**A Thesis for the Degree of Master of Science**

# Plasmid-encoded MCP is involved in virulence, motility, and biofilm formation of *Cronobacter sakazakii* ATCC 29544

크로노박터 사카자키의 새로운 병원성 플라스미드 분리와
그 병원성 유전자 MCP의 특성 연구

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농학석사학위논문

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이 논문을 석사학위논문으로 제출함

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# ABSTRACT

The aim of this study was to elucidate the function of the plasmid-

encoded *mcp* (methyl-accepting chemotaxis protein) gene that plays pleiotropic roles in *C. sakazakii* ATCC 29544. By searching for virulence factors using a random transposon insertion mutant library, I identified and sequenced a new plasmid, pCSA2, in *C. sakazakii* ATCC 29544. *In silico* analysis of pCSA2 revealed that it encoded 6 open reading frames, and one of them was *mcp*. The *mcp* mutant was defective for invasion into and adhesion to epithelial cells, and the virulence of the *mcp* mutant was attenuated in rat pups. In addition, I showed that putative MCP regulates the motility of *C. sakazakii* ATCC 29544 and the expression of the flagellar genes was enhanced in the absence of a functional *mcp* gene. Furthermore, lack of the *mcp* gene also impaired the ability of *C. sakazakii* to form biofilm. Our results demonstrate a regulatory role for MCP in diverse biological processes including virulence of *C. sakazakii* ATCC 29544. To the best of our knowledge, this study is the first to elucidate a potential function of a plasmid-encoded MCP homolog in *C. sakazakii* ST8

Keywords: *Cronobacter sakazakii,* virulence, methyl-accepting chemotaxis protein, pCSA2, D-galactose-binding periplasmic protein

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# Ⅰ. INTRODUCTION

*Cronobacter* spp. is a gram negative, motile, non-spore forming, facultative anaerobic microorganism (1, 2) that has been isolated from a wide range of environments including water, soil, and a variety of fresh and processed foods such as powdered milk formula for infants (3-9). The organism is considered to be an opportunistic pathogen and has been linked to life-threatening diseases including necrotizing enterocolitis, septicemia, and meningitis in humans. In particular, this organism has also been linked to a high mortality rate (40-80%) in low birth weight neonates (5, 9-11). A few reports describe the transmission and virulence of *Cronobacter* spp.; however, we are still far from completely understanding these mechanisms. *Cronobacter* spp. are able to form biofilm on surfaces such as glass, stainless steel, polyvinyl chloride, silicone, and enteral feeding tubes, and this biofilm formation could be a vehicle of infection (12-14). The outer membrane proteins OmpA and OmpX from *C. sakazakii* were reported to be involved in invasion/adhesion to human enterocyte-like Caco-2 and intestinal INT407 epithelial cells (15-17). A LysR-type transcriptional regulator (LTTR) was reported to play a role in various phenotypes that might be important for the transmission and pathogenesis of *C. sakazakii*, suggesting a role as a global

regulator (18).

Bacterial plasmids are self-replicating, extra-chromosomal replicons that can encode a diverse assortment of virulence factors including antibiotic resistance, toxins, adherence factors, and secretion systems (19-22). Plasmid-borne virulence gene clusters of one species have been found in plasmids of other species or pathogenic groups, suggesting acquisition by horizontal gene transfer (20, 21). Recently, two closely related plasmids pESA3 (131 kb) and pCTU1 (138 kb) were identified in *C. sakazakii* ATCC BAA-894 (23) and C. *turicensis* z3032 (24), respectively. These two plasmids encode common virulence factors, an aerobactin-like siderophore and an ABC ferric-iron transporter eitABCE (25). pESA3 also encodes an outer membrane protease shown to provide serum resistance to *C. sakazakii* and enhance host invasion (26).

Methyl-accepting chemotaxis proteins (MCPs) mediate many of the chemotactic behaviors of bacteria and archaea. Bacteria respond to various environmental signals (27-29) that activate the corresponding MCPs such as Tar (taxis towards aspartate and maltose, away from nickel and cobalt), Tsr (taxis towards serine, away from leucine, indole, and weak acids), Trg (taxis towards galactose and ribose), and Tap (taxis towards dipeptides) in *Escherichia coli* (29-32). The ability of MCPs to adapt to the chemical

environment via methylation allows changes in the organism's motility and feedback adaptation (33, 34). In addition to chemotaxis, MCPs have been implicated in the virulence of certain pathogens such as *Treponema pallidum*, *Vibrio cholera*, and *Pseudomonas aeruginosa* (35-37).

Putative virulence trait in *Cronobacter* includes attachment and invasion of host cells. While screening the *C. sakazakii* type strain, ATCC 29544 random mutant library for invasion-related virulence factors, I identified a putative methyl-accepting chemotaxis protein (MCP) that is encoded by a novel plasmid, pCSA2. Complete sequencing and annotation of pCSA2 were performed, and putative MCP in pCSA2 was confirmed to be involved in adhesion and invasion in cultured mammalian cells, and regulation of motility and biofilm formation in *C. sakazakii* ATCC 29544. Our data imply a regulatory role for MCP in diverse biological processes including the virulence of *C. sakazakii* ATCC 29544.

# Ⅱ. 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. Bacteria were grown at 37°C in Tryptic Soy Broth (TSB, Difco, Detroit, MI) in aerobic conditions. When necessary, ampicillin, chloramphenicol, and kanamycin were used at 50 μg/ml, 25 μg/ml, and 50 μg/ml, respectively.

## 2.2. Random mutagenesis and screening.

Random mutagenesis was performed using the EZ-Tn5$^{TM}$pMOD$^{TM}$-3<R6K$_{\gamma}$ori/MCS> transposon system (Epicentre, Madison, WI) according to the manufacturer's instructions. Briefly, the transposon construct was released by restriction digestion with PvuII and then electroporated (V = 1.8 kV) (MicroPulser, Bio-Rad, Hercules, CA) into competent *C. sakazakii* ATCC 29544. Transformants were selected on Tryptic Soy Agar (TSA, Difco, Detroit, MI) plates containing kanamycin (50 μg/ml). The resulting colonies were individually cultured and stored at -80°C in TSB containing 15% (v/v) glycerol.

**2.3. Determination of the transposon insertion site.**

To locate the transposon insertion site, genomic DNA was isolated from candidate clones that were defective in invasion (see below for invasion assay). After self-ligation of restriction enzyme-digested DNA according to the manufacturer's protocol (Epicentre), the ligation mixture was electroporated into EC100D$^{TM}$ *pir*$^{+}$, and the transformants were rescued on TSA containing kanamycin (50 μg/ml). The self-ligated vector was recovered by using a plasmid DNA purification kit (DNA-spin$^{TM}$, INtRON, Korea) and sequenced with Tn5-specific primers provided by the manufacturer (pMOD$^{TM}$<MCS> Forward Sequencing Primer and pMOD$^{TM}$<MCS> Reverse Sequencing Primer).

**2.4. Complete nucleotide sequencing and bioinformatics.**

*C. sakazakii* ATCC 29544 plasmid DNA was prepared by using the plasmid DNA purification kit (DNA-spin$^{TM}$, INtRON, Korea). DNA sequence information obtained from transposon insertion site identification was used for primer walking to complete the whole sequence of the plasmid. Primer walking has been requested at Macrogen, Korea. The complete DNA

sequence of the plasmid was assembled using the SeqMan II sequence analysis software (DNASTAR Inc., Madison, WI). The open reading frames (ORFs) were identified with the ORF Finder at the National Center of Bioinformatics site (http://www.ncbi.nlm.gov/gorf) and GenMark Hmm prokaryotic version 2.5 (http://opal.biology.gatech.edu/GeneMark/genemark_prok_gms_plus.cgi). The functional analyses of ORFs were conducted using BLASTP and InterProScan (38, 39).

**2.5. Nucleotide sequence accession number.**

The GenBank accession number for the complete DNA sequence and annotation information for pCSA2 is KC663407.

**2.6. Cell culture.**

Human enterocyte-like epithelial Caco-2 (ATCC, Manassas, VA) cells were maintained in Eagle's Minimum Essential Medium (EMEM with L-Glutamine, ATCC) containing 20% fetal bovine serum (FBS, Invitrogen). Trypsin-treated cells were seeded (approximately 2 x $10^5$ cells per well) into

24-well tissue culture plates (TPP, Switzerland) and grown at 37°C under 5% $CO_2$. Medium was replaced every 2 days. Cell viability was determined by trypan blue staining.

**2.7. Site-specific mutagenesis of *C. sakazakii* ATCC29544.**

The one-step gene inactivation method was used to replace the *mcp* gene in pCSA2 with the kanamycin resistance ($Km^r$) cassette (35). The $Km^r$ cassette from plasmid pKD13 (40) was amplified using primers mcp-lamb-F and mcp-lamb-R. Primer mcp-lamb-F (5'-ACCATGGCAGTACTTACATTATCCGGGCAATCTGATTCATTGTAGG CTGGAGCTGCTTCG-3') carries the sequence immediately upstream of the start codon of the *mcp* gene followed by the priming site 1 sequence (underlined) of pKD13 (40). Primer mcp-lamb-R (5'-ATCTGTGATGTTCGACAGATATGGGGATATGCACCATGGCATTCCGG GGATCCGTCGACC-3') harbors the sequence immediately downstream of the stop codon of the *mcp* gene linked to the priming site 4 sequence (underlined) of pKD13 (40). The resulting PCR product was transformed into *C. sakazakii* ATCC 29544 and selected for kanamycin resistance. Insertion of the $Km^r$ cassette into the corresponding gene was verified by

colony PCR using primers K1 (5'-CAGTCATAGCCGAATAGCCT-3'), mcp-confirm-F (5'-GGTCACCACCATCGTATATTCT-3'), and mcp-confirm-R (5'-GATAAGGCTACACTGAAAGGAC-3').

**2.8. Construction of the complementation strain.**

Plasmid pMCP, which contains the MCP coding sequence and its own promoter, was constructed for complementation of the *mcp* mutant. The *mcp* gene was amplified by PCR using primers mcp-pACYC-F (5'-GAGTGTTTTCCGGATCCCGGAT -3') and mcp-pACYC-R (5'-GATGGTCGACAGATATGGGGATAT-3') and *C. sakazakii* ATCC 29544 genomic DNA as a template. The product was introduced between the BamHI and SalI restriction sites of pACYC184 (41). The sequence of the *mcp* coding region in the recombinant plasmid was confirmed by nucleotide sequencing (Macrogen, Korea).

**2.9. Invasion assay.**

The invasion assay was conducted as described previously (18) with modifications. Caco-2 cells were grown in EMEM supplemented with 20% FBS. Prior to bacterial infection, a monolayer of $2x10^5$ Caco-2 cells was prepared in a 24-well tissue culture plate. Bacteria were prepared by

transferring a 1% inoculum from overnight culture into fresh, pre-warmed TSB and incubating for 3 h (OD = 1.5). Bacterial cells were collected by centrifugation (at 10,000 x g for 3 min at 4°C), washed with phosphate-buffered saline (PBS, pH 7.4), re-suspended in EMEM with 10% 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 one times with pre-warmed PBS to remove extracellular bacteria and then incubated for 1.5 h with the pre-warmed medium supplemented with 100 μg/ml of gentamicin to kill extracellular bacteria. Afterward, the wells were washed three times with PBS, lysed in 1% Triton X-100 for 15 min, and then serially diluted in PBS. A dilution of the suspension was plated on TSA medium to enumerate the colony-forming units (CFUs).

**2.10. Adhesion assay.**

To assess adhesive ability, the epithelial cells were treated with 0.8 μg/ml of cytochalasin D (Sigma) for 30 min. Cytochalasin D depolymerizes actin filaments and inhibit the internalization of bacteria (31). Before bacterial infection, the wells were washed with PBS, and fresh EMEM media was added. Then, *C. sakazakii,* which was prepared similar to the invasion

assay, was applied onto the Caco-2 cell monolayer at a MOI of 100 and incubated for 45 min. The plates were washed three times with PBS, lysed in 1% Triton X-100, and then serially diluted in PBS. A dilution of the suspensions was plated on TSA medium to enumerate the CFUs of adhesive bacteria.

### 2.11. *In vivo* rat pup virulence assay by competitive index analysis.

Bacterial cells grown for 3 h in TSB medium were pelleted, washed, and re-suspended in PBS. Three-day-old Sprague-Dawley female rat pups were used to assess the virulence of the *C. sakazakii* strains. A mixed inoculum of $10^7$ CFU of each strain in 50 µl of PBS was administered orally into groups of rat pups (5 rats/group). To analyze bacterial colonization in organs, the rat pups were sacrificed at 24 h after infection, and the spleens and livers were removed aseptically. The organs were homogenized in 1 ml of ice-cold PBS and serially diluted. Bacterial loads were determined by plating the diluents on TSA agar plates in the presence or absence of appropriate antibiotics. The results are presented as competitive index (CI) values, which were calculated by dividing the CFUs of the mutant strain by the CFUs of the wild-type strain.

**2.12. Motility assay.**

A 1-µl aliquot of subculture grown for 3 h in TSB was spotted in the middle of a swim plate (TSA, 0.3% agar) and allowed to dry for 1 h at room temperature. The plates were incubated at 37°C for 8 h.

**2.13. RNA isolation and qRT-PCR.**

For the extraction of RNA from *C. sakazakii*, bacteria were grown at 37°C for 3 h in TSB. RNA was extracted using the RNeasy Mini Kit (Qiagen) followed by treatment with RNase-free DNase (Ambion). cDNA was synthesized using Omnitranscript reverse transcription reagents (Qiagen) and random hexamers (Invitrogen) and quantified by using the 2X iQ SYBR green Supermix (Bio-Rad). Real-time amplification of the PCR products was performed using the iCycler iQ real-time detection system (Bio-Rad). The calculated threshold cycle ($C_T$) corresponding to a target gene was normalized to the $C_T$ of the control gene coding 16s ribosomal RNA (rRNA) (42). The primers were designed using a PCR primer design tool, Primer3 Plus. The sequences of the primers used in the quantitative reverse transcription-PCR (qRT-PCR) analysis are listed in Table 2.

**2.14. Biofilm assay.**

The experiment was performed as previously described (18) with modifications. *C. sakazakii* was inoculated into 3 ml TSB and incubated at 37°C with aeration until the cell density reached 2.5 x $10^8$ CFU/ml. The culture was diluted 1:100 in TSB, and 500 µl portions were loaded in triplicate into a 24-well polystyrene plate (SPL Life Sciences, Korea) and incubated at 37°C for 48 h without shaking. For fixation of the biofilm, 100 µl of 99% methanol was added for 15 min, the supernatants were removed, and the plates were air-dried. Then, 500 µl of crystal violet (CV) solution was added. After 20 min, the excess CV was removed, and the plates were 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 micro plate reader (Tecan, Austria).

**2.15. Two-dimensional gel electrophoresis (2DGE)**

The wild type and the mutant were grown to mid-log phase in 50 ml TSA broth in Erlenmeyer flasks (250 ml) at 37°C with rotation at 220 rpm for 1.5 h. Protein was prepared from the wild type and the mutant as

described by Yoon *et al.(43)*. Protein concentrations were determined by the Bradford assay. For first-dimension separation, 400 or 900 μg of protein from each sample was separated by isoelectric focusing using 13-cm IPG strips (GE Healthcare). For second-dimension separation, proteins were separated based on molecular weight by sodium dodecyl sulfated-polyacrylamide gel electrophoresis (SDS-PAGE) using 10 or 12% acrylamide gels. Gels were visualized by colloidal Coomassie blue staining (Invitrogen). Protein spots showing different levels were excised from the stained gels, which was followed by in-gel digestion using the method (43). Protein identification was analyzed by MALDI TOF-TOF mass spectrometry (http://kiki.snu.ac.kr).

**2.16. Statistical analysis.**

Statistical analyses were conducted using the GraphPad Prism program (version 5.0). All results were analyzed by the unpaired *t*-test. Data were represented as mean ± standard deviation. A *P* value of $<0.05$ was considered statistically significant.

.

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

| Strain or plasmid | Description | Reference of source |
|---|---|---|
| *C. sakazakii* | | |
| ATCC 29544 | Wild-type | ATCC |
| HR101 | *mcp*::$Km^r$ | This study |
| HR102 | HR101 with pPMCP | This study |
| HR103 | Δ*mglB* | This study |
| HR104 | Δ*mglB*, Δ*mcp* | This study |
| *E. coli* | | |
| DH5α | *supE44 hsdR17 recA1 gyrA96 thi-1 relA1* | (44) |
| EC100D™ | *pir*$^+$ | Epicentre |
| Plasmids | | |
| pACYC184 | $rep_{p15A}$ $Cm^r$ $Tet^r$ | (41) |
| pKD13 | $repR_{6K\gamma}$ $Ap^r$-FRT $Km^r$-FRT | (40) |
| pKD46 | $rep_{pSC101}$(Ts) $Ap^r$ $P_{araBAD}$ *γ β exo* | (40) |
| pMCP | pACYC184-*mcp* | This study |

# Ⅲ. RESULTS

## 3.1. A plasmid-encoded gene discovered in an invasion-attenuated mutant.

To identify genes related to the virulence of *C. sakazakii*, transposon-mediated random mutant libraries were constructed in *C. sakazakii* ATCC 29544. Invasion-defective clones were screened by invasion assay into epithelial cells (Fig. 1). I found the location of transposon insertion site and sequenced the boundary region between the transposon and the *C. sakazakii* genome with Tn5-specific primers provided by the manufacturer. A mutant had the transposon inserted into an unknown region of DNA. A nucleotide blast search of the boundary region showed no homology with *Cronobacter* spp. but identified part of the *mobA* gene, which is related to plasmid conjugation (45), suggesting that this mutant might have a transposon in a novel plasmid of *C. sakazakii* ATCC 29544.

In order to confirm the presence of the boundary region sequence in the plasmid of *C. sakazakii* ATCC 29544, primers were designed using the boundary sequence from the transposon insertion mutant. The expected size of the PCR amplicon was identified only in the plasmid fraction (data not shown). Taken together, these data implied that there is an unreported plasmid in *C. sakazakii* ATCC 29544 that might be related to its virulence.

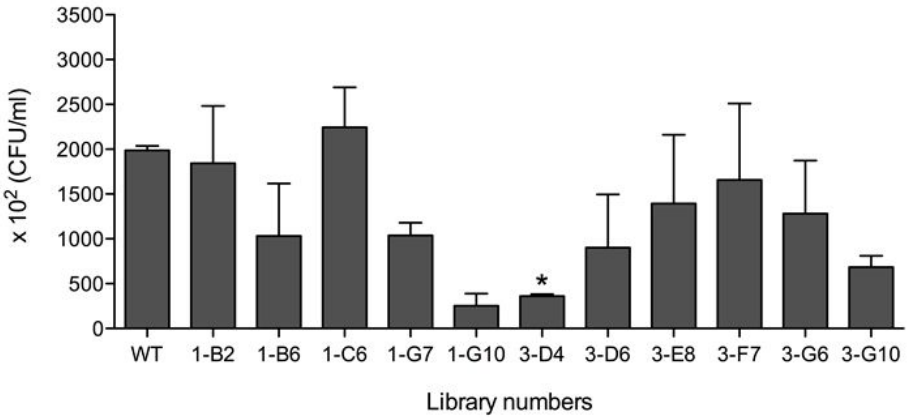


**Figure 1. Invasion assay for virulent gene screening.** Transposon-mediated random mutant libraries were screened by invasion assay into epithelial cells.

### 3.2. pCSA2 is a novel plasmid in *C. sakazakii* ATCC 29544 that contains 6 open reading frames.

By using primer walking, I obtained the complete plasmid sequence and named it pCSA2, which stands for plasmid of *C. sakazakii*. The pCSA2 comprises 5,103 bp with overall G+C content of 55.01%. Six ORFs were identified on pCSA2: four genes predicted to encode relaxases (mobA, mobB, mobC, and mobD), a putative DNA replication protein-encoding gene, and a putative methyl-accepting chemotaxis protein (MCP)–encoding gene (Fig. 2A). In the pCSA2 DNA sequence, the region containing the relaxase genes and the DNA replication gene is highly similar (97% identities) to that of the *Enterobacter cloacae* plasmid pS51B which is 4854 bp in size (Fig. 2B), which belongs to the ColE1 superfamily of mobilizable plasmids and is commonly detected in *Enterobacteriaceae* (46). A relaxase is a single-strand DNA trans-esterase enzyme produced by some prokaryotes and viruses (45). Relaxases are responsible for site- and strand-specific nicks in double-stranded DNA. Mob relaxases nick at the origin of transfer (oriT) to initiate the process of DNA mobilization and transfer known as bacterial conjugation (47). Therefore, the four *mob* genes might have a role in the conjugation of pCSA2. The DNA replication protein is required for the replication of the plasmid (48)

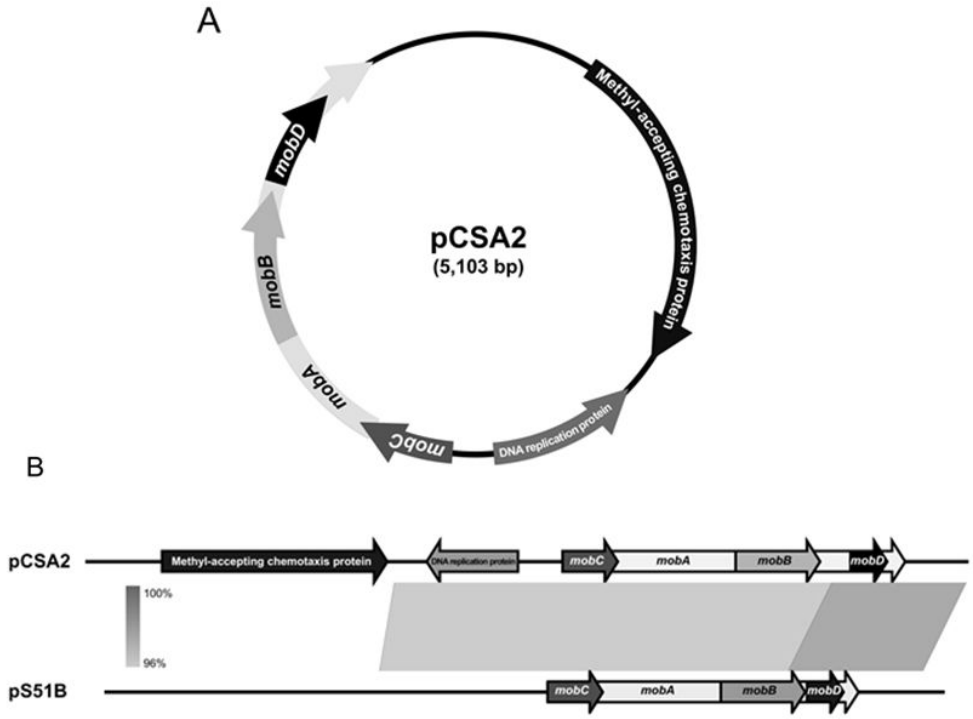


**Figure 2. Identification of pCSA2.** (A) Schematic representation of pCSA2 in *C. sakazakii* ATCC 29544. The transposon (Tn5) insertion is designated. (B) Linear comparison figures showing BLAST matches between pCSA2 and pS51B using Easyfig (Ver. 2.1).

### 3.3. Mcp protein is comprised of two sensory PAS domain and MCP domain.

Despite lack of DNA sequence information, its protein sequences were well-conserved. The deduced protein harbors an MCP domain and two sensory PAS (Per-Arnt-Sim sensory) domains (Fig. 3A). Therefore, I named this gene *mcp* (methyl-accepting chemotaxis protein). MCP domains share similar topology and signaling mechanisms. MCPs either bind ligands directly or interact with ligand-binding proteins, transducing the signal to downstream signaling proteins in the cytoplasm (29, 30). The PAS domain is responsible for sensing the input signal and protein-protein interaction. PAS domains have been implicated in diverse biological processes including global regulation of metabolism, nitrogen fixation, aerotaxis, hypoxia responses, and ion channel function in both prokaryotes and eukaryotes (49-52). *C. sakazakii* is likely to encounter numerous suboptimal conditions during its transition from the environment to the host (8, 9, 53). Therefore, elucidation of the mechanisms used by *C. sakazakii* to regulate environmental signals will be important for the development of control and treatment strategies. In this respect, the *mcp* gene was a reasonable target for further study.

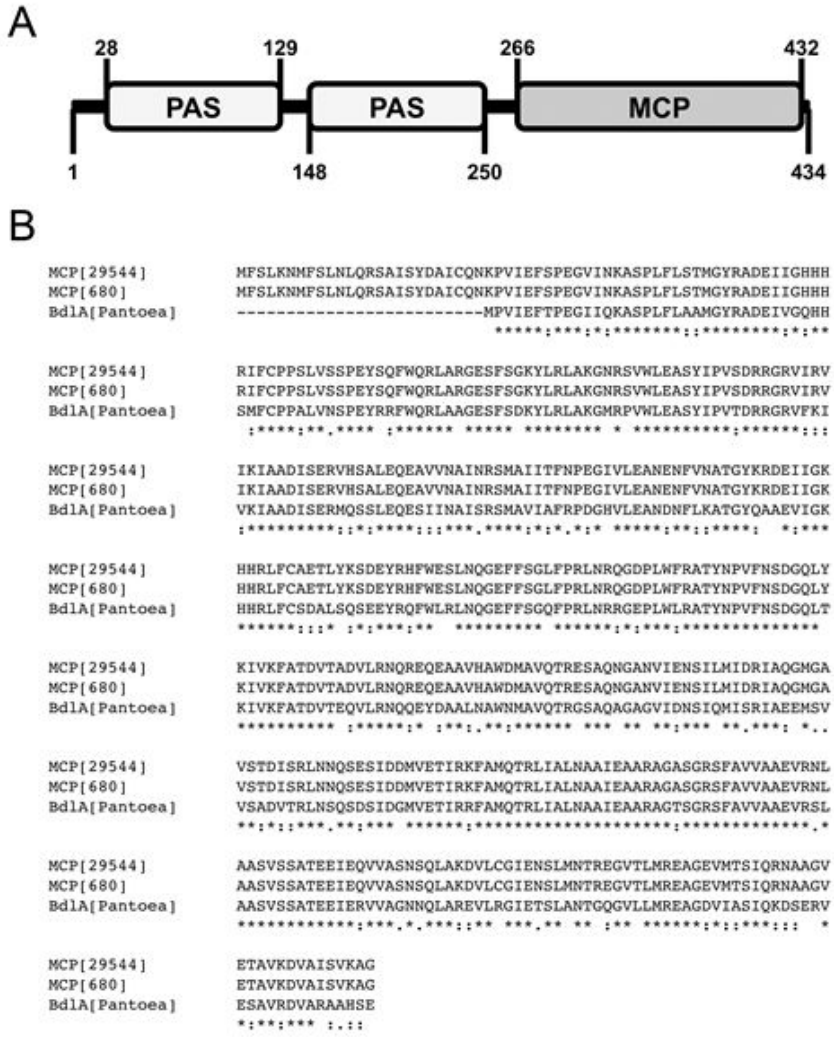


**Figure 3. Deduced sequence analysis of MCP.** (A) Domain structures of MCP. PAS, Per-Arnt-Sim sensory domain; MCP, Methyl-accepting Chemotaxis Protein domain. (B) Alignment of amino acid sequences of MCP of *C. sakazakii* ATCC 29544, MCP of *C. sakazakii* 680 (ZP_19182295.1), and BdlA of Pantoea vagans C9-1 (YP_003930749.1) by Clustal Omega (ver. 1.1.0).

### 3.4. Plasmid-encoded putative MCP affects adhesion/invasion.

To explore whether the *mcp* gene could contribute to *C. sakazakii* ATCC 29544 virulence, I constructed a strain in which the entire *mcp* gene was replaced with a gene encoded kanamycin resistance gene using lambda red recombination. As the *mcp* gene is encoded in the plasmid, I did not remove the inserted $Km^r$ cassette for the maintenance of the plasmid. Due to the plasmid copy number, I sub-cultured the mutant on TSA plates containing kanamycin until there was no *mcp* PCR product (Fig. 4A). Removal of the *mcp* gene from the plasmid presented no significant growth defect compared to the wild-type strain in TSB media (data not shown). After construction of the *mcp* mutant, the gentamicin protection assay confirmed the observations in the random mutant library screening. The mutant had a significantly lower invasion rate (approximately 20% compared to 100% of WT) compared to WT in the invasion assay (Fig. 4B). This result was comparable to the library screening results. The phenotypic defect of the *mcp* mutant was indeed due to *mcp* function because expression of the *mcp* gene from a complement strain enabled the *mcp* mutant to invade Caco-2 cells at a similar level to the wild-type strain (Fig. 4B).

For successful invasion into epithelial cells, *C. sakazakii* needs to bind to the surface of epithelial cells (15, 17). In the adhesion assay, I pre-

treated Caco-2 cells with cytochalasin D (CD), an agent that causes microfilament depolymerization in eukaryotic cells and thus inhibits *C. sakazakii* invasion (17). The inhibitory effect of CD on the internalization of *C. sakazakii* to Caco-2 cells was confirmed by the wild-type strain (data not shown). Only 25% of *mcp* mutants were recovered from the adhesion assay compared to 100% in WT (Fig. 4C). Again, the adhesive defect of the *mcp* mutant was restored by the complementary plasmid expressing *mcp* (Fig. 4C). These results indicate that the MCP encoded in pCSA2 is important for the infection of *C. sakazakii*.

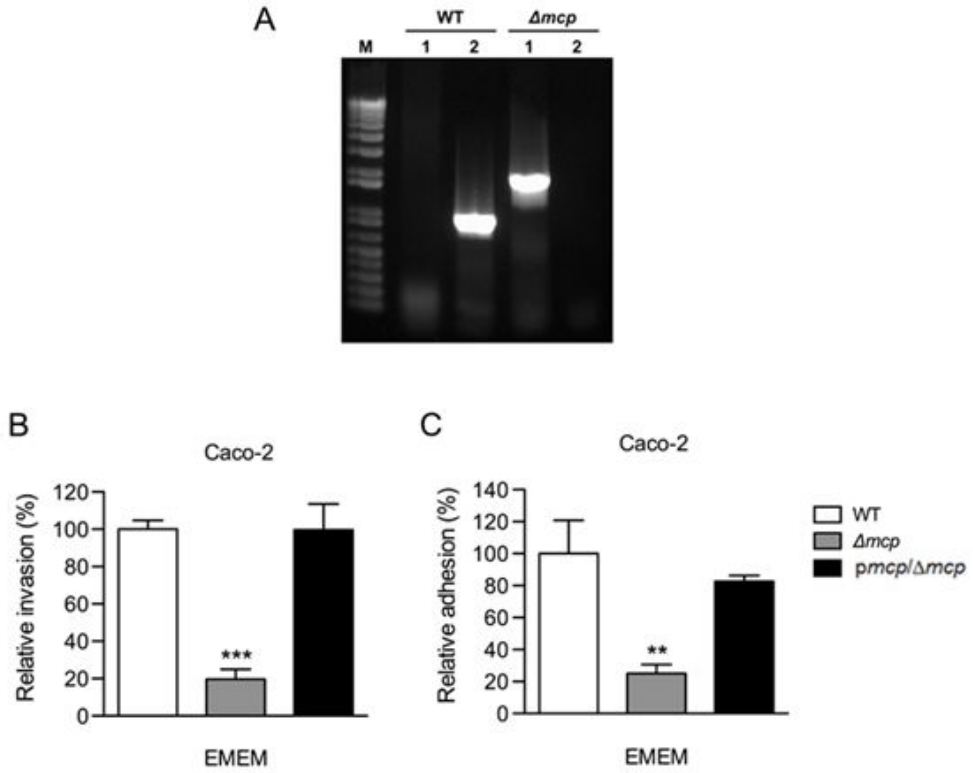
A
WT
Δmcp
M
1
2
1
2
B
Caco-2
Relative invasion (%)
120
100
80
60
40
20
0
***
EMEM
C
Caco-2
Relative adhesion (%)
140
120
100
80
60
40
20
0
**
EMEM
WT
Δmcp
pmcp/Δmcp

**Figure 4. Construction of the *mcp* deletion mutant by lambda recombination and the contribution of the *mcp* gene to *C. sakazakii* virulence.** (A) Confirmation of the wild-type (WT) strain and the *mcp* mutant (Δ*mcp*) by PCR. Lane M, the nucleotide size maker; Lane 1, primer sets (K1 and mcp-confirm-R) for the Kmr cassette; Lane 2, primer sets (mcp-confirm-F and mcp-confirm-R) for the *mcp* gene. (B) Caco-2 epithelial cells were infected with the wild-type (WT) strain (ATCC 29544), the *mcp* deletion mutant (HR101), or strain HR101 harboring the pMCP plasmid (*Δmcp*/pMCP). The numbers of intracellular bacteria were determined at 1.5 h after infection by using the gentamicin protection assay. Means and standard deviation from at least three independent experiments are shown. Triple asterisks indicate that the numbers of bacteria were significantly different ($P<0.001$) compared to the WT strain. (C) The CD-pretreated Caco-2 epithelial cells were infected with the *C. sakazakii* strains similar to the invasion assay. The numbers of intracellular bacteria were determined at 45 min after infection without using the gentamicin. Means and standard deviation from at least three independent experiments are shown. Double asterisks indicate that the numbers of bacteria were significantly different ($P<0.01$) from those of the WT strain.

### 3.5. *C. sakazakii* lacking a plasmid-encoded putative MCP showed attenuated virulence in rat pups.

The effect of the *mcp* deletion mutation on *C. sakazakii* virulence was further analyzed using a newborn rat model (54, 55). Comparison of the CI(competitive index) values of wild-type to the *mcp* deletion mutant revealed lower numbers of the *mcp* deletion mutant cells than wild-type cells at 24 h post infection in both the liver and the spleen of rat pups (Fig. 5). This phenotypic difference was due to the absence of MCP function because the *mcp* deletion strain carrying the complementary plasmid expressing *mcp* was able to colonize the liver and spleen as efficiently as the wild-type strain (Fig. 5). Taken together, these results suggest that putative MCP plays a crucial role in the pathogenesis of *C. sakazakii* ATCC 29544.

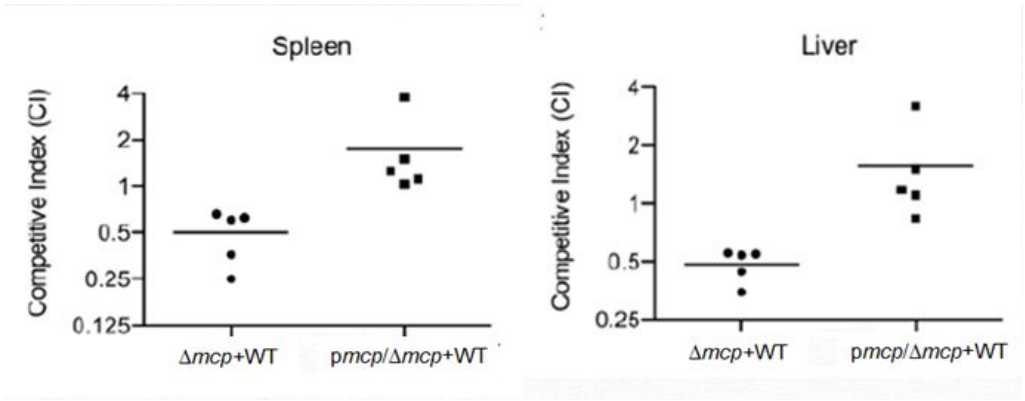


**Figure 5.** ***In vivo*** **rat pup assay.** Groups of SD rats (5 rats/group) were infected orally with a mixed inoculum of approximately $10^7$ CFU of each wild-type and mutant strain. At 24 h after infection, the numbers of bacteria in the liver and spleen were determined. The competitive index (CI) values were calculated by dividing the number of mutant strain CFUs by the number of wild-type strain CFUs.

### 3.6. Plasmid-born MCP regulates motility of *C. sakazakii* ATCC 29544.

As *mcp* is a putative MCP-coding gene and chemotactic regulation in bacteria results in general changes in flagellar rotation (27), I assessed the motility of the WT and the *mcp* mutant. The wild-type strain was motile but showed low motility (~15.7 mm in diameter) under standard motility assay conditions. On the other hand, the *mcp* mutant was hypermotile (~31.5 mm) (Fig. 6A). Introduction of the plasmid expressing the *mcp* gene reduced the motility (~17.3 mm) close to that of the wild-type strain, suggesting that this gene is related to flagellar regulation.

Next, I evaluated the expression level of several flagellar biosynthesis-related genes in the *mcp* mutant and wild-type strain by qRT-PCR. I chose four genes, *flhD*, *flhC*, *fliA*, and *fliC,* that represent the regulation of flagellar assembly (56). Due to lack of information on the genome sequence of *C. sakazakii* ATCC 29544, I determined the sequences of those genes from the genome sequence data of *C. sakazakii* BAA-894 and then confirmed the existence of those genes in ATCC 29544 (data not shown). Interestingly, the mRNA levels of the *fliA* and *fliC* genes in the *mcp* deletion mutant increased by ~5 and ~3 fold, respectively, compared with the wild-type strain. The complementary plasmid reduced the expression of both *fliA* and *fliC*; however, the expression level was not as low as in the wild-type

strain (Fig. 6B). This result might be due to the low copy number of the backbone plasmid pACYC184 (41) for the complementary plasmid, pMCP. The *fliA* gene encodes an alternative sigma factor, which is responsible for the transcription of class III flagellar genes including the filament structure genes and the genes for the chemosensory pathway (56, 57). Because the *fliC* gene is one of the class III flagellar genes (56), the enhanced expression of *fliC* might be due to the large amount of FliA. These regulations corresponded to the increase of motility in the *mcp* mutant. Moreover, two flagellar master regulators, *flhD* and *flhC* genes, were not changed in their expression in the *mcp* mutant (Fig. 6B), suggesting that MCP affects the expression of class III flagellar genes via *fliA*.

Motility is a well-known virulence factor in many pathogenic bacteria (58, 59). The motility phenotype is reported to be coupled to the expression of multiple virulence factors, and the negative effects of hypermotility on virulence have been reported in *Vibrio cholera* (60, 61) and *Ralstonia solanacearum* (62). As attachment of *C. sakazakii* to the cell surface is a critical step for host invasion (17), disruption of the adherence of the bacteria to the host cell (Fig. 4C) due to the hypermotility of the *mcp* mutant.

**Table 2. Primers used in qRT-PCR analysis**

| Primers | Target genes | Sequences (5′ to 3′) |
|---|---|---|
| fliA-RT-F | *fliA* | GCAGGAACTGGGACGTAACG |
| fliA-RT-R | *fliA* | GTGTCGAGCAACATCTGACGAT |
| fliC-RT-F | *fliC* | CGTATCGCTGGTGGTGCTAA |
| fliC-RT-R | *fliC* | CAGCGCCAACCTGAATTTTC |
| flhD-RT-F | *flhD* | AAGCGTCTGCGATGTTTCG |
| flhD-RT-R | *flhD* | CAGCCAGTTTCACCATTTGC |
| flhC-RT-F | *flhC* | GCAACTTAGCCGCGGTAGAC |
| flhC-RT-R | *flhC* | TGAACCAGTCCGTGGAAAAGG |
| control-RT-F | 16s rRNA | GGGCCTCATGCCATCAGAT |
| control-RT-R | 16s rRNA | TCTCAGACCAGCTAGGGATCGT |

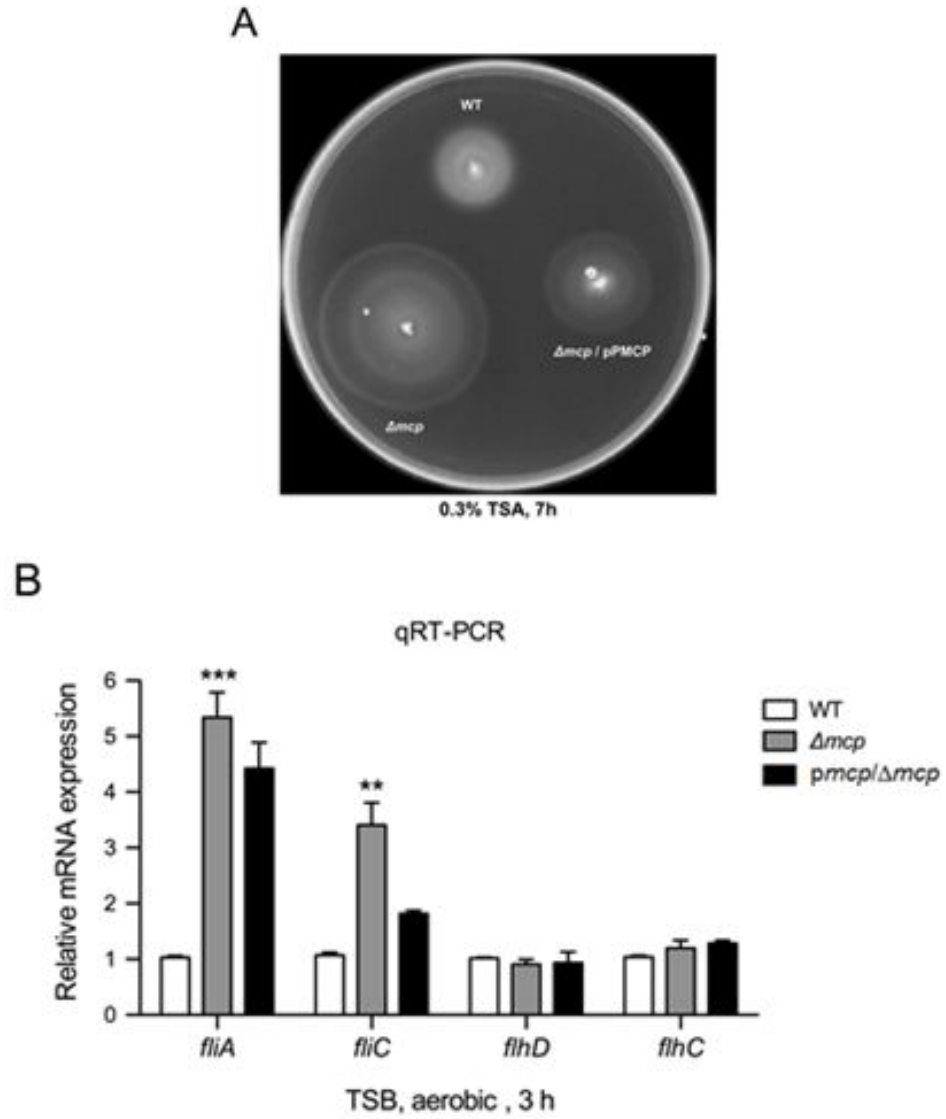
A
WT
Δmcp / pPMCP
Δmcp
0.3% TSA, 7h
B
qRT-PCR
Relative mRNA expression
0
1
2
3
4
5
6
***
**
WT
Δmcp
pmcp/Δmcp
fliA
fliC
flhD
flhC
TSB, aerobic , 3 h

**Figure 6. The effect of the *mcp* gene on the regulation of motility in *C. sakazakii*.** (A) A 1-μl aliquot of subculture was spotted onto the middle of a swim plate (TSA, 0.3% agar). (B) The transcription levels of the *fliA*, *fliC*, *flhD*, and *flhC* genes in *C. sakazakii* were determined via qRT-PCR. The bacterial RNA was isolated from the wild-type (WT) strain (ATCC 29544), the *mcp* deletion mutant (HR101), or strain HR101 harboring the pMCP plasmid (*Δmcp*/pMCP). To obtain the relative mRNA expression values on the *y*-axis, the mRNA level of each gene was divided by the mRNA level of the 16s rRNA-coding gene. The mRNA expression values were further normalized by the transcription levels displayed by the wild-type (WT) strain. Means and standard deviations from three independent experiments are shown. Asterisks indicate significant differences (***, $P<0.001$; **, $P<0.01$).

### 3.7. Biofilm formation is affected by the plasmid-encoded putative MCP.

In the blast results, MCP of *C. sakazakii* ATCC 29544 showed 74% identity with biofilm dispersion protein BdlA of *Pantoea* spp. (Fig. 3B). In *Pseudomonas aeruginosa*, BdlA, a chemotaxis transducer protein, is essential for biofilm dispersion (63-65). According to the proposed model of BdlA regulation in *P. aeruginosa* biofilm dispersion, an active form of BdlA is required for the transition from surface-attached biofilm to the motile lifestyle, and BdlA is intact but inactive under the planktonic conditions (64). Therefore, I hypothesized that deletion of *mcp* might affect biofilm formation in *C. sakazakii*. As expected, the *mcp* deletion mutant resulted in ~2-fold reduction of biofilm formation (Fig. 7). The expression of the *mcp* gene from a complementary plasmid enabled the *mcp* deletion mutant to form biofilm (~1.6-fold increase compared with the mutant), albeit at a lower level than the wild-type strain (Fig. 7).

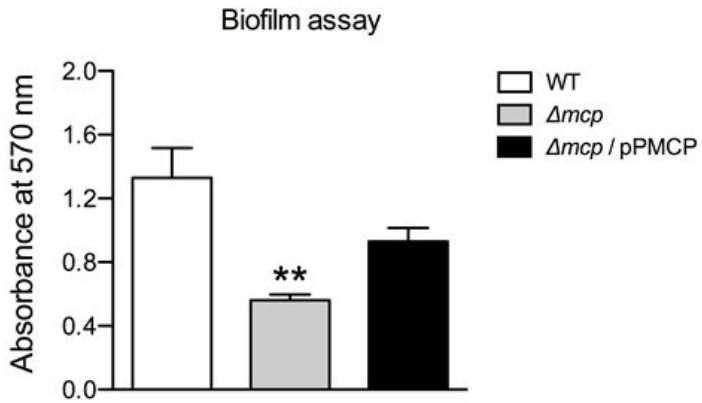


**Figure 7. Biofilm formation**. The crystal violet biofilm assay was performed in triplicate and repeated three times. Biofilm formation is indicated by the absorbance at 570 nm from the wild-type (WT) strain (ATCC 29544), the *mcp* deletion mutant (HR101), or strain HR101 harboring the pMCP plasmid (*Δmcp*/pMCP). Double asterisks indicate that the absorbance was significantly different ($P<0.01$) compared to the WT strain.

### *3.8.* Lack of the *mcp* gene affected the expression of multiple genes in *C. sakazakii*

Profiles of protein expression for the WT and the mutant were compared by using 2 dimensional gel electrophoresis (2DGE). As shown in Fig.8 (A) and (B), some spots showed differences in expression between the WT and mutant, and 4 protein spots were selected and identified by matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) analyses after digestion with trypsin. 4 proteins (MglB, RbsA, MreB, Prs) were up-regulated in the mutant. Proteins involved in rod shape-determining protein (MreB), ribose import ATP-binding protein (RbsA), D-galactose-binding periplasmic protein (MglB), Ribose-phosphate pyrophosphokinase (Prs).

To confirm the expression of the identified genes, quantitative real-time PCR was performed (Fig. 8C). The transcriptional expression level of *mglB*, *rbsB*, *mreB*, *prs* genes was increased about 96.3, 1.8, 2.8 and 5.4 –fold, respectively. The results obtained with the transcriptional data support the proteomic data in demonstration the negative regulatory role of *mcp* in *C. sakazakii* ATCC 29544.f

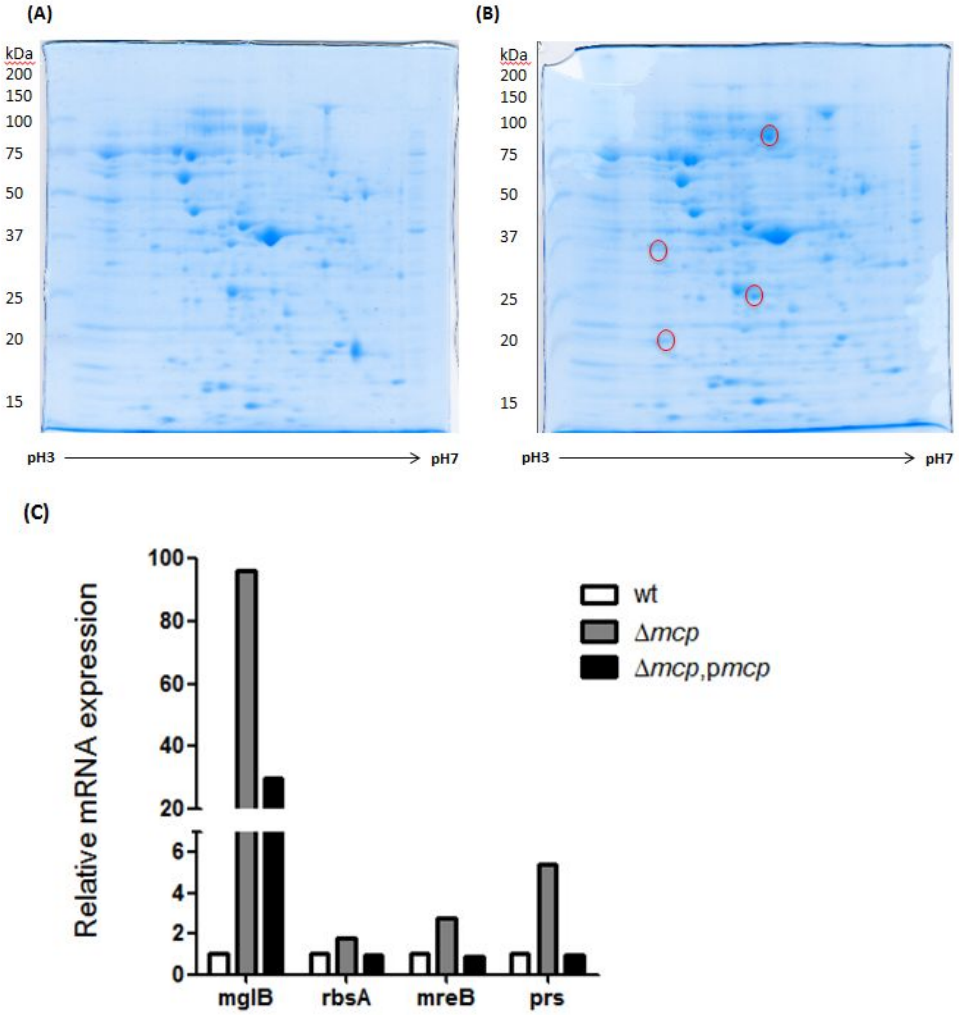


**Figure 8.** (A),(B) 2DEG images of whole-cell lysates from wild type and *mcp* deletion mutant. Proteins were separated by 2DEG using IPG strips, pH 4 to 7. (C) Confirmation of expression levels by quantitative real time PCR. The transcriptional levels of genes that exhibited differential expression in the wild type and the mutant lacking the methyl-accepting chemotaxis protein as determined by proteomic analysis were confirmed by qRT-PCR

### 3.9. *MglB* mutant has similar effect with *mcp* mutant.

*mglB* was chosen as second target gene because it showed a dramatic increase when *mcp* gene was deleted. In *E. coli* and other enteric bacteria, MglB is a periplasmic, high-affinity receptor for galactose (and glucose) and a component of an ATP-binding cassette (ABC) operon involved in galactose/glucose import (66). Upon binding carbohydrate, MglB also interacts specifically with the *E. coli* sensory transducer Trg (a methyl-accepting chemotaxis protein) to induce a chemotactic response (67).

I constructed *mglB* deletion mutant and *mglB*, *mcp* double mutant. Motility assay was performed with those strains. All the mutants showed hypermotility compare to WT. However, *ΔmglB* and *ΔmglBΔmcp* double mutant are somewhat reduced motility than *mcp* mutant (Fig 9A). In the case of invasion assay, *ΔmglB* and *ΔmglBΔmcp* double mutant had a significantly lower invasion rate (approximately 20% compared to 100% of WT) compared to WT (Fig.9B). Those two mutant strains have same phenotype demonstrating that *mcp* gene phenotype might be hidden due to *mglB* gene. Further study will be needed to understand role of *mglB* in virulence of *C. sakazakii*

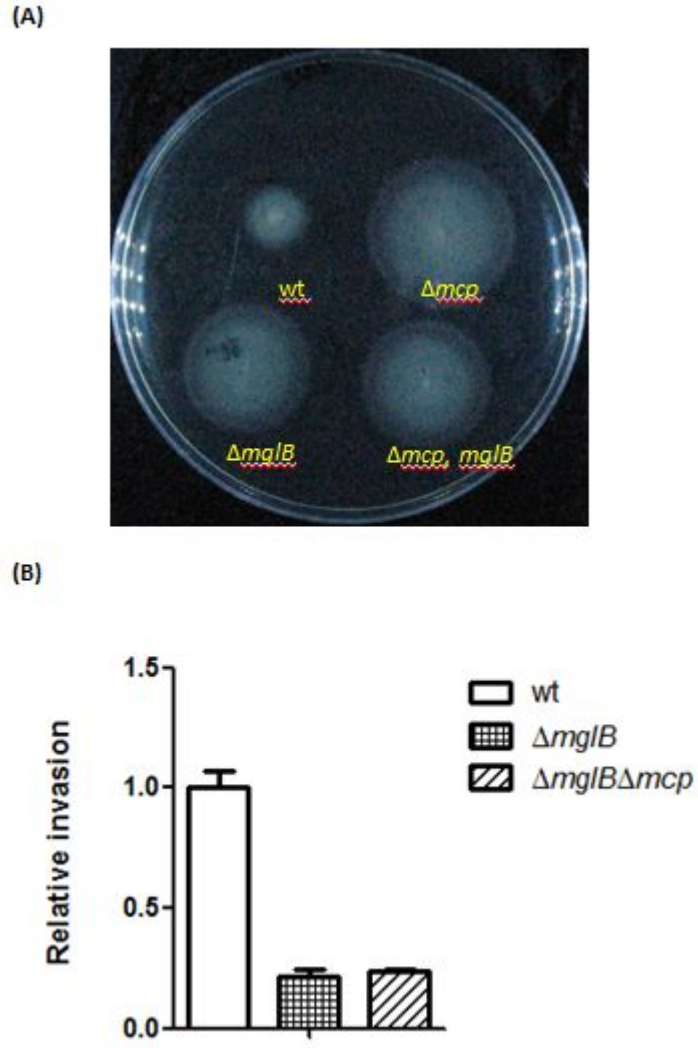


**Figure 9. The phenotypes characteristic of *ΔmglB* and *ΔmglBΔmcp* double mutant.** (A) A 1-μl aliquot of subculture was spotted onto the middle of a swim plate (TSA, 0.3% agar) (B) Caco-2 epithelial cells were infected with the wild-type (WT) strain (ATCC 29544), the *mglB* deletion mutant and *ΔmglBΔmcp* double mutant.

# Ⅳ. DISCUSSION

*Cronobacter sakazakii* is comsidered to be an emerging pathogen cuased bacteremia, meningitis, and local necrotizing enterocolitis in premature infants. The survival infants from *C. sakazakii* infection have severe neurological malfunction including brain abscesses, development delays, and impaired sight and hearing. Although the incidence of neonatal infections by *C. sakazakii* has increased, the pathogenicity of *C. sakazakii* is virtually unknown.

In this study, I identified new plasmid, named pCSA2, which is associated virulence for the first time in *C. sakazakii* ATCC 29544. According to *Cronobacter* spp. plasmid analysis by PCR using primers to detect the *repA* gene by Franco group, 97% of 229 *Cronobacter* species isolates were found to possess a homologous RepFIB plasmid(25). *C. sakazakii* ATCC 29544 strain was also PCR positive for repA gene of pCTU3, pESA3/pCTU1 (data not shown). Consequently, *C. sakazakii* ATCC 29544 might have another two virulence plasmids possessed a homologous RepFIB besides pCSA2.

Multilocus sequence typing (MLST) discriminates *Cronobacter* genus. Comparing 7 putative housekeeping genes (*atpD, fusA, glmS, gltB,*

*gyrB, infB* and *pps*) DNA sequences distributes the species and strain's ST. Among more than 50 STs, ST8 which is primarily composed of isolates from clinical sources comprises *C. sakazakii* ATCC 29544 and *C. sakazakii* 680(68-70). The *mcp* gene sequence from *C. sakazakii* ATCC 29544 is matched 100% by *C. sakazakii* 680, which is not found in any other lineages (70), suggesting that the MCP related to virulence is probably specific to the ST8 lineage.

A new plasmid, pCSA2, in *C. sakazakii* ATCC 29544 was identified and completely sequenced. I demonstrated that the putative MCP encoded in pCSA2 is important for invasion/adhesion of *C. sakazakii* ST8 into host cells and for its movement into deeper organs such as the liver and spleen. MCP also regulated two other phenotypes, motility and biofilm formation. Additionally, to check oxidative stress response of *mcp* gene gentamicin protection assay was conducted. Monolayer of $5x10^5$ RAW 264.7, mouse leukaemic monocyte macrophage cell line, maintained in DMEM supplemented with 10% FBS. After infection for 45 min, 100 μg/ml of gentamicin is added and they are incubated for 45 min, 8, 24, 48 hours. Next the macrophage cells are lysed using Triton X-100, and they are plated on TSA agar medium. In the case of survival assay, no statistical significance was found between WT and *mcp* mutant strain (data not shown). I also

checked hydrogen peroxide sensitivity assay. The overnight cultured WT, *mcp* mutant and complementation strains were serially diluted and dotted on TSA agar containing 250 μM $H_2O_2$. There was no difference between them (data not shown). It is suggested that *mcp* is not involves tolerance to oxidative stress in *C. sakazakii*. These diverse effects of putative MCP should be further studied, especially for the global regulations of *C. sakazakii* ATCC 29544.

I also report here that MglB is important for the invasion of *C. sakazakii* into the host cells. MglB interacts with the sensory transducer Trg to induce a chemotactic response in *E. coli(67)*. It is thus plausible that the mgl operon plays a central role in the physiology of virulent *C. skakazakii* by comprising a (the) major uptake pathway for a vital energy source. The previous study showed the existence of a mglBAC operon in *Treponema pallidum* may have important implications with respect to *T. pallidum* survival, tissue dissemination, and sensory transduction during virulence expression (71). These findings potentially have important implications for *C. sakazakii* pathogenesis, particularly as they may pertain to chemotactic responses by *C. sakazakii*.

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# 국문 초록

크로노박터 사카자키는 그람 음성균으로 다양한 환경에 분포하며 특히 분유에서 검출되어 영유아가 섭취하였을 때 높은 치사율을 일으키는 세균입니다. 아직까지 사카자키 균의 병원성에 대해 연구된 바가 미흡하여 병원성 유전자에 대한 연구를 수행하였습니다. 병원성 유전자 탐색을 위해 무작위로 세균의 유전자를 망가뜨린 후 사람 유래 소장 상피 세포에 감염시켰고 세포 내로 침투하는 능력이 현저히 떨어지는 군을 선별하였습니다. 그 결과 세균 DNA 이외의 작은 원형의 DNA 분자인 플라스미드에 있는 유전자 Methyl-accepting chemotaxis (MCP)가 망가져 있었고 그 플라스미드는 아직 학계에 보고되어 있지 않은 종류였습니다. pCSA2라고 명명한 플라스미드의 전체 DNA 시퀀스를 밝혔고 미국 국립생물정보센터(NCBI)에 최초로 등재하였습니다. pCSA2는 크기가 약 5kb였으며 총 6개의 유전자를 가지고 있었습니다. 그 중 병원성 유전자로 파악되는 *mcp* (methyl-accepting chemotaxis)의 영향을 알아보기 위해 *mcp*를 항생제 유전자로 치환시킨 돌연변이를 만들어 실험에 돌입했습니다. 이 *mcp* 유전자가 없으면 사카자키균은 소장 상피 세포의 부착능이 떨어지고 따라서 침투능력도 감소하는 것으로 나타났습니다. 출생 3일 된 새끼 쥐에 균

을 주입하는 동물 실험을 통해 in vivo상에서도 소장에서 다른 기관으로의 전이가 방해받는다는 사실을 입증했습니다. 그 밖에도 *mcp*는 균의 운동성과 바이오필름 형성을 조절하는 다면발현성을 보였습니다. 이런 다양한 효과들로 인해서 *mcp*는 사카자키균의 병원성에 중요한 인자임을 확인할 수 있었습니다.

주요어 : 크로노박터 사카자키, methyl-accpeting chemotaxis protein, 침투능, 부착능, 바이오필름, 운동성, 병원성 인자

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