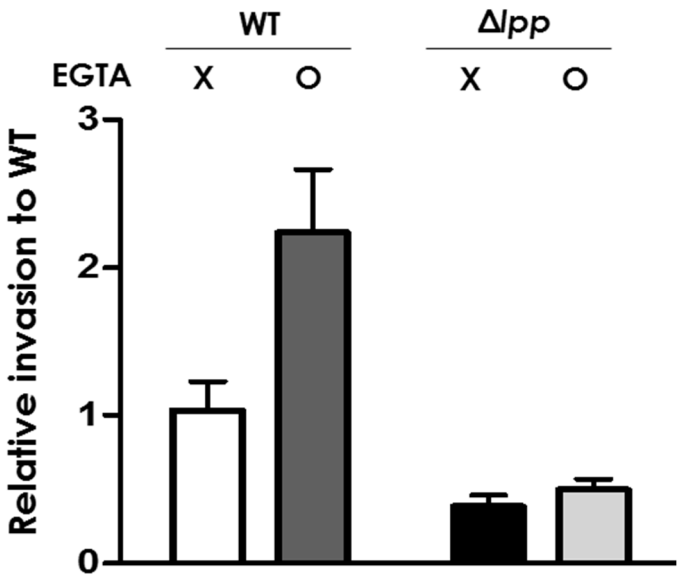


Fig. 6. Invasion of *C. sakazakii* into Caco-2 cells after pretreatment with EGTA. Caco-2 cells were pretreated with 5 mM EGTA (or not pretreated) and used for an invasion assay Relative invasion was determined by comparison to the non-EGTA-treated wild type. Error bars represent the means and SEM from three independent experiments.

3.7. Recombinant Lpp inhibits adherence of *C. sakazakii* to Caco-2 cells

To determine whether *lpp* in C. sakazakii affects its adhesive ability, we performed adhesion assay using Caco-2 cells. As expected, the binding of the *lpp* mutant to Caco-2 cells was significantly reduced compared with the wild type (Fig. 7A). The complemented strain restored the adhesive ability of the *lpp* mutant to wild type levels (Fig. 7A).

To determine the role of Lpp in *C. sakazakii* adherence to epithelial cells, I tested if recombinant Lpp would inhibit with the adherence of *C. sakazakii* ATCC 29544 to Caco-2 cells by mixing the cells with different concentrations of recombinant Lpp. The protein was isolated using metal affinity resin (Fig. 7B). The adhesion ability of wild type treated with the recombinant Lpp decreased in a dose-dependent manner (Fig. 7C). In addition, the adhesion ability of *lpp* mutant treated with the recombinant Lpp did not change compared to the *lpp* mutant without recombinant Lpp (data not shown). The result that purified recombinant Lpp interferes with the adherence of *C. sakazakii* ATCC 29544 to Caco-2 cells suggests that Lpp may bind to unknown host part(s) of the Caco-2 cells. The recombianat

Lpp is not effect to Caco-2 cells, confirmed by the LDH release assay kit (Fig. S2).

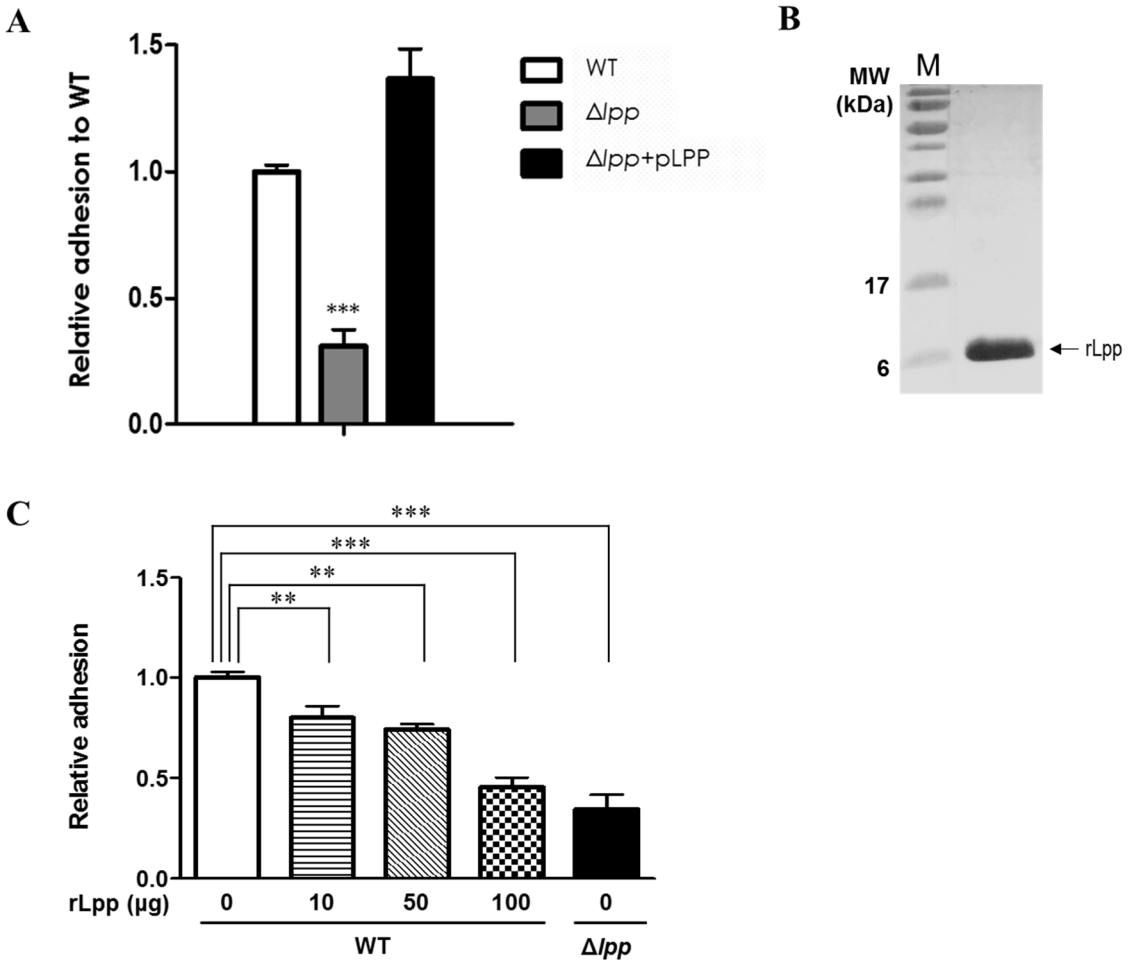

A
Relative adhesion to WT
1.5
1.0
0.5
0.0
***
WT
Δlpp
Δlpp+pLPP
B
MW
(kDa)
M
17
6
rLpp
C
Relative adhesion
***
***
**
**
1.5
1.0
0.5
0.0
rLpp (μg)
0
10
50
100
0
WT
Δlpp

Fig. 7. The effect of *lpp* mutation on the host cell adhesion and inhibition of adhesion by purified protein. (A) CD-pretreated Caco-2 epithelial cells were infected with *C. sakazakii* strains. The numbers of intracellular bacteria were determined 45 min after infection without the use of gentamicin. (B) The recombinant Lpp protein was overexpressed in *E. coli* BL21(DE3). The purified protein was resolved by 15% SDS-PAGE. Lane 1, size marker proteins; lane 2, recombinant Lpp. (C) In adhesion assay, various concentration (5, 10, 50, and 100 μg) of purified Lpp protein was mixed with *C. sakazakii* ATCC 29544 or the *lpp* mutant, and was added to Caco-2 cells. Error bars represent the means and SEM from three independent experiments. The asterisks indicate a significant difference (***, $P < 0.001$; **, $P < 0.05$).

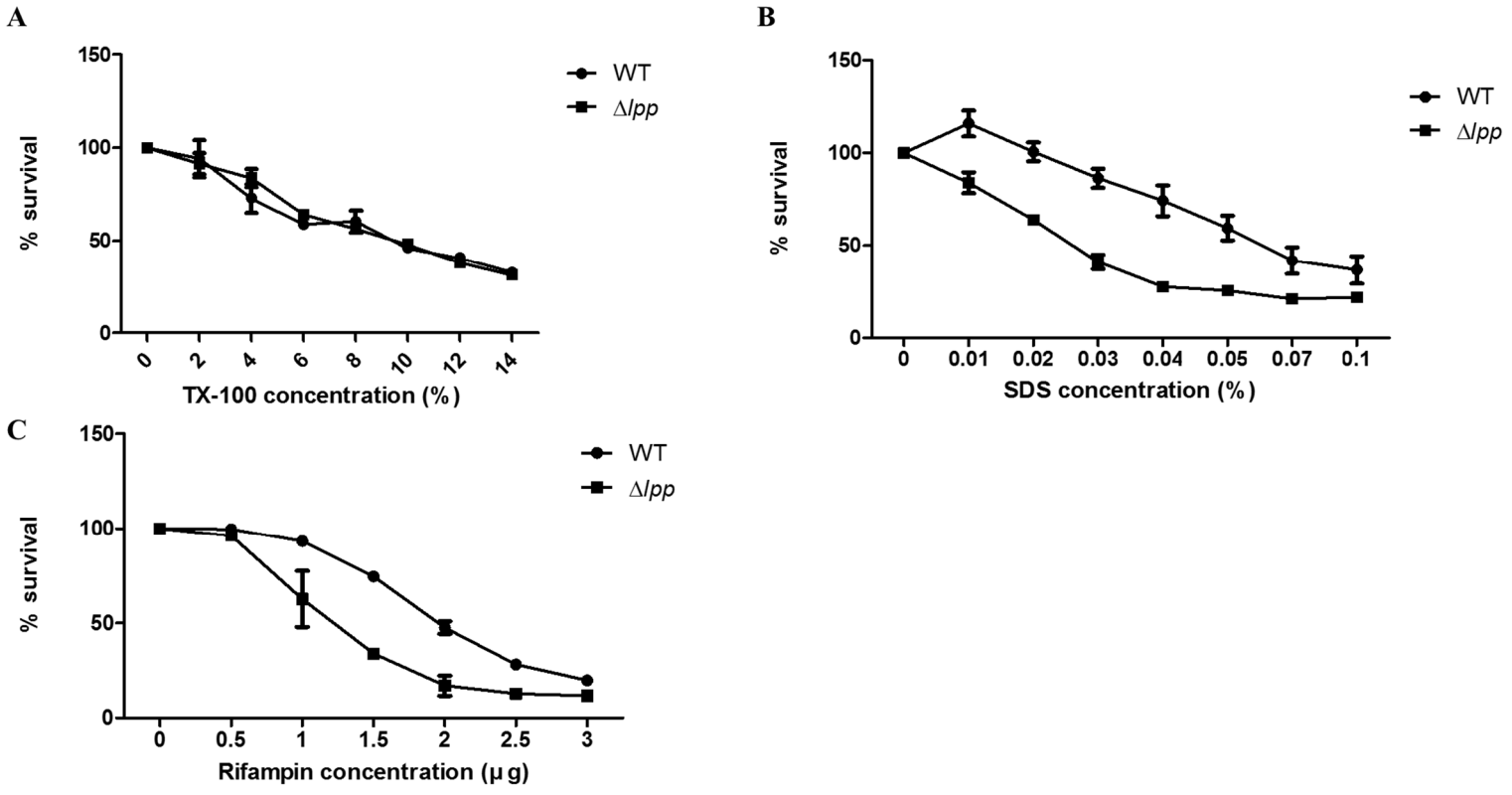


Fig. S1. Integrity of the membrane in the *lpp* mutant of *C. sakazakii*. Sensitivity to detergents, such as Triton X (TX-100) (A), SDS (B), and to rifampin (C).

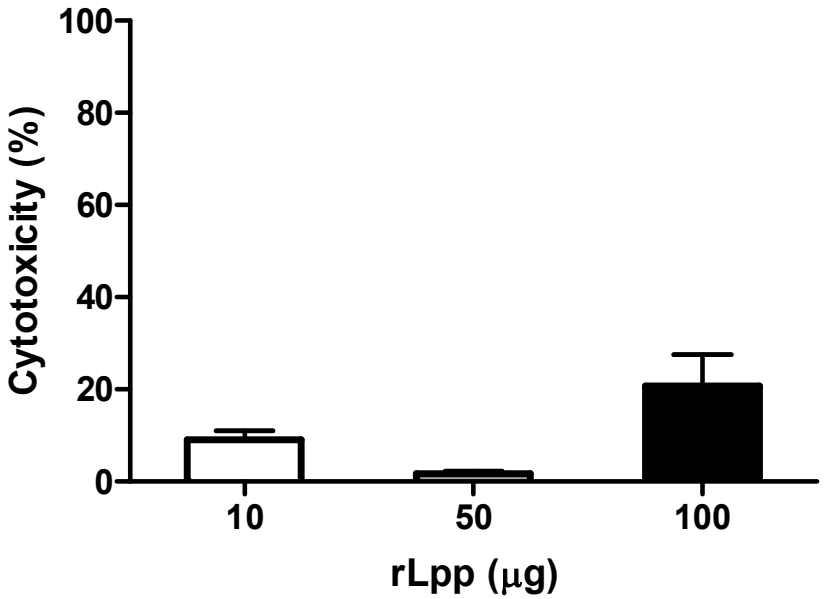


Fig. S2. Cytotoxicity of recombinant Lpp (rLpp) protein. rLpp cytotoxicity was confirmed by the LDH release assay kit.

4. DISCUSSION

In this study, I suggested that the Lpp belong to the virulence of *C. sakazakii* ATCC 29544. Lpp is the forth putative outer membrane protein in *C. sakazakii*, after OmpA, OmpX, and Inv (Dilini Chandrapala, 2014; Kim et al., 2010). A loss of *lpp in C. sakazakii* causes defective in growth kinetics, outer membrane permeability, invasion/adhesion into epithelial cells, survival within macrophages. Particularly, invasion assay with EGTA treatment, which can disrupted the tight junction and then exposed the basolateral region, showed that Lpp was important for basolateral invasion of Caco-2 cells in *C. sakazakii*. In addition, the result of the Western blot analysis about OmpA and OmpX suggested that Lpp plays a role in the invasion into Caco-2 cells.

The importance of bacterial flagella as a virulence factor has previously been reported, including biofilm formation, invasion, adhesion, and colonization. The *lpp* double-knockout mutant was non-motile in *S.* Typhimurium (Sha et al., 2004). In addition, the motility of *lpp* mutant in *Y. pseudotuberculosis* was significantly decreased (Sha et al., 2008). In contrast, in this study, *lpp* mutant was shown to be more motile than wild type (Fig. 3A, B). The

number of flagella could have been affected in *lpp* mutant, as previously reported for the *hfq* mutant of *C. sakazakii* ATCC 29544, which was confirmed by TEM. According to a previous study, increased motility in *E. coli* 43895OR strain has been described by *lpp* mutation (Uhlich et al., 2009). They suggested that *cpxR* is increased in *lpp* mutant, and phosphorylated CpxR inhibits motility by suppressing transcription of *motAB* and *cheAW* (Uhlich et al., 2009). However, no significant difference in transcription level of *cpxR*, *motAB*, and *cheAW* was observed between wild type and *lpp* mutant (Fig. 3D). Future studies are needed to investigate the role of *lpp* in mechanisms of flagellar regulation in *C. sakazakii*.

Likewise, the flagella are important for adhesion to Caco-2 intestinal epithelial cells in *C. sakazakii* (Hartmann et al., 2010). Interestingly, although *lpp* mutant showed increased motility due to hyper-expression of flagella, the adhesive ability was decreased by *lpp* mutation in this study (Fig. 7A). It was hypothesized that the Lpp was required for the adhesion to Caco- 2 intestinal epithelial cells directly or indirectly, beyond the role of flagella. To test this hypothesis, the recombinant Lpp was added to Caco-2 monolayer with wild-type strain. The adhesive ability was decreased in a dose-dependent manner by the addition of purified recombinant

Lpp (Fig. 7C). The result that the recombinant Lpp interferes with the adherence of *C. sakazakii* to Caco-2 cells suggests that Lpp proteins can adhered an unknown host receptor(s) on Caco-2 cells. This result indicated that Lpp protein, which may be the free-form, behaves as a true adhesin. Further work is needed to determine where recombinant Lpp binds to receptors(s) on Caco-2 cells.

The *lpp* can also be expected to useful tools in the development of vaccines as targets or vaccine candidates, and *lpp* mutant may serve as attenuated vaccine candidates. First, studies on cell surface lipoproteins have continued because of their role as vaccine targets. Although the role of free-form Lpp is poorly studied, the evidence of the surface-exposed form (free-form) Lpp has recently been discovered by immunogold labeling/electron microscopy in 2011 (Cowles et al., 2011). As other surface-exposed lipoproteins, future studies are needed to investigate the role as vaccine targets against *C. sakazakii* infection. Also, previous studies have addressed the role of *lpp* as promising vaccine candidates. As well as Gram-negative bacteria, the Lpp was considered as vaccine candidates against Gram-positive bacteria such as *S. aureus* (Nguyen and Gotz, 2016). In addition, a previous research showed demonstrated that because *lpp* mutant elicited

robust immune response in mice, the *lpp* mutant showed potential as viable vaccine candidate against *Salmonella* infection (Erova et al., 2016).

In conclusion, I have showed here that *lpp* is essential for virulence of *C. sakazakii* ATCC 29544. Our results suggested that the Lpp is crucial role in maintaining the outer membrane integrity, and contributes to the invasive/adhesive ability into epithelial cells, persistence into macrophages. Also, this report first suggested that free-form Lpp plays role in the adhesion of host cells as adhesin. Further studied are required to investigate the mechanisms of the interactions between Lpp in *C. sakazakii* and host cell receptors, especially for the regulation of *C. sakazaii* ATCC 29544.

Reference

Bar-Oz, B., Preminger, A., Peleg, O., Block, C., Arad, I., 2001. *Enterobacter sakazakii* infection in the newborn. Acta Paediatrica 90, 356–358.

Choi, Y., Kim, S., Hwang, H., Kim, K.P., Kang, D.H., Ryu, S., 2015. Plasmid-encoded MCP is involved in virulence, motility, and biofilm formation of *Cronobacter sakazakii ATCC* 29544. Infection and Immunity 83, 197–204.

Cowles, C.E., Li, Y., Semmelhack, M.F., Cristea, I.M., Silhavy, T.J., 2011. The free and bound forms of Lpp occupy distinct subcellular locations in *Escherichia coli*. Molecular Microbiology 79, 1168–1181.

D. Drudy, N.R.M., T. Quinn, P. G. Wall, S. Fanning, 2006. *Enterobacter sakazakii*: An Emerging Pathogen in Powdered Infant Formula. FOOD SAFETY 42, 996–1002.

Daniel W. Yem, H.C.W., 1978. Physiological characterization of an *Escherichia coli* mutant altered in the structure of murein lipoprotein. Journal of Bacteriology 133, 1419–1426.

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of Sciences 97, 6640–6645.

Diao, J., Bouwman, C., Yan, D., Kang, J., Katakam, A.K., Liu, P., Pantua, H., Abbas, A.R., Nickerson, N.N., Austin, C., Reichelt, M., Sandoval, W., Xu, M., Whitfield, C., Kapadia, S.B., 2017. Peptidoglycan Association of Murein

Lipoprotein Is Required for KpsD-Dependent Group 2 Capsular Polysaccharide Expression and Serum Resistance in a Uropathogenic *Escherichia coli* Isolate. MBio 8.

Dilini Chandrapala, K.K., Younho Choi, Amal Senevirathne, Dong-Hyun Kang, Sangryeol Ryu, Kwang-Pyo Kima, 2014. 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. Infection and Immunity 82, 1755-1765.

Egan, A.J.F., 2018. Bacterial outer membrane constriction. Molecular Microbiology 107, 676-687.

Erova, T.E., Kirtley, M.L., Fitts, E.C., Ponnusamy, D., Baze, W.B., Andersson, J.A., Cong, Y., Tiner, B.L., Sha, J., Chopra, A.K., 2016. Protective Immunity Elicited by Oral Immunization of Mice with *Salmonella enterica* Serovar Typhimurium Braun Lipoprotein (Lpp) and Acetyltransferase (MsbB) Mutants. Front Cell Infect Microbiol 6, 148.

Fadl, A.A., Sha, J., Klimpel, G.R., Olano, J.P., Galindo, C.L., Chopra, A.K., 2005. Attenuation of *Salmonella enterica* Serovar Typhimurium by altering biological functions of murein lipoprotein and lipopolysaccharide. Infection and Immunity 73, 8433-8436.

Fadl, A.A., Sha, J., Klimpel, G.R., Olano, J.P., Niesel, D.W., Chopra, A.K., 2005. Murein lipoprotein is a critical outer membrane component involved in *Salmonella enterica* serovar typhimurium systemic infection. Infection and Immunity 73, 1081-1096.

H Zhang, J.W.P., D W Niesel, G R Klimpel, 1997. Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. The Journal of Immunology 159, 4868-4878.

HANAHAN, D., 1983. Studies on Transformation of Escherichia coli with Plasmids. Journal of Molecular Biology 166, 557-580.

Hartmann, I., Carranza, P., Lehner, A., Stephan, R., Eberl, L., Riedel, K., 2010. Genes involved in *Cronobacter sakazakii* biofilm formation. Applied and Environmental Microbiology 76, 2251-2261.

Hideho Suzuki, Y.N., Seiichi Yasuda, Akiko Nishimura, Masao Yamada, and, Hirota, Y., 1978. Murein-Lipoprotein of *Escherichia coli*: A Protein Involved in the Stabilization of Bacterial Cell Envelope. Molec. gen. Genet 167.

Hsieh, P.F., Liu, J.Y., Pan, Y.J., Wu, M.C., Lin, T.L., Huang, Y.T., Wang, J.T., 2013. *Klebsiella pneumoniae* peptidoglycan-associated lipoprotein and murein lipoprotein contribute to serum resistance, antiphagocytosis, and proinflammatory cytokine stimulation. Journal of Infectious Diseases 208, 1580-1589.

Hunter, C.J., Petrosyan, M., Ford, H.R., Prasadarao, N.V., 2008. *Enterobacter sakazakii*: an emerging pathogen in infants and neonates. Surgical infections 9, 533-539.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R., Joosten, H., 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of

Cronobacter sakazakii gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter genomospecies* 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. International Journal of Systematic and Evolutionary Microbiology 58, 1442-1447.

Iversen, C., Waddington, M., On, S.L., Forsythe, S., 2004. Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* Species. Journal of Clinical Microbiology 42, 5368-5370.

Jøker, R., Nørholm, T., Siboni, K., 1965. A case of neonatal meningitis caused by a yellow enterobacter. Danish Medical Bulletin 12, 128-130.

Kim, K., Kim, K.P., Choi, J., Lim, J.A., Lee, J., Hwang, S., Ryu, S., 2010. Outer membrane proteins A (OmpA) and X (OmpX) are essential for basolateral invasion of *Cronobacter sakazakii*. Applied and Environmental Microbiology 76, 5188-5198.

Kim, S., Hwang, H., Kim, K.P., Yoon, H., Kang, D.H., Ryu, S., 2015. Hfq plays important roles in virulence and stress adaptation in *Cronobacter sakazakii* ATCC 29544. Infection and Immunity 83, 2089-2098.

Kim, S., Yoon, H., Ryu, S., 2018. New virulence factor CSK29544_02616 as LpxA binding partner in *Cronobacter sakazakii*. Sci Rep 8, 835.

Klaus Hantke, V.B., 1973. Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the mureinlipoprotein

of the *Escherichia coli* outer membrane. European Journal of Biochemistry 34, 284–296.

Mullane, N., Iversen, C., Healy, B., Walsh, C., Whyte, P., Wall, P., Quinn, T., Fanning, S., 2007. *Enterobacter sakazakii* an emerging bacterial pathogen with implications for infant health. Minerva Pediatrica 59, 137–148.

Nguyen, M.T., Gotz, F., 2016. Lipoproteins of Gram-Positive Bacteria: Key Players in the Immune Response and Virulence. Microbiology and Molecular Biology Reviews 80, 891–903.

Sha, J., Agar, S.L., Baze, W.B., Olano, J.P., Fadl, A.A., Erova, T.E., Wang, S., Foltz, S.M., Suarez, G., Motin, V.L., Chauhan, S., Klimpel, G.R., Peterson, J.W., Chopra, A.K., 2008. Braun lipoprotein (Lpp) contributes to virulence of *yersiniae*: potential role of Lpp in inducing bubonic and pneumonic plague. Infection and Immunity 76, 1390–1409.

Sha, J., Fadl, A.A., Klimpel, G.R., Niesel, D.W., Popov, V.L., Chopra, A.K., 2004. The two murein lipoproteins of *Salmonella enterica* serovar Typhimurium contribute to the virulence of the organism. Infection and Immunity 72, 3987–4003.

Sutcliffe, J.G., 1978. pBR322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. Nucleic Acids Research 5, 2721–2728.

Townsend, S.M., Hurrell, E., Gonzalez-Gomez, I., Lowe, J., Frye, J.G., Forsythe, S., Badger, J.L., 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.

Uhlich, G.A., Gunther, N.W.t., Bayles, D.O., Mosier, D.A., 2009. The CsgA and Lpp proteins of an *Escherichia coli* O157:H7 strain affect HEp-2 cell invasion, motility, and biofilm formation. Infection and Immunity 77, 1543-1552.

Willis, J., Robinson, J.E., 1988. *Enterobacter sakazakii* meningitis in neonates. The Pediatric infectious disease journal 7, 196-199.

Wilson, M.M., Bernstein, H.D., 2016. Surface-Exposed Lipoproteins: An Emerging Secretion Phenomenon in Gram-Negative Bacteria. Trends in Microbiology 24, 198-208.

국문초록

Cronobacter sakazakii (크로노박터 사카자키)는 괴사성 장염, 패혈증, 뇌수막염과 같은 질병을 유발하는 식중독균으로, 특히 영유아에게 있어 높은 치사율을 나타낸다. 치명적인 병원성에도 불구하고, *C. sakazakii* 의 병원성과 관련된 메커니즘과 그 인자들에 대한 연구는 아직 미비한 실정이다. 따라서 *C. sakazakii* 의 기준주인 *C. sakazakii* ATCC 29544 균주를 이용하여, 병원성과 관련된 새로운 인자를 규명하고자 하였다. 본 연구에서는 선행 연구에 의해서 스크리닝 된 인자들 중, *C. sakazakii* 의 외막 단백질 중 하나인 murein lipoprotein (Lpp)을 타깃으로 선정하였으며, Lpp 의 *C. sakazakii* 내 병원성 관련 역할을 규명하였다. *lpp* 유전자의 역할을 규명하기 위하여, Lambda-red recombination 방법으로 구축된 *lpp* 유전자가 결여된 균주를 이용하여, phenotype 을 확인, 비교하는 실험을 진행하였다. *lpp* 유전자가 결여된 균주는 Cell membrane permeability assay 결과에서, membrane integrity 가 다소 감소하는 결과를 보였다. 또한, *lpp* 결여 균주는 0.3% 한천 배지에서 wild type 보다 더 높은 운동성을 보였으며, 편모 합성 관련 유전자의 전사체 발현 정도가 증가하였다. TEM 관찰 결과, 편모의 수가 wild type 과 비교하여 증가하였음을 확인할 수 있었다. *lpp* 결여 균주를 이용하여 인간 유래 장 상피 세포인 Caco-2 로의 침투와 부착

능력을 확인해본 결과, 모두 현저하게 감소하였다. 뿐만 아니라, 대식세포인 RAW 264.7 내의 생존 능력도 wild type 과 비교하였을 때 크게 떨어지는 것을 확인하였다. 특히, EGTA 를 이용하여 tight junction 이 제거되어도, *lpp* 결여 균주의 Caco-2 내로의 침투 능력이 wild type 만큼 증가하지 않았다. 이는 *C. sakazakii* 의 Lpp 가 세포 기저부로의 침투에 있어 중요한 역할을 함을 의미한다. 또한, 정제한 recombinant Lpp 단백질을 wild type 과 함께 처리하였을 때에, 농도 의존적으로 Caco-2 로의 부착 능력이 감소하는 것을 확인하였다. 이를 통해, Lpp 단백질이 숙주 세포 표면의 어떤 부분에 부착한다는 것을 알 수 있으며, adhesin 으로서의 역할을 함을 나타낸다. 결론적으로, 본 연구 결과는 Lpp 가 *C. sakazakii* ATCC 29544 의 병원성에 중요한 역할을 한다는 것을 의미한다. .

주요어: 크로노박터 사카자키 (*Cronobacter sakazakii*), murein lipoprotein (Lpp), 병원성, Caco-2, adhesin

학번: 2016-21720