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Plasmid-Encoded MCP Is Involved in Virulence, Motility, and Biofilm Formation of *Cronobacter sakazakii* ATCC 29544

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The aim of this study was to elucidate the function of the plasmid-borne *mcp* (methyl-accepting chemotaxis protein) gene, which plays pleiotropic roles in *Cronobacter sakazakii* ATCC 29544. By searching for virulence factors using a random transposon insertion mutant library, we identified and sequenced a new plasmid, pCSA2, in *C. sakazakii* ATCC 29544. An *in silico* analysis of pCSA2 revealed that it included six putative 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, we demonstrated that putative MCP regulates the motility of *C. sakazakii*, and the expression of the flagellar genes was enhanced in the absence of a functional *mcp* gene. Furthermore, a lack of the *mcp* gene also impaired the ability of *C. sakazakii* to form a biofilm. Our results demonstrate a regulatory role for MCP in diverse biological processes, including the 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 the *C. sakazakii* sequence type 8 (ST8) lineage.

Cronobacter spp. are Gram-negative, motile, non-spore-forming, facultative anaerobic microorganisms (1, 2) that have 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, with a high mortality rate (40 to 80%) in low-birthweight neonates (7, 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. can form a 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* are reportedly involved in invasion/adhesion to human enterocyte-like Caco-2 and intestinal INT407 epithelial cells (15–17). A LysR-type transcriptional regulator (LTTR) reportedly plays 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 and extrachromosomal 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, the genomes of two *Cronobacter* species, *Cronobacter sakazakii* ATCC BAA-894 and *C. turicensis* z3032, have been completely sequenced and shown to possess two and three plasmids, respectively (23, 24). In particular, pESA3 (131 kb) of *C. sakazakii* ATCC BAA-894 (23) and pCTU1 (138 kb) of *C. turicensis* z3032 (24) were found to be closely related. Franco et al. reported that 97% of 220 *Cronobacter* species isolates had a homologous RepFIB plasmid, and these two plasmids contain a single RepFIB-like origin of the replication gene *repA* and 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* BAA-894 and enhance host invasion (26). Likewise, *C. sakazakii* 680 and *C. sakazakii* ATCC 29544, which belong to the ST8 lineage, reportedly contain a pESA3/pCTU1-like plasmid according to a comparative analysis (23, 27).

Methyl-accepting chemotaxis proteins (MCPs) mediate many of the chemotactic behaviors of bacteria and archaea. Bacteria respond to various environmental signals (28–30) that activate the corresponding MCPs, such as Tar (taxis toward aspartate and maltose, away from nickel and cobalt), Tsr (taxis toward serine, away from leucine, indole, and weak acids), Trg (taxis toward galactose and ribose), and Tap (taxis toward dipeptides) in *Escherichia coli* (30, 31). The ability of MCPs to adapt to the chemical environment via methylation allows changes in the organism's motility and feedback adaptation (32–34). In addition to chemotaxis, MCPs have been implicated in the virulence of certain pathogens, such as *Treponema pallidum*, *Vibrio cholerae*, and *Pseudomonas aeruginosa* (35–37).

C. sakazakii is known to cause a systemic infection via translocation from the intestinal lumen into the blood circulation by actively invading various epithelial and endothelial cells of human

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
C. sakazakii		
ATCC 29544	Wild type	ATCC
HR101	*mcp*::Km^r	This study
HR102	HR101 with pPMCP	This study
E. coli		
DH5α	*supE44 hsdR17 recA1 gyrA96 thi-1 relA1*	68
EC100DTM	*pir*$^+$	Epicentre
Plasmids		
pACYC184	rep$_{p15A}$ Cm^r Tet^r	43
pKD13	repR$_{6K\gamma}$ Ap^r -FRT Km^r -FRT	42
pKD46	rep$_{pSC101}$(Ts) Ap^r P$_{araBAD}$ γ β *exo*	42
pPMCP	pACYC184-*mcp*	This study

and animal origin (16, 17, 38). While screening the *C. sakazakii* ATCC 29544 random mutant library for invasion-related virulence factors, we identified a putative MCP that is encoded by a novel plasmid, pCSA2. pCSA2 was completely sequenced and annotated, and the putative MCP in pCSA2 was confirmed to be involved in adhesion and invasion in cultured mammalian cells, organ colonization in rat pups, and the 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 the *C. sakazakii* ST8 lineage, which comprises *C. sakazakii* ATCC 29544 and 680 (27, 39).

MATERIALS AND METHODS

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) under aerobic conditions. When necessary, ampicillin, chloramphenicol, and kanamycin were used at 50 μg/ml, 25 μg/ml, and 50 μg/ml, respectively.

Random mutagenesis and screening. Random mutagenesis was performed using the EZ-Tn5pMOD-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 (1.8 kV) (MicroPulser; Bio-Rad, Hercules, CA) into competent *C. sakazakii* ATCC 29544. The transformants were selected on tryptic soy agar (TSA; Difco) plates containing kanamycin (50 μg/ml). The resulting colonies were individually cultured and stored at −80°C in TSB containing 15% (vol/vol) glycerol.

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 the self-ligation of restriction enzyme-digested DNA according to the manufacturer's protocol (Epicentre), the ligation mixture was electroporated into EC100D *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; INtRON, South Korea) and sequenced with Tn*5*-specific primers provided by the manufacturer (pMOD<MCS> forward sequencing primer and pMOD<MCS> reverse sequencing primer).

Complete nucleotide sequencing and bioinformatics. *C. sakazakii* ATCC 29544 plasmid DNA was prepared using a plasmid DNA purification kit (DNA-spin; INtRON). The DNA sequence information obtained from transposon insertion site identification was used for primer walking to complete the whole sequence of the plasmid. Primer walking was performed by Macrogen, South Korea. The complete genome of the plasmid was assembled using 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.nih.gov/projects/gorf/) and GeneMark.hmm prokaryotic (http://exon.gatech.edu/GeneMark/gmhmmp.cgi). The functional analyses of ORFs were conducted using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) (40, 41).

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 10% fetal bovine serum (FBS; Invitrogen). Trypsin-treated cells were seeded (approximately 5 × 10^4 cells per well) into 24-well tissue culture plates (TPP, Switzerland) and grown at 37°C under 5% CO_2. The medium was replaced every 2 days. The cell viability was determined with trypan blue staining.

Site-specific mutagenesis of *C. sakazakii* ATCC 29544. The one-step gene inactivation method was used to replace the *mcp* gene in pCSA2 with the kanamycin resistance (Km^r) cassette (42). The Km^r cassette from plasmid pKD13 (42) was amplified using primers mcp-lamb-F and mcp-lamb-R. Primer mcp-lamb-F (5′-ACCATGGCAGTACTTACATTATCCGGGCAATCTGATTCATTGTAGGCTGGAGCTGCTTCG-3′) carries the sequence immediately upstream of the start codon of the *mcp* gene, followed by the priming site 1 sequence (underlined) of pKD13 (42). Primer mcp-lamb-R (5′-ATCTGTGATGTTCGACAGATATGGGGATATGCACCATGGCATTCCGGGGATCCGTCGACC-3′) harbors the sequence immediately downstream of the stop codon of the *mcp* gene linked to the priming site 4 sequence (underlined) of pKD13 (42). The resulting PCR product was transformed into *C. sakazakii* ATCC 29544 and selected for kanamycin resistance. The 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′).

Construction of the complementation strain. The plasmid pPMCP, which contains the MCP coding sequence and its own promoter, was constructed to complement the *mcp* mutant. The *mcp* gene was amplified by PCR using the primers mcp-pACYC-F (5′-GAGTGTTTTCCGGATCCCGGAT-3′) and mcp-pACYC-R (5′-GATGGTCGACAGATATGGGGATAT-3′) (underlining indicates restriction enzyme sites BamHI and SalI, repectively) and *C. sakazakii* ATCC 29544 genomic DNA as a template. The product was introduced between the BamHI and SalI restriction sites of pACYC184 (43). The sequence of the *mcp* coding region in the recombinant plasmid was confirmed by nucleotide sequencing (Macrogen, South Korea).

Invasion assay. The invasion assay was conducted as described previously (18), with modifications. Caco-2 cells were grown in EMEM supplemented with 10% FBS. Prior to bacterial infection, a monolayer of 2 × 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, prewarmed TSB and incubating the resultant mixture for 3 h (optical density [OD] = 1.5). The bacterial cells were collected by centrifugation (at 10,000 × *g* for 3 min at 4°C), washed with phosphate-buffered saline (PBS; pH 7.4), resuspended 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 three times with prewarmed PBS to remove extracellular bacteria and then incubated for 1.5 h with the prewarmed medium supplemented with 100 μg/ml of gentamicin to kill extracellular bacteria. Subsequently, the wells were washed three times with PBS, lysed in 1% Triton X-100 for 30 min, and then serially diluted in PBS. A dilution of the suspension was plated on TSA medium to enumerate the CFU.

Adhesion assay. To assess the adhesive ability, the epithelial cells were treated with 0.8 μg/ml of cytochalasin D (CD; Sigma) for 30 min to inhibit the internalization of bacteria (44, 45). The internalization of wild-type (WT) *C. sakazakii* into Caco-2 cells was inhibited by approximately 70%

TABLE 2 Primers used in qRT-PCR analysis

Primer	Target gene	Sequence (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

in the presence of 0.8 μg/ml of cytochalasin D. Prior to bacterial infection, the wells were washed with PBS, and fresh EMEM was added. Subsequently, *C. sakazakii*, which was prepared in a manner similar to that for the invasion assay, was applied 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, and then serially diluted in PBS. A dilution of the suspensions was plated on TSA medium to enumerate the CFU of adhesive bacteria.

***In vivo* rat pup virulence assay by CI analysis.** Bacterial cells grown for 3 h were pelleted, washed, and resuspended in PBS. Three-day-old Sprague-Dawley female rat pups were used to assess the virulence of the *C. sakazakii* WT and HR101 strains. A mixed inoculum of 5×10^9 CFU of WT ATCC 29544 and HR101 in 50 μl of PBS was administered orally to groups of rat pups (5 rats/group). To analyze bacterial colonization in organs, the rat pups were sacrificed 20 h after infection, and the spleen and liver were aseptically removed. The organs were homogenized in 1 ml of ice-cold PBS and serially diluted. The bacterial loads were determined by plating the diluents on TSA plates in the presence or absence of kanamycin. The results are presented as competitive index (CI) values, which were calculated as $(\text{mutant}_{\text{output}}/\text{wild type}_{\text{output}})/(\text{mutant}_{\text{input}}/\text{wild type}_{\text{input}})$.

Motility assay. A 1-μl aliquot of a 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.

RNA isolation and qRT-PCR. To extract RNA from *C. sakazakii*, bacteria were grown at 37°C for 3 h in TSB. RNA was extracted using an RNeasy minikit (Qiagen), followed by treatment with RNase-free DNase (Ambion). cDNA was synthesized using Omnitranscript reverse transcription (RT) reagents (Qiagen) and random hexamers (Invitrogen) and quantified using 2× iQ SYBR green Supermix (Bio-Rad). The 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 the 16S rRNA (46). The primers were designed using a PCR primer design tool, Primer3 Plus. The sequences of the primers used in the

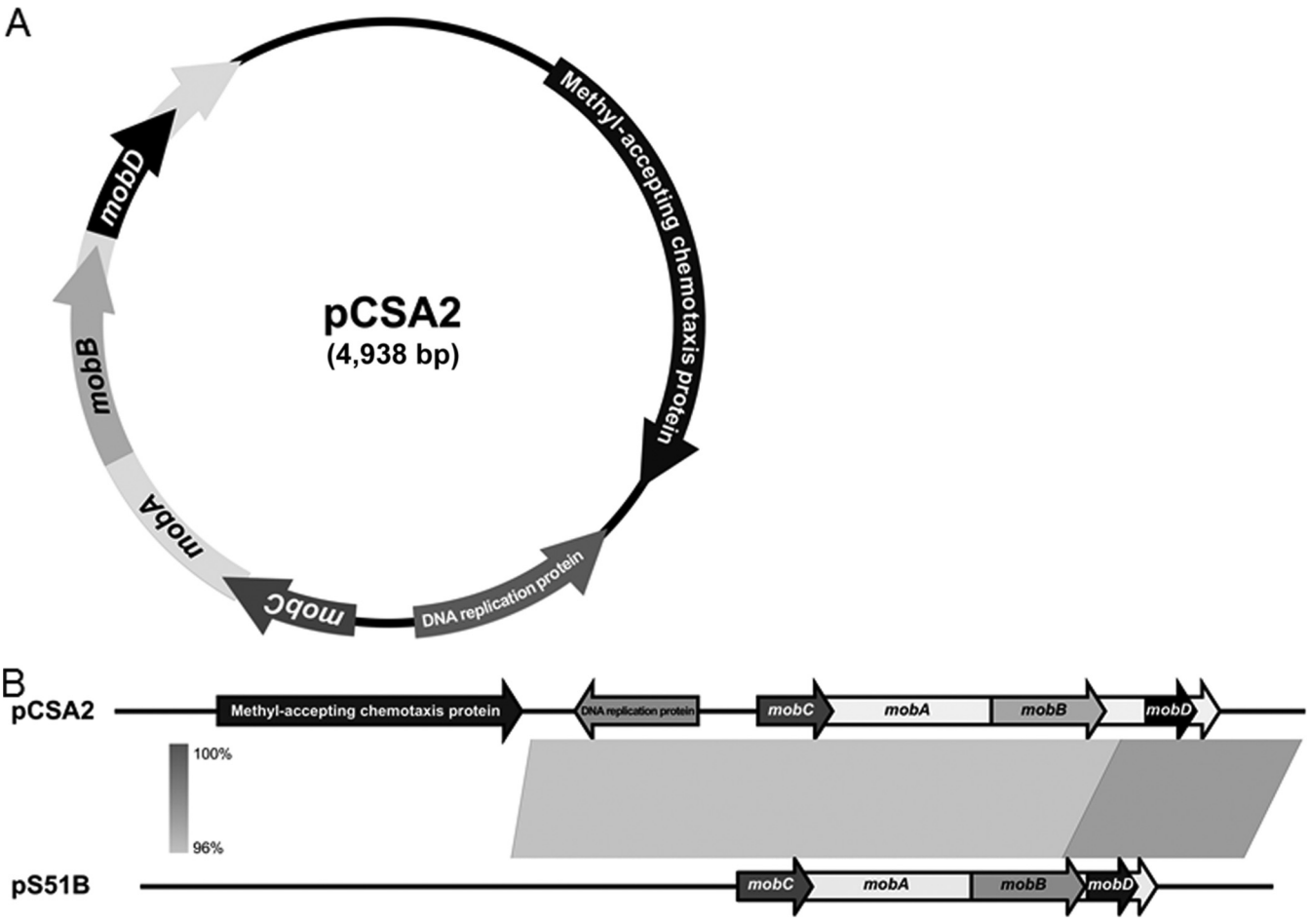

FIG 1 Identification of pCSA2 and construction of the *mcp* deletion mutant by lambda recombination. (A) Schematic representation of pCSA2 in *C. sakazakii* ATCC 29544. The transposon (Tn5) insertion is designated. (B) Linear comparison diagrams showing BLAST matches between pCSA2 and pS51B, obtained from Easyfig (version 2.1).

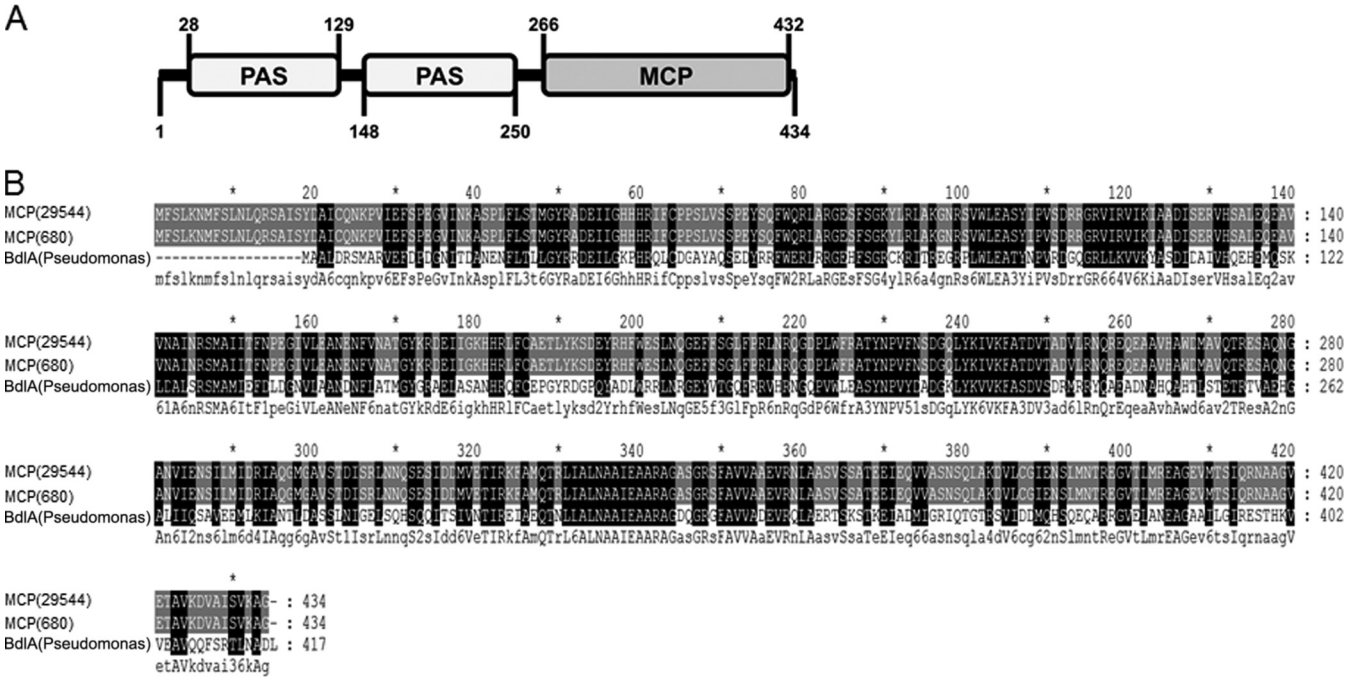

FIG 2 Deduced sequence analysis of MCP. (A) Domain structures of MCP. PAS, Per-Arnt-Sim sensory domain; MCP, methyl-accepting chemotaxis protein domain. (B) Alignment of amino acid sequences of MCP of *C. sakazakii* ATCC 29544, MCP of *C. sakazakii* 680 (ZP_19182295.1), and BdlA of *P. aeruginosa* PAO1 (NP250114.1) by Clustal Omega (version 1.2.1) and Genedoc. Capital letters in the consensus sequence indicate conserved amino acids appearing in all aligned sequences, and lowercase letters indicate conserved amino acids appearing in at least two sequences.

quantitative reverse transcription-PCR (qRT-PCR) analysis are listed in Table 2.

Biofilm assay. The experiment was performed as previously described (18), with modifications. *C. sakazakii* was inoculated into 3 ml of TSB and incubated at 37°C with aeration until the cell density reached 2.5×10^8 CFU/ml. The culture was diluted 1:100 in TSB, and 500-μl portions were loaded in triplicate into a 24-well polystyrene plate (SPL Life Sciences, South Korea) and incubated at 37°C for 48 h without shaking. To fix the biofilm, 100 μl of 99% methanol was added for 15 min, the supernatants were removed, and the plates were air dried. Subsequently, 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, the bound CV was released by adding 250 μl of 95% ethanol (Merck). The absorbance was measured at 570 nm using a Sunrise basic microplate reader (Tecan, Austria).

Statistical analysis. Statistical analyses were conducted using the GraphPad Prism program (version 5.0). All results were analyzed with the unpaired *t* test. The data are represented as the means ± standard deviations. A *P* value of <0.05 was considered statistically significant.

Ethics statement. This study was carried out according to the recommended protocol for the care and use of laboratory animals from the Institute of Laboratory Animal Resources at Seoul National University, which is based on the Korean Animal Protection Law and Korea Food and Drug Administration regulations on laboratory animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Seoul National University (Institutional Animal Care and Use Committee permit number SNU-130214-1-3).

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

RESULTS AND DISCUSSION

A plasmid-borne gene discovered in an invasion-attenuated mutant. To identify genes related to the virulence of *C. sakazakii*, a transposon-mediated random mutant library was constructed in *C. sakazakii* ATCC 29544, and an invasion-defective clone was screened. Three hundred clones were screened with an invasion assay, and one clone showing the most defective invasion ability was selected and analyzed further. After plasmid recovery and sequencing of the boundary region between the transposon and the *C. sakazakii* genome, we found that one mutant had the transposon inserted into an unknown region of DNA. A nucleotide BLAST search of the transposon-flanking region showed no homology with *Cronobacter* spp. but identified part of the *mobA* gene, which is related to plasmid conjugation (47). This finding suggested that this mutant contained a transposon in a novel plasmid of *C. sakazakii* ATCC 29544.

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 *C. sakazakii* ATCC 29544 contains an unreported plasmid that might be related to its virulence.

pCSA2 is a novel plasmid in *C. sakazakii* ATCC 29544 that contains 6 open reading frames. We obtained the complete plasmid sequence using primer walking and named it pCSA2, which stands for plasmid of *C. sakazakii* ATCC 29544. The pCSA2 comprises 4,938 bp with an overall G+C content of 54.88%. Six ORFs were identified on pCSA2: four genes predicted to encode relaxases (*mobA*, *mobB*, *mobC*, and *mobD*), a gene encoding a putative DNA replication protein, and a gene encoding a putative methyl-accepting chemotaxis protein (MCP) (Fig. 1A). We attempted to determine the plasmid profiles of *C. sakazakii* ATCC 29544 with a *repA*-targeted PCR assay (25) and found that *C. sakazakii* ATCC

29544 was PCR positive for pCTU3 and pESA3/pCTU1 but not pESA2/pCTU2 (data not shown). To corroborate the PCR result, the whole genome of *C. sakazakii* ATCC 29544 was sequenced and compared with that of published plasmids. The resultant comparison of the DNA sequence suggests that *C. sakazakii* ATCC 29544 contains pCTU3-like, pESA3/pCTU1-like, and pCSA2 sequences (unpublished data). The DNA sequences of the region containing the genes for relaxase and the DNA replication protein are highly similar (97% identities) to those of the *Enterobacter cloacae* plasmid pS51B, which belongs to the ColE1 superfamily of mobilizable plasmids commonly detected in *Enterobacteriaceae* (Fig. 1B) (48). A relaxase is a single-stranded DNA transesterase enzyme produced by some prokaryotes and viruses (47). 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 (49). Therefore, the four *mob* genes might play a role in the conjugation of pCSA2. The DNA replication protein is required for the replication of the plasmid (50).

The putative MCP-encoding gene lacked identity to any other gene in *C. sakazakii* strains except for a recently sequenced *C. sakazakii* 680 genome (27), which contains a gene with 100% identical DNA sequences. The deduced protein of the putative MCP gene contains an MCP domain and two sensory PAS (*P*er-*A*rnt-*S*im sensory) domains and high sequence similarity with the biofilm dispersion protein BdlA of *Pseudomonas aeruginosa* (Fig. 2). Therefore, we named this gene *mcp* (for methyl-accepting chemotaxis protein). MCP domains share a 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 (30, 31). The PAS domain is responsible for sensing the input signal and protein-protein interaction. PAS domains have been implicated in diverse biological processes, including the global regulation of metabolism, nitrogen fixation, aerotaxis, hypoxia responses, and ion channel function, in both prokaryotes and eukaryotes (51–54). *C. sakazakii* is likely to encounter numerous suboptimal conditions during its transition from the environment to the host (55). Therefore, elucidating the mechanisms used by *C. sakazakii* to regulate environmental signals will be important to understand the pathogenesis of *C. sakazakii* ATCC 29544. For this, the *mcp* gene was a reasonable target for further study.

Plasmid-encoded putative MCP affects adhesion/invasion. To explore the ability of the *mcp* gene to contribute to *C. sakazakii* ATCC 29544 virulence, we constructed a strain in which the entire *mcp* gene was replaced with a gene encoding kanamycin resistance using lambda red recombination (data not shown). Because the *mcp* gene is carried in the plasmid, we did not remove the inserted Km^r cassette for the maintenance of the plasmid. Due to the plasmid copy number, we subcultured the mutant on TSA plates containing kanamycin until the *mcp* PCR product was no longer detected (data not shown). Removing the *mcp* gene from the plasmid did not result in a significant growth defect compared to growth of the wild-type strain in TSB media (data not shown). After constructing the *mcp* mutant, the gentamicin protection assay confirmed the observations in the random mutant library screening. The invasion rate of the mutant was significantly lower than that of the WT (approximately 20%, compared to 100%) in the invasion assay (Fig. 3A). This result was comparable to the library screening results. The phenotypic defect of the *mcp* mutant was indeed due to *mcp* function, because the expression of the *mcp* gene from a complement strain enabled the *mcp* mutant to invade Caco-2 cells at a level similar to that of the wild-type strain (Fig. 3A).

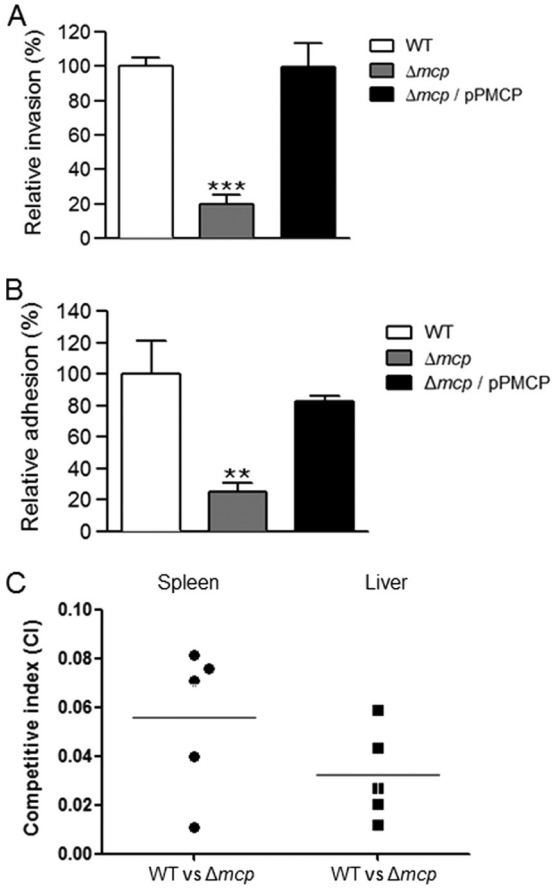

FIG 3 Contribution of the *mcp* gene to *C. sakazakii* virulence. (A) Caco-2 epithelial cells were infected with the wild-type (WT) strain (ATCC 29544), the *mcp* deletion mutant (HR101), or strain HR101 harboring the pPMCP plasmid (Δ*mcp*/pPMCP). The numbers of intracellular bacteria were determined 1.5 h after infection using the gentamicin protection assay. The means and standard deviations from at least three independent experiments are shown. Triple asterisks indicate that the numbers of bacteria were significantly different ($P < 0.001$) from those of the WT strain. (B) CD-pretreated Caco-2 epithelial cells were infected with *C. sakazakii* strains similar to those used in the invasion assay. The numbers of intracellular bacteria were determined 45 min after infection without the use of gentamicin. The means and standard deviations 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. (C) Groups of SD rats (5 rats/group) were infected orally with a mixed inoculum of approximately 5×10^9 CFU of the WT and mutant strain. Twenty hours after infection, the numbers of bacteria in the liver and spleen were determined. The competitive index (CI) values were calculated as ($\text{mutant}_{\text{output}}$/$\text{wild type}_{\text{output}}$)/($\text{mutant}_{\text{input}}$/$\text{wild type}_{\text{input}}$); thus, a competitive index of <1.0 indicates a strain with a competitive disadvantage.

C. sakazakii needs to bind to the surfaces of epithelial cells to successfully invade them (15). For the adhesion assay, we pretreated Caco-2 cells with cytochalasin D (CD), an agent that causes microfilament depolymerization in eukaryotic cells and thus inhibits *C. sakazakii* invasion (17, 44). Only 25% of *mcp* mutants were recovered from the adhesion assay, compared to 100% for the WT (Fig. 3B). Again, the complementary plasmid

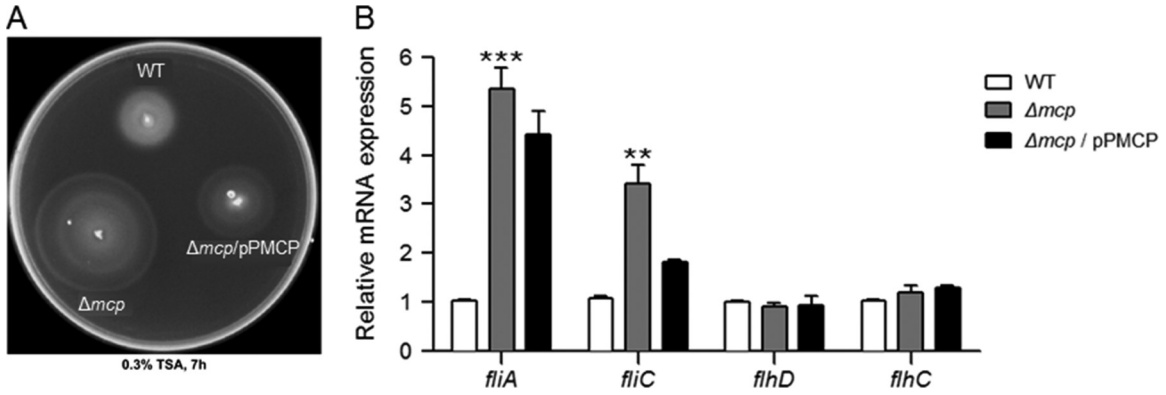

FIG 4 Effect of the *mcp* gene on the regulation of motility in *C. sakazakii* ATCC 29544. (A) A 1-μl aliquot of a subculture was spotted onto the middle of a swim plate (TSA, 0.3% agar). (B) The transcription levels of the *fliA*, *fliC*, *flhD*, and *flhC* genes in *C. sakazakii* were determined via 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 pPMCP plasmid (Δ*mcp*/pPMCP). 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 strain. The means and standard deviations from three independent experiments are shown. Asterisks indicate significant differences (***, $P < 0.001$; **, $P < 0.01$).

expressing *mcp* restored the adhesive defect of the *mcp* mutant (Fig. 3B). These results indicate that the MCP encoded in pCSA2 is important for the infection of *C. sakazakii* ATCC 29544.

***C. sakazakii* lacking a plasmid-encoded putative MCP showed attenuated virulence in rat pups.** The effects of the *mcp* deletion mutation on *C. sakazakii* virulence were further analyzed using a newborn rat model (56, 57). A comparison of the CI values of the *mcp* deletion mutant to those of the wild type revealed an approximately 100-fold-reduced ability to translocate into deep organs (the liver and spleen) in the *mcp* deletion mutant 20 h after infection in both the livers and spleens of rat pups (Fig. 3C). Taken together, these results suggest that the putative MCP plays a crucial role in the pathogenesis of *C. sakazakii* ATCC 29544, which was originally isolated from throat culture of a patient with whooping cough (2).

Plasmid-borne MCP regulates the motility of *C. sakazakii*. Because *mcp* is a putative MCP-coding gene and chemotactic regulation in bacteria results in general changes in flagellar rotation (28), we 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 assay conditions. Conversely, the *mcp* mutant was hypermotile (~31.5 mm) (Fig. 4A). Introducing the plasmid expressing the *mcp* gene reduced the motility (~17.3 mm) to close to that of the wild-type strain, suggesting that this gene is related to flagellar regulation.

Next, we evaluated the expression level of several flagellar biosynthesis-related genes in the *mcp* mutant and wild-type strain by qRT-PCR. We selected four genes, *flhD*, *flhC*, *fliA*, and *fliC*, that represent the regulation of flagellar assembly (58). These genes from strains BAA-894 and 680 have more than 99% identity except for *fliA*, which has 92% identity. Due to a lack of information on the genome sequence of *C. sakazakii* ATCC 29544, we determined the sequences of these genes from the genome sequence data of *C. sakazakii* BAA-894 and then confirmed the existence of these genes in ATCC 29544, with high sequence identities (data not shown). Interestingly, the mRNA levels of the *fliA* and *fliC* genes in the *mcp* deletion mutant increased ~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 that of the wild-type strain (Fig. 4B). This result might be due to the low copy number of the backbone plasmid pACYC184 (43) in the complementary plasmid, pPMCP. 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 (58, 59). Because the *fliC* gene is one of the class III flagellar genes (58), the enhanced expression of *fliC* might be due to the large amount of FliA. These regulations corresponded to the increase in the motility of the *mcp* mutant. Moreover, the expression levels of two flagellar master regulator genes, *flhD* and *flhC*, did not change in the *mcp* mutant (Fig. 4A), suggesting that MCP affects the expression of class III flagellar genes via *fliA*.

Motility is a well-known virulence factor in many pathogenic bacteria (60, 61). The motility phenotype is reportedly coupled to the expression of multiple virulence factors, and the negative effects of hypermotility on virulence have been reported for *Vibrio cholerae* (62, 63) and *Ralstonia solanacearum* (64). In *Vibrio cholerae*, hypermotility caused poorer toxin production and defects in colonization *in vivo* (63, 64). Moreover, the hyperflagellated strain showed a lower ability to form biofilm in *Ralstonia solanacearum*. Because the attachment of *C. sakazakii* to the cell surface is a critical step for host invasion (17), the disruption of the adherence of *C. sakazakii* ATCC 29544 to the host cell (Fig. 3B) due to the hypermotility of the *mcp* mutant may have caused the colonization defect *in vivo*, as shown in Fig. 3C.

Biofilm formation is affected by the plasmid-encoded putative MCP. In the BLAST results, the MCP of *C. sakazakii* ATCC 29544 showed 63% similarity with the biofilm dispersion protein BdlA of *P. aeruginosa* (Fig. 2B). In *Pseudomonas aeruginosa*, BdlA, a chemotaxis transducer protein, is essential for biofilm dispersion (65–67). 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 planktonic conditions (66). Therefore, we hypothesized that the deletion of *mcp* might affect biofilm formation in *C. sakazakii*. As expected, the *mcp* deletion mutant resulted in an ~2-fold reduction of biofilm

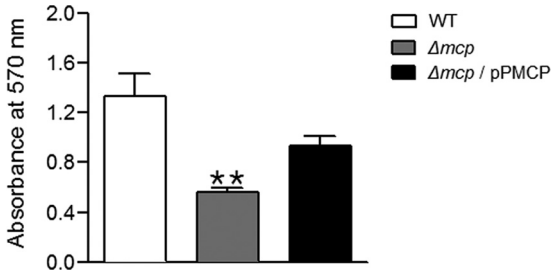

FIG 5 Biofilm formation. The crystal violet biofilm assay was performed in triplicate and repeated three times. The biofilm formation is indicated by the absorbance at 570 nm from the wild-type (WT) strain (ATCC 29544), the *mcp* deletion mutant (HR101), or strain HR101 harboring the pPMCP plasmid (Δ*mcp*/pPMCP). Double asterisks indicate that the absorbance was significantly different ($P < 0.01$) from that of the WT strain.

formation (Fig. 5). 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 for the wild-type strain (Fig. 5).

Multilocus sequence typing (MLST) discriminates the *Cronobacter* genus via a comparison with 7 putative housekeeping genes (*atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB*, and *pps*), which are necessary for their biological roles in DNA repair, replication, and amino acid biosynthesis (39). Among the many serotypes, ST8 comprises *C. sakazakii* ATCC 29544 and *C. sakazakii* 680 (27, 39). Only *C. sakazakii* 680 matches the MCP sequence 100%, and the MCP sequence is not found in any other lineages, suggesting that the MCP association with virulence is likely specific to the ST8 lineage. However, we demonstrated that the putative MCP encoded in pCSA2 is important for the invasion/adhesion and colonization of *C. sakazakii* ATCC 29544. MCP also regulated two other phenotypes, motility and biofilm formation. These diverse effects of putative MCP should be further studied, especially for the global regulations of *C. sakazakii* ATCC 29544.

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