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hfq Plays Important Roles in Virulence and Stress Adaptation in *Cronobacter sakazakii* ATCC 29544

Seongok Kim,[a] Hyelyeon Hwang,[a] Kwang-Pyo Kim,[b] Hyunjin Yoon,[c] Dong-Hyun Kang,[a] Sangryeol Ryu[a]

Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Research Institute for Agriculture and Life Sciences, and Center for Food and Bioconvergence, Seoul National University, Seoul, South Korea[a]; Department of Food Science and Technology, College of Agriculture and Life Sciences, Chonbuk National University, Jeonju, South Korea[b]; Department of Molecular Science and Technology, Department of Applied Chemistry and Biological Engineering, Ajou University, Suwon, South Korea[c]

***Cronobacter* spp. are opportunistic pathogens that cause neonatal meningitis and sepsis with high mortality in neonates. Despite the peril associated with *Cronobacter* infection, the mechanisms of pathogenesis are still being unraveled. Hfq, which is known as an RNA chaperone, participates in the interaction with bacterial small RNAs (sRNAs) to regulate posttranscriptionally the expression of various genes. Recent studies have demonstrated that Hfq contributes to the pathogenesis of numerous species of bacteria, and its roles are varied between bacterial species. Here, we tried to elucidate the role of Hfq in *C. sakazakii* virulence. In the absence of *hfq*, *C. sakazakii* was highly attenuated in dissemination *in vivo*, showed defects in invasion (3-fold) into animal cells and survival (10^3-fold) within host cells, and exhibited low resistance to hydrogen peroxide (10^2-fold). Remarkably, the loss of *hfq* led to hypermotility on soft agar, which is contrary to what has been observed in other pathogenic bacteria. The hyperflagellated bacteria were likely to be attributable to the increased transcription of genes associated with flagellar biosynthesis in a strain lacking *hfq*. Together, these data strongly suggest that *hfq* plays important roles in the virulence of *C. sakazakii* by participating in the regulation of multiple genes.**

The genus *Cronobacter* (formerly *Enterobacter sakazakii*) comprises Gram-negative, rod-shaped, peritrichous, and yellow-pigmented facultative anaerobes belonging to the family *Enterobacteriaceae*. This family encompasses the well-known enteric pathogens *Salmonella* and pathogenic *Escherichia coli*, but *Cronobacter* spp. are most closely related to the genera *Enterobacter* and *Citrobacter*. Based on the original 16 biogroups of Farmer et al., *E. sakazakii* has been reclassified into several *Cronobacter* spp. according to genotypic and phenotypic evaluations (1–4). *Cronobacter* spp. are present in a wide range of environments, including water, soil, processed foods, and facilities (5), and are composed of seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti* (3, 4, 6).

C. sakazakii is a foodborne opportunistic pathogen that causes bacteremia, meningitis, and necrotizing enterocolitis, particularly in premature infants (7–9). Although the incidence rate is quite low, mortality is high, ranging from 40 to 80% (10–12). Despite the serious perils associated with *C. sakazakii*, little is known at the molecular level about the mechanism of its pathogenicity and about its virulence factors. A cell-bound zinc-containing metalloprotease, encoded by *zpx*, caused rounding of Chinese hamster ovary (CHO) cells, which might be important in dissemination of the pathogen into the systemic circulation (13). The outer membrane proteins A (OmpA) and OmpX are reported to be essential for adhesion to/invasion of Caco-2 and INT-407 cells (14, 15). Studies have shown that *C. sakazakii* can invade and translocate efficiently across cultured human intestinal epithelial cells, as well as endothelial cells, experimentally mimicking the potential path of meningitis (14, 16). A plasmid-borne outer membrane protease (Cpa) is reported to be involved in *C. sakazakii* protection against complement-dependent serum killing and efficient invasion by activating plasminogen into plasmin (17). Furthermore, plasmid-borne iron acquisition systems, such as an aerobacterin-like siderophore (cronobactin) and an ABC ferric-iron transporter (*eitABCE*), might enable *Cronobacter* spp. to obtain iron in a highly iron-restricted environment (18). Flagella from *Cronobacter* spp. can induce an inflammatory response dependent on Toll-like receptor 5 (TLR5) recognition, so they could contribute to the pathogenesis of the bacteria (19). As a global regulator, LysR-type transcriptional regulator (LTTR) in *C. sakazakii* impacts pathogenesis, invasion, biofilm formation, and *in vivo* challenge (20).

The Hfq protein, which was first discovered in *E. coli* nearly half a century ago as a host bacterial factor required for the RNA synthesis of bacteriophage Qβ (21), is widely conserved as an RNA chaperone in many bacterial species (22). It oligomerizes into a hexameric ring structure (23), enhances the formation of small-RNA (sRNA)–mRNA duplexes, and contributes to RNA regulation by interacting with RNA turnover enzymes, RNase E, polynucleotide phosphorylase, and poly(A) polymerase (24). Thus, Hfq is regarded as a posttranscriptional global regulator involved in the biogenesis of outer membrane proteins (OMPs) (25), quorum sensing (26), and various stress responses (27). In addition, recent studies have demonstrated the importance of Hfq in the

Received 31 December 2014 **Returned for modification** 30 January 2015
Accepted 26 February 2015

Accepted manuscript posted online 9 March 2015

Citation 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. Infect Immun 83:2089–2098. doi:10.1128/IAI.03161-14.

Editor: B. A. McCormick

Address correspondence to Sangryeol Ryu, sangryu@snu.ac.kr.

doi:10.1128/IAI.03161-14

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or characteristics[a]	Reference or source
C. sakazakii		
ATCC 29544	Wild-type strain	15
ES1001	29544 harboring pKD46	15
SK001	29544 harboring pACYC184	This study
SK002	29544 harboring pBAD18	This study
SK003	Δ*hfq*	This study
SK004	*hfq*::*kan* harboring pACYC184	This study
SK005	Δ*hfq* harboring pACYC184	This study
SK006	Δ*hfq* harboring pBAD18	This study
SK007	Δ*hfq* harboring pHFQ	This study
SK008	Δ*hfq* harboring pHFQ-A	This study
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	36
Plasmids		
pKD13	*oriR6K* Amp^r FRT Kan^r FRT	35
pKD46	*oriR101 repA101*(Ts) Amp^r *ara BADpgam-bet-exo*	35
pCP20	oripSC101(TS) Amp^r Cm^r cI857λ $P_R$flp	35
pACYC184	Tet^r Cm^r p15A *ori*	35
pBAD18	Amp^r *araC* P_{BAD} pBR322 *ori*; expression vector	37
pHFQ	pACYC184-*hfq*	This study
pHFQ-A	pBAD18-*hfq*	This study

[a] FRT, FLP recombination target.

pathogenesis of various bacteria, including *Listeria monocytogenes*, *Yersinia pseudotuberculosis*, *Francisella tularensis*, *Salmonella enterica* serovar Typhimurium, and *E. coli* (28–32). For example, a lack of Hfq in *S.* Typhimurium caused attenuated virulence *in vivo*, reduced host cell adhesion and invasion *in vitro*, defectiveness in secretion of effector proteins, chronic envelope stress (accumulation of periplasmic and outer membrane proteins), loss of motility, and reduced survival within cultured macrophages (31). Such pleiotropic functions of the *hfq* gene product were also observed in other bacterial species (29, 33), but the effects of *hfq* deletion could be different from one species to another; the deletion of *hfq* in *S.* Typhimurium and *Brucella abortus* reduced bacterial growth and survival within the host cells, but not in *L. monocytogenes* and *F. tularensis* (28, 30, 34).

The aim of this study was to elucidate the function of the *hfq* homolog of *C. sakazakii* in virulence or stress responses, such as motility, invasion of host epithelial cells, intracellular survival in macrophages, and resistance to hydrogen peroxide. Here, we demonstrate that Hfq is essential for virulence and stress adaptation in *C. sakazakii* ATCC 29544.

MATERIALS AND METHODS

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

Construction of a Δ*hfq* deletion mutant using the Lambda-Red recombination method. The whole genome sequence of *C. sakazakii* BAA-894 was obtained from GenBank (18) and adopted to manipulate the *C. sakazakii* ATCC 29544 genome. Site-specific mutation of *C. sakazakii* ATCC 29544 was generated by the Lambda-Red recombination method, as described by Kim et al. and Datsenko and Wanner (15, 35). Briefly, the kanamycin resistance cassette from plasmid pKD13 was amplified using the following primers (Table 2): for *hfq* deletion mutant construction, *hfq*-lamb-F (5′-*ATG GCT AAG GGG CAA TCT TTG CAA GAT CCG TTC CTC AAC GCG CTG CGT CGT* GTA GGC TGG AGC TGC TTC G-3′) and *hfq*-lamb-R (5′-*TTA CTC GGC GTC TTC GCT ATC CTG AGA GGC AGC GGA AGA TGG CTG TGC GT*A TTC CGG GGA TCC GTC GAC C-3′). (The nucleotide sequences originating from pKD13 are underlined, and those from the *C. sakazakii* gene of interest are shown in italics.)

The PCR products were transformed into the wild-type (WT) strain, *C. sakazakii* ATCC 29544, harboring the pKD46 plasmid, by electroporation, and cells were selected for kanamycin-resistant transformants, *hfq*::*kan*. Finally, the kanamycin resistance cassette was removed using the pCP20 plasmid, as described previously (20).

Growth curves. *C. sakazakii* strains ATCC 29544 (SK001), Δ*hfq* (SK005), and Δ*hfq* harboring pHFQ (SK007) were cultured overnight in LB medium at 37°C and subcultured with a 1% overnight culture in 50 ml LB medium. The cultures were incubated with shaking at 220 rpm in 250-ml Scott flasks at 37°C for 14 h. Every 1 h, the optical density was measured at 600 nm (OD_{600}).

Complementation study. Two *hfq* complementation plasmids (a pACYC184 derivative, pHFQ, with its own *hfq* promoter and a pBAD18 derivative, pHFQ-A, under an arabinose-inducible promoter) were constructed. To make pHFQ, the entire 309-bp *hfq* coding sequence (accession number KC866358) and 782 bp of upstream sequence comprising *miaA* (accession number KC866359), which includes promoter regions of *hfq*, were amplified from *C. sakazakii* ATCC 29544 chromosomal DNA (33) with primer sets *hfq*-pACYC184-SphI-F (5′-AAA GCA TGC AGC GAT GGC GGA GAT TGT CG-3′) and *hfq*-pACYC184-SalI-R (5′-AAA GTC GAC GTA AAA TAG ATG TGT ACC AG-3′) (the artificial restriction enzyme digestion site is underlined) and cloned into pACYC184 (36). For pHFQ-A, *hfq*-pBAD18-EcoRI-F (5′-AAA GAA TTC GGT TCA AGA GTA TAA ACA AC-3′) and *hfq*-pACYC184-SalI-R (5′-AAA GTC GAC GTA AAA TAG ATG TGT ACC AG-3′) were used to amplify the *hfq* coding region into the pBAD18 vector (the underlined nucleotide sequences are artificially added restriction recognition sites) (37). The plasmids were used to transform the mutant to generate Δ*hfq* harboring pHFQ and Δ*hfq* harboring pHFQ-A.

Gentamicin protection (invasion) assay. To determine bacterial invasion of mammalian Caco-2 cells, a gentamicin protection assay was performed as described previously (15). Briefly, bacteria were prepared by transferring a 1% inoculum from overnight cultures into fresh LB medium, followed by incubation for 2.5 h at 37°C with constant shaking. *C. sakazakii* cells were collected by centrifugation at 20,000 × *g*, washed with phosphate-buffered saline (PBS) (pH 7.4), and resuspended in 1 ml of prewarmed fresh Eagle's minimum essential medium (EMEM) (ATCC). Mammalian cells were seeded in EMEM supplemented with 20% fetal bovine serum (FBS) (Gibco, Invitrogen) in 24-well tissue culture plates with a cell density of 2×10^5 per well. The cell monolayers were incubated for 1 day, infected with bacteria at a multiplicity of infection (MOI) of 100, and incubated for 1.5 h in the presence of 5% CO_2. After the cells were washed once with PBS, fresh medium containing gentamicin (100 μg/ml; Sigma) was added, and the plates were further incubated for 1.5 h, followed by three washes with PBS. Then, 500 μl of 1% Triton X-100 was added, and the plates were incubated for a further 15 min before the bacteria were collected and plated on tryptic soy agar (TSA).

Motility assay. The *C. sakazakii* ATCC 29544 (SK002) and Δ*hfq* (SK006) strains and the complemented strain (SK008) were cultured overnight in LB medium at 37°C, which was used to subculture (1% inoculum) fresh LB medium. Bacteria were grown to midexponential phase and then diluted to adjust the OD_{600} to 1.5. Aliquots (2 μl) of each strain were injected onto soft-agar motility plates (LB medium containing 0.3% agar) and incubated at 37°C for 7 h. A GelDoc EZ imager (Bio-Rad) was used to take a photograph.

TABLE 2 Oligonucleotides used in this study

Oligonucleotide name	Oligonucleotide sequence (5′ to 3′)	Reference or source
Mutant construction		
hfq-lamb-F	ATG GCT AAG GGG CAA TCT TTG CAA GAT CCG TTC CTC AAC GCG CTG CGT CGT GTA GGC TGG AGC TGC TTC G	This study
hfq-lamb-R	TTA CTC GGC GTC TTC GCT ATC CTG AGA GGC AGC GGA AGA TGG CTG TGC GTA TTC CGG GGA TCC GTC GAC C	This study
hfq-lamb-conF	GTA CAG CGA TGT GTT ACA GGT	This study
hfq-lamb-conR	CCA ACA AAA TAC TTT GGG TGC G	This study
Cloning		
hfq- pACYC184-R-SalI	AAA GTC GAC GTA AAA TAG ATG TGT ACC AG	This study
hfq-pACYC184-F-SphI	AAA GCA TGC AGC GAT GGC GGA GAT TGT CG	This study
pACYC184-seq-F	GGC TCA TGA GCG CTT GTT TC	This study
pACYC184-seq-R	TGT CCT ACG AGT TGC ATG ATA	This study
hfq-pBAD18-F-EcoRI	AAA GAA TTC GGT TCA AGA GTA TAA ACA AC	This study
pBAD18-seq-F	GTC CAC ATT GAT TAT TTG CAC G	This study
pBAD18-seq-R	CAG GCT GAA AAT CTT CTC TCA T	This study
qRT-PCR		
Control-RT-F	GGG CCT CAT GCC ATC AGA T	70
Control-RT-R	TCT CAG ACC AGC TAG GGA TCG T	70
flhC-RT-F	GCA ACT TAG CCG CGG TAG AC	This study
flhC-RT-R	TGA ACC AGT CCG TGG AAA AGG	This study
fliA-RT-F	GCA GGA ACT GGG ACG TAA CG	This study
fliA-RT-R	GTG TCG AGC AAC ATC TGA CGA T	This study
flgK-RT-F	CGC TAT GAG CAG ATG TCG AAA AT	This study
flgK-RT-R	GTC TGC AGG CTT TTG AAG AAA TC	This study
fliC-RT-F	CGT ATC GCT GGT GGT GCT AA	This study
fliC-RT-R	CAG CGC CAA CCT GAA TTT TC	This study
miaA-RT-F	GAG CAG CGT TTT CAC CAG AT	This study
miaA-RT-R	AGG CAT GTC CGT ATG CAA AT	This study
hflX-RT-F	TGT CGA AGC ATT ACA GGT GAT T	This study
hflX-RT-R	AAT CTC AAC GGC TTT ACC TTC A	This study

Bacterial survival assay in animal cells. A long-term survival assay was performed as described previously (38). Briefly, RAW264.7 murine macrophage-like cells were seeded in Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen) supplemented with 10% FBS in 24-well plates at a density of 5×10^5 cells per well and incubated for 1 day at 37°C with 5% CO_2. *C. sakazakii* strains were prepared as described for the invasion assay, and cell monolayers were infected with bacteria at an MOI of 100 for 45 min at 37°C. The medium was replaced with DMEM (plus 10% FBS) supplemented with 100 μg/ml of gentamicin, and the cells were incubated for an additional 45 min. The plates were washed twice with PBS, and the cells were lysed with 1% Triton X-100, followed by serial dilution and plating onto TSA to determine the numbers of intracellular bacteria at various time points. For further study of bacterial persistence (long-term survival), the cells were replenished daily with fresh medium containing 10 μg/ml of gentamicin, and the numbers of intracellular bacteria were determined at 3, 8, 24, 48, 72, and 96 h.

Bacterial resistance against hydrogen peroxide. A hydrogen peroxide assay was performed as described previously with minor modifications (29). A *C. sakazakii* culture was prepared as for the motility assay. Hydrogen peroxide (Sigma) was added to the culture to a final concentration of

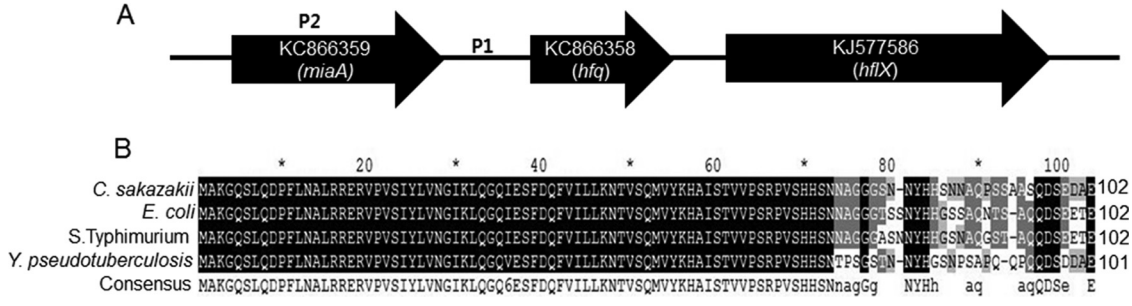

FIG 1 *hfq* homolog in *C. sakazakii* ATCC 29544. (A) Genomic organizations of *hfq* (KC866358), *miaA* (KC866359), and *hflX* (KJ577586) in *C. sakazakii* ATCC 29544. The promoter regions of *hfq* are indicated as P1 and P2. (B) Multiple-sequence alignment of *hfq* sequences in Gram-negative pathogens with a functionally identified *hfq* gene 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 acids appearing in at least two sequences. Identical and conserved residues are in black and shaded gray (dark gray, >70% conserved; light gray, >40% conserved), respectively. Every 10 amino acids are indicated by asterisks.

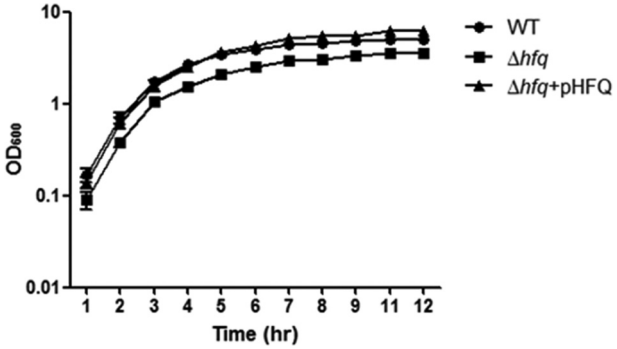

FIG 2 Growth of *C. sakazakii* Δ*hfq* in LB medium. *C. sakazakii* wild-type and Δ*hfq* strains and Δ*hfq* harboring pHFQ were cultured in LB broth at 37°C. The OD_{600} value of each strain harboring the pACYC184 control plasmid or the complementation plasmid pHFQ was measured hourly. The error bars represent means and standard errors of the mean (SEM) from three independent biological replicates.

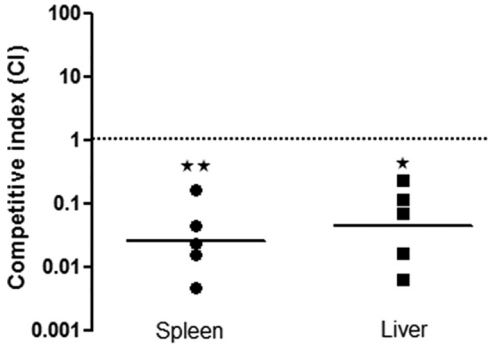

FIG 3 Diminished virulence of *C. sakazakii* due to the absence of *hfq in vivo*. Two- to 3-day-old rat pups were orally administered a mixture of wild-type *C. sakazakii* harboring pACYC184 (SK001) and an *hfq*::*kan* strain (SK004), with approximately 5×10^8 CFU/ml of each. To determine the bacterial loads in the spleen and liver, the organs were removed aseptically, homogenized at 20 h postinfection, and plated onto selective agar. The CI was calculated as follows: $(\Delta hfq_{output}/\Delta hfq_{input})/(WT_{output}/WT_{input})$. A CI of <1 indicates attenuation of virulence. Each circle or square represents the CI for a single animal in the spleen and liver of each group, respectively. The geometric means of the CIs for all the rat pups are shown as solid lines, and the asterisks indicate significant differences (**, $P < 0.05$; *, $P < 0.1$). CI values of less than 1 indicate that *hfq*::*kan* (SK004) strains were outcompeted by the wild type (SK001).

100 mM, and the culture was incubated at 37°C for 10 min, followed by counting of viable cells on TSA. For quantitative real-time (qRT)-PCR analysis (see below), each sample was exposed to 100 mM H_2O_2 for 5 min at 37°C.

qRT-PCR. A *C. sakazakii* culture was prepared as for the motility assay. Five hundred microliters of the culture was mixed with 1 ml of RNA Protect Bacteria Reagent (Qiagen), and total RNA was isolated using an RNeasy minikit (Qiagen). The RNA sample was then treated with RNase-free DNase (Ambion) to remove residual DNA. cDNA was synthesized using Omnitranscript Reverse Transcription reagents (Qiagen) and random hexamers (Invitrogen), and quantification of the cDNA was performed using 2× iQ SYBR green Supermix (Bio-Rad). The real-time amplification of the PCR products was performed using the CFX Connect Real-Time PCR Detection system (Bio-Rad). The mRNA level of each gene was divided by the mRNA level of the 16S rRNA. The mRNA expression values in strains SK005 and SK007 were further normalized to the transcription levels in the wild-type strain.

Animal study *in vivo*. Three- to 4-day-old Sprague-Dawley (SD) rat pups were used to assess the virulence of the WT (SK001) and the *hfq* mutant (SK004) strains. Midexponential-phase bacteria were collected, washed, and resuspended in PBS. For competitive assays, a mixed inoculum of 1×10^9 CFU/ml of the fully virulent WT (SK001) and the *hfq* mutant (SK004) harboring a *kan* cassette was orally administered to rat pups. To analyze bacterial colonization in organs, all the rat pups were killed with CO_2 at 20 h postinfection. The spleens and livers were aseptically removed, homogenized, and serially diluted. Bacterial loads were determined for the WT and mutant by plating onto TSA in the presence or absence of kanamycin. The results are presented as competitive-index (CI) values, which were calculated as follows: $(hfq_{output}/hfq_{input})/(WT_{output}/WT_{input})$.

TEM analysis. Flagellar morphology was visualized by negative staining, followed by transmission electron microscopy (TEM). After being negatively stained with 2% uranyl acetate for 1 min on carbon-Formvar copper grids, flagella were examined with an energy-filtering transmission microscope (E-TEM; Libra 120, Germany) at a voltage of 120 kV.

Western blot analysis. *C. sakazakii* ATCC 29544 was cultured overnight in LB medium at 37°C, which was used to subculture (1% inoculum) fresh LB medium. The bacteria were grown to midexponential phase, collected, and disrupted in B-PER (Thermo Scientific) according to the manufacturer's instructions. The protein samples were loaded on a 12% SDS-polyacrylamide gel. The separated proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat dry milk–1× 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 and then treated with anti-mouse IgG conjugated with peroxidase (Santa Cruz Biotechnology) as the secondary antibody in all of the immunoblot experiments. The chemiluminescent signals were developed with a West-Zol plus Western blot detection system (Intron Biotechnology, South Korea).

Polyclonal antibody production against OmpA or OmpX. BALB/c mice were immunized with recombinant OmpA or OmpX that was prepared as described previously (15). Anti-OmpA or anti-OmpX sera were obtained by bleeding, stored, in a refrigerator overnight, and centrifuged at 5,000 × *g* for 10 min.

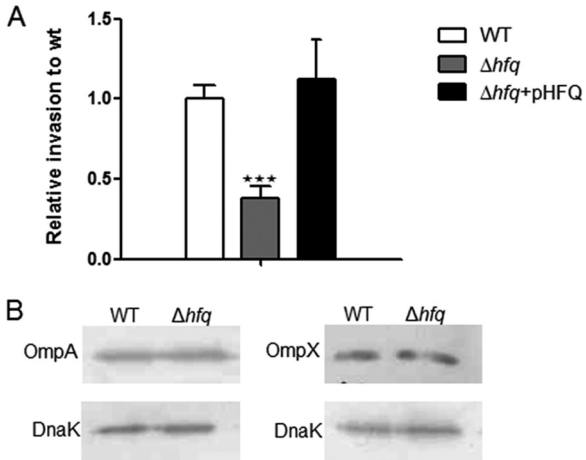

FIG 4 Loss of Hfq impairs the invasion of epithelial cells. (A) Confluent monolayers of eukaryotic cells were infected with *C. sakazakii* grown to the exponential phase and incubated for 1.5 h, followed by gentamicin treatment (100 μg/ml) for a further 1.5 h. The Caco-2 cells were lysed with 1% Triton X-100 to recover the intracellular bacteria. The data are representative of 3 independent experiments. The bars represent the means and SEM from independent experiments performed in triplicate. The asterisks indicate significant differences (***, $P < 0.001$). (B) Levels of OmpA and OmpX were assessed by immunoblotting in exponentially grown cells. The blots are representative of 3 independent experiments. DnaK was used as a loading control.

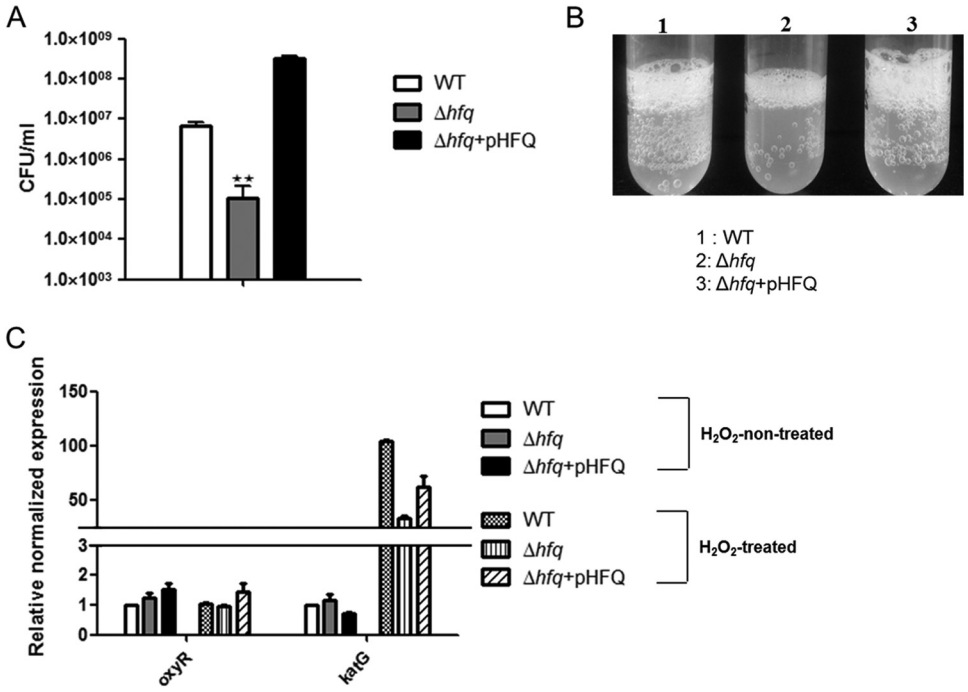

FIG 5 Stress tolerance is attenuated in *C. sakazakii* in the absence of Hfq. (A) Survival of *C. sakazakii* strains harboring the pACYC184 control plasmid or a complementation plasmid, pHFQ, upon treatment with 100 mM H_2O_2 for 10 min. (B) Indirect catalase activities of strains treated with H_2O_2. 1, wild type (SK001); 2, Δ*hfq* (SK005); 3, Δ*hfq* harboring pHFQ (SK007). The bubbles indicate oxygen gas, produced by decomposition of H_2O_2. (C) qRT-PCR analysis of *oxyR* and *katG* expression levels. To obtain the expression level relative to the WT on the *y* axis, the mRNA level of each gene was divided by the mRNA level of the 16S rRNA. The mRNA expression values in strains SK005 and SK007 were further normalized to the transcription levels of the wild type. The bars represent means and SEM from independent experiments performed in triplicate. The data are representative of 3 independent experiments. The asterisks indicate significant differences (**, $P < 0.05$).

Statistical analysis. Statistical analysis was conducted using the GraphPad Prism program (version 5.01). All results were analyzed by Student's unpaired *t* test. The data are presented as means and 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, 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).

RESULTS AND DISCUSSION

Construction and growth characteristics of the *hfq* mutant in *C. sakazakii*. As numerous studies have demonstrated that Hfq contributes to the pathogenesis of many bacteria differently (28, 30, 34), we studied the effects of an Hfq-like protein on the pathogenesis of *C. sakazakii* ATCC 29544. The gene (KC866358) homologous to the *hfq* gene is located in a clockwise orientation in the genome of *C. sakazakii* ATCC 29544, as shown in Fig. 1A, and is also found in *C. sakazakii* ATCC BAA 894 (YP_001436319.1) and *C. turicensis* z3032 (YP_003212051.1) (39, 40). The gene (KC866358) showed high protein sequence similarity to the *hfq* genes of *E. coli* K-12 (89%), *S.* Typhimurium (91%), and *Y. pseudotuberculosis* (84%) (Fig. 1B). In most bacteria, the N-terminal domain of Hfq is highly conserved and is responsible for RNA binding and protein-protein interactions among Sm (the core of small nuclear ribonucleoprotein particles [snRNPs]) and Sm-like proteins (41, 42), whereas the C-terminal fragments are variable in length and amino acid composition (43). The C-terminal domain of Hfq is reported to play roles in gene regulation and protein stability (43, 44), suggesting that the variable regions at the C termini may be responsible for the differential gene regulation by Hfq observed in diverse bacterial species. To understand the roles of *hfq* in *C. sakazakii* ATCC 29544 pathogenesis, we generated an unmarked mutant lacking the entire *hfq* gene using the Lambda-Red recombination technique.

The loss of *hfq* was verified by sequencing, and we confirmed no polar effect on *hflX*, located downstream of *hfq*, by qRT-PCR (data not shown). When we compared the growth of the strains in LB medium with constant aeration at 37°C, the deletion mutant showed a slightly lower growth rate than the wild type (Fig. 2). The plasmid carrying the *hfq* gene complemented the reduced growth of the Δ*hfq* strain (Fig. 2).

The loss of *hfq* attenuates colonization by *C. sakazakii* in rat pups. Hfq is a global regulator known to modulate physiological fitness and pathogenesis at multiple levels in various bacteria (29, 31, 33, 45). A *Klebsiella pneumoniae* mutant lacking *hfq* was unable to disseminate into extraintestinal organs (spleen and liver) and was attenuated in induction of a systemic infection in a mouse model (33). Similarly, *Vibrio cholerae hfq* mutants were highly attenuated in virulence in the suckling mouse model of cholera, showing 1,000-fold less efficacy than wild-type bacteria at colonizing the murine small intestine (45).

To examine whether the *hfq* gene in *C. sakazakii* contributes to pathogenesis, an *in vivo* challenge assay using newborn rats was

conducted as performed in other studies (38, 46). To minimize individual variations between infant rats, competitive assays were carried out by orally inoculating the bacterial mixture containing equal numbers of Δ*hfq* and wild-type strains. At 20 h postinfection, the *hfq* deletion mutant was outcompeted by the wild type, with CIs of 0.05 ± 0.06 and 0.08 ± 0.09 in the spleen and liver, respectively (Fig. 3). These results suggest that *hfq* is critical for the ability of *C. sakazakii* to disseminate into deeper organs. The attenuated virulence of the Δ*hfq* mutant might be attributable to multiple defects during the infection processes, including decreased invasion into host cells and impaired resistance against environmental stresses. To define the roles of Hfq in virulence regulation in *C. sakazakii*, a variety of approaches were applied, as described below.

Hfq is involved in the invasion of *C. sakazakii* into human epithelial cells. The loss of *hfq* caused a reduction in invasion ability in *S.* Typhimurium and *E. coli* (31, 47). To explore whether *hfq* in *C. sakazakii* affects its invasion ability, a gentamicin protection assay was performed using Caco-2 cells. As expected, the Δ*hfq* strain showed approximately 3-fold-reduced invasion into these cells compared with the WT. Complementation with the plasmid harboring the *hfq* fully restored the invasion ability of the Δ*hfq* strain to wild-type levels (Fig. 4A). Additionally, we checked the invasiveness of the strains in HepG2 cells, which are derived from liver epithelia, and the result was comparable to that with Caco-2 cells (data not shown), suggesting that impaired invasiveness may be common for the epithelium-derived cell lines.

In *Salmonella*, Hfq controls *Salmonella* pathogenicity island I (SPI1), which encodes a type III secretion system (T3SS) and several effector proteins that are involved in the invasion of *Salmonella* into host cells (31). A secretion system equivalent to the T3SS has not been identified in *Cronobacter*; rather, it invades host cells via a receptor-mediated mechanism (14). Previously, outer membrane proteins (OmpA and OmpX) were reported to be involved in *C. sakazakii* invasion of human intestinal Caco-2 cells as invasins (15). In this study, the effect of Hfq on the expression of OmpA and OmpX was studied with Western blot analysis using anti-OmpA and anti-OmpX polyclonal antibodies. However, we did not observe substantial differences in OmpA and OmpX expression (Fig. 4B), suggesting that Hfq might not be associated with outer membrane protein expression. However, the possibility that Hfq may regulate other, yet-unidentified invasins in *C. sakazakii* ATCC 29544 cannot be ruled out.

Hfq is required for oxidative-stress resistance in *C. sakazakii*. In aerobic environments, many bacteria generate or encounter reactive oxygen species (ROS) (superoxide anion, hydrogen peroxide, and hydroxyl radicals), which damage lipids, proteins, and nucleic acids (48). It is well known that pathogenic bacteria have the ability to cope with oxidative stresses, which is essential for their pathogenesis (49). Here, we tested the role of Hfq in bacterial resistance to oxidative stress by exposing wild-type and Δ*hfq* strains of *C. sakazakii* to H_2O_2. The *hfq* mutant showed significantly (100-fold) lower survival than the WT in the presence of 100 mM H_2O_2 after 10 min of exposure (Fig. 5A). In agreement with this result, the Δ*hfq* strain exhibited a reduction in bubble formation, which is indicative of decomposition of H_2O_2 into water and oxygen (Fig. 5B). *katG* is one of the H_2O_2-inducible genes and encodes hydroperoxidase I or catalase, a primary detoxifier of H_2O_2 in many bacteria, including *C. sakazakii* (50, 51). As *katG* is positively regulated by OxyR, which is a H_2O_2-inducible regulator in *E. coli* and *S.* Typhimurium (50, 52), both *oxyR* and

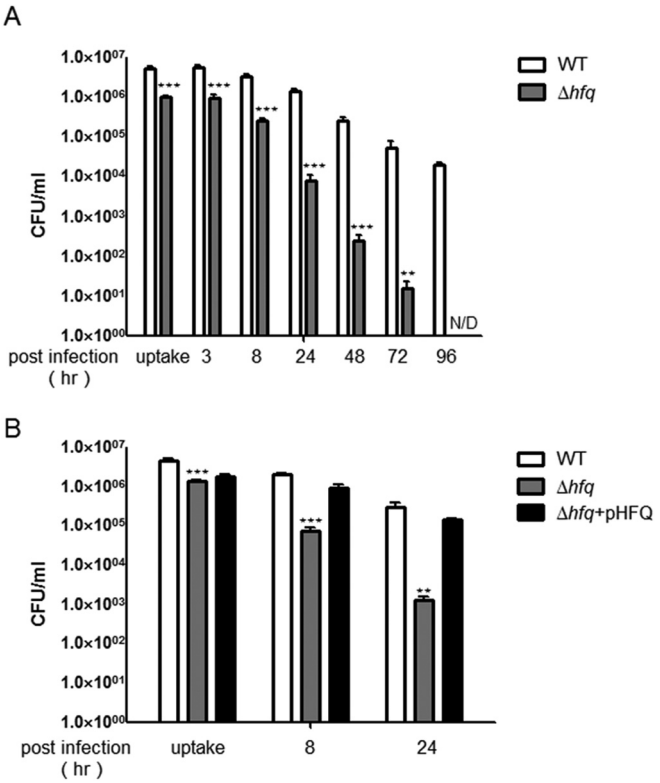

FIG 6 Lack of Hfq results in reduced intracellular survival of *C. sakazakii* in macrophage-like cells. The *C. sakazakii* strains harboring the pACYC184 control plasmid or a complementation plasmid, pHFQ, and the wild type or *hfq* mutant (SK003) were incubated with RAW264.7 macrophage-like cells. (A) Intracellular bacteria of the wild type and *hfq* mutant (SK003) were evaluated for the number of CFU at uptake (1.5 h) and at 8, 24, 48, 72, and 96 h postinfection. (B) Intracellular bacteria of *C. sakazakii* strains harboring the pACYC184 control plasmid or a complementation plasmid, pHFQ, were evaluated for the number of CFU at uptake (1.5 h) and at 8 and 24 h postinfection. The bars represent means and SEM from independent experiments performed in triplicate. The data are representative of 3 independent experiments. The asterisks indicate significant differences (***, $P < 0.001$; **, $P < 0.05$).

katG expression levels were monitored by qRT-PCR. The addition of H_2O_2 significantly increased *katG* expression in wild-type bacteria, but the absence of Hfq decreased its expression approximately 3-fold (Fig. 5C), implying that Hfq plays an important role in inducing *katG* expression for protection of *C. sakazakii* against oxidative stress. Complementation with a plasmid containing *hfq* in *trans* restored the impaired resistance of the Δ*hfq* strain to H_2O_2 (Fig. 5C). Interestingly, *oxyR* expression was not induced by H_2O_2 in both strains, suggesting that OxyR-independent *katG* induction may be possible, as has been reported in *Neisseria gonorrhea* and *Bradyrhizobium japonicum* (53, 54).

Loss of the *hfq* gene reduces intracellular survival of *C. sakazakii* in macrophage-like cells. A previous study showed that *Cronobacter* spp. were able to persist or replicate within macrophages (38), and *Salmonella hfq* was shown to be critical for bacterial survival in cultured host macrophages (31). Thus, we examined the impact of *hfq* deletion on the survival of *C. sakazakii* within cultured macrophages.

Although the initial uptake of Δ*hfq* bacteria by macrophages was 5-fold lower than that of the WT, clearance of the mutant (2-log-unit and 5-log-unit decreases in survival after 24 and 72 h,

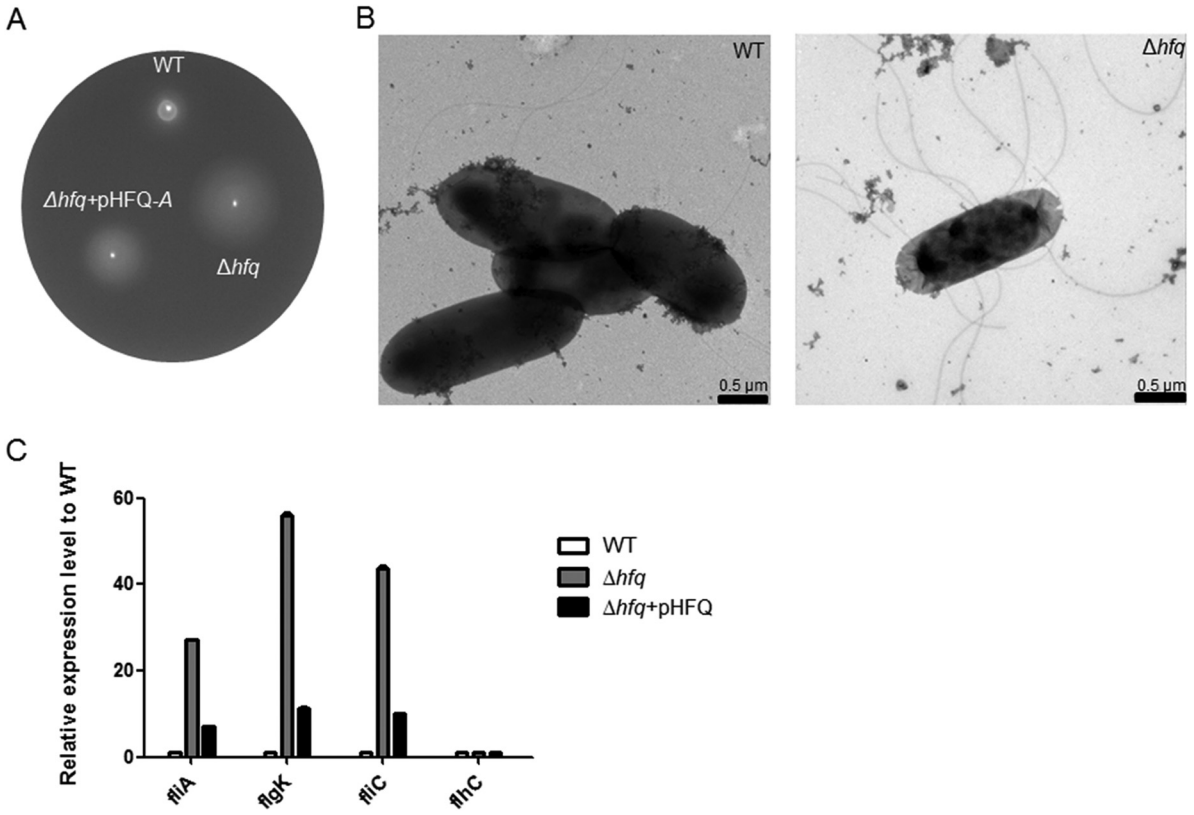

FIG 7 Loss of Hfq causes enhanced motility. (A) Motility of the *C. sakazakii* strains harboring the pBAD18 control plasmid or the complementation plasmid pHFQ-A on semisolid agar plates. The strains were cultured on semisolid agar plates containing 1.33 mM arabinose at 37°C, and motility was monitored. The image is representative of several experiments. (B) Morphology of wild-type and Δ*hfq* (SK003) strains imaged by transmission electron microscopy. (C) qRT-PCR of *fliA*, *flgK*, *fliC*, and *flhC* transcripts from WT (SK001) and Δ*hfq* (SK005) strains and Δ*hfq* harboring pHFQ (SK007). To obtain the expression level relative to that of the WT on the *y* axis, the mRNA level of each gene was divided by the mRNA level of the 16S rRNA. The mRNA expression values in strains SK005 and SK007 were further normalized to the transcription levels of the wild type. The bars represent means and SEM from independent experiments performed in triplicate. The data are representative of 3 independent experiments.

respectively) was significantly faster than for the WT (0.5 and 1.5 log units at the respective time points) on average (Fig. 6A). Furthermore, the *hfq* deletion mutants were cleared completely after 72 h postinfection, whereas WT strains (approximately 4 log CFU/ml) were still able to persist within macrophages at 96 h (Fig. 6A). A plasmid containing *hfq* complemented the ability to survive within macrophages (Fig. 6B). These data suggest that *hfq* is necessary for intracellular survival of *C. sakazakii* in host macrophages.

F. tularensis lacking *katG* caused increases in hydrogen peroxide production and tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) expression within macrophages, leading to attenuation of bacterial survival inside host cells (55). In this regard, the accelerated clearance of the Δ*hfq* strain within host cells might be attributable, at least in part, to decreased *katG* expression (Fig. 5C), which causes H_2O_2 accumulation and stimulates inflammatory cytokine production. Another probable explanation for the attenuated Δ*hfq* persistence inside the macrophage-like cells is that flagellar synthesis is altered by the removal of *hfq*, as described below.

A *C. sakazakii* Δ*hfq* strain is hypermotile due to higher expression of flagella. As motility is an important virulence determinant (56), we examined the effect of Hfq on the swimming motility of *C. sakazakii* using 0.3% soft-agar plates. The absence of the *hfq* gene resulted in increased motility (Fig. 7A), and the transmission electron microscope image showed that the *hfq* mutant was more flagellated than the WT (Fig. 7B). These results suggested the possibility of negative regulation of flagellar synthesis by Hfq. Therefore, the expression of flagellum-associated genes was studied in the *hfq* mutant by qRT-PCR (Fig. 7C). The transcription of *fliA*, which encodes an alternative sigma factor specific for the late operons of flagellar regulons, including *flgK* and *fliC* (57), was increased 30-fold in the *hfq* mutant compared with the wild type. In accordance with the increased *fliA* transcription, genes under the control of FliA, including *flgK* and *fliC*, encoding a flagellar-hook-associated protein and a flagellin protein, respectively, showed increases in transcription in the absence of Hfq. On the other hand, the transcriptional level of *flhDC*, encoding a master regulator of flagellar regulons, was not affected by the lack of Hfq. Interestingly, introducing a plasmid harboring *hfq* into the *hfq* mutant could partially complement the transcription levels of *fliA*, *flgK*, and *fliC* (Fig. 7C) but could not recover the hypermotility (data not shown). An *Acinetobacter baylyi* Δ*hfq* strain showing a retarded growth and abnormal morphology was fully complemented with the introduction of *hfq* and its cognate promoter region into the chromosome, while a low-copy-number plasmid harboring *hfq* under a leaky inducible promoter could not complement it, indicating that fine-tuning of *hfq* expression is impor-

tant for its tight regulation (58). The failure of the pHFQ plasmid to complement the hypermotility of the *hfq* strain might be caused by the imbalance of Hfq production from the low-copy-number plasmid pACYC184 (59). To rule out that possibility, the pHFQ-A plasmid, in which *hfq* expression is under an arabinose-inducible promoter (37), was used to optimize Hfq expression and accomplished partial restoration of motility with 1.33 mM arabinose (Fig. 7A). Taken together, these data suggest that *hfq* in *C. sakazakii* is involved in the negative regulation of flagellar biosynthesis.

It is interesting that the *C. sakazakii hfq* mutant showed an increased motility phenotype, unlike other bacteria, i.e., *Salmonella* and uropathogenic *E. coli* (31, 32). One explanation for this phenomenon might be the variable region at the C terminus of Hfq, as shown in Fig. 1B. According to a previous report, an Hfq variant lacking its C terminus was defective in autoregulation and riboregulation (44). Therefore, C-terminal variation might enable differential gene regulation from species to species. Alternatively, the hypermotility of the *hfq* strain may be associated with Hfq-mediated RpoS regulation in flagellar biosynthesis. It has been reported that Hfq is required for efficient translation of the *rpoS* sigma factor (60, 61), and RpoS negatively regulates operons involved in flagellar biosynthesis and functions in *E. coli* by decreasing the amount of RpoF, which competes with RpoS in binding to the core RNA polymerase (62, 63). In the absence of RpoS, the expression of RpoF (FliA) and FliC was increased, and the surface of the bacteria was found to be more flagellated than that of the WT strain. In this regard, it is plausible that the *hfq* null mutation caused poor translation of *rpoS* in *C. sakazakii* and that the lowered level of RpoS resulted in higher flagellar biosynthesis and formation. Further study would be needed to shed light on the mechanisms of flagellar regulation mediated by *hfq*.

The ability of pathogenic microbes to survive within cultured phagocytic cells is associated with the ability to escape or remodel an innate immune response that employs antimicrobial defense mechanisms, such as oxidative burst and acidic compartmentalization (38). To establish a good niche within the host, bacteria should have the ability to modulate immune system activation by harnessing cytokine production (64, 65). It has been reported that *C. sakazakii* flagella and flagellin proteins stimulate the production of inflammatory cytokines (IL-8, TNF-α, and IL-10) in a dose-dependent manner (19). To dampen cytokine stimulation, pathogens, such as *Pseudomonas* and *Salmonella*, reduce flagellar synthesis after entering host cells (65–67). Bacterial flagella activate the signaling pathway by binding to TLR5 during infection (68). Indeed, secreted flagellin from *Pseudomonas* triggered the expression of NF-κB and secretion of IL-8. To avoid the activation of TLR5, the enzyme AprA, which cleaves flagellin, is produced (67). Likewise, flagellin from *Salmonella* also stimulates the activation of immune cells, leading to proinflammatory cytokine expression. However, intracellular *Salmonella* represses flagellar expression to modulate the immune response, whereas extracellular bacteria express flagella. Overall, it is important for pathogenic bacteria to harness flagellar expression for successful infection (65, 67). In this context, hyperflagellation of the *hfq* mutant (Fig. 7) might result in higher production of inflammatory cytokines. Accordingly, the increase in cytokine production might elicit an immune response, leading to accelerated clearance of intracellular bacteria or causing local inflammation and necrosis (69).

Conclusions. In the present study, we demonstrated that the loss of Hfq remarkably attenuated *C. sakazakii* virulence in a rat pup model of infection due to defects in growth, invasion ability, resistance to oxidative stress, and intracellular survival. In addition, to our surprise, the Hfq-deficient strain showed hypermotility, which is a distinctive phenotype among bacteria.

ACKNOWLEDGMENT

This research was supported by a grant (14162MFDS972) from the Ministry of Food and Drug Safety, South Korea, 2015.

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