A Thesis for the Degree of Master of Science

Isolation and characterization of bacteriophages specific to *Cronobacter sakazakii* and construction of phage cocktail targeting two different host receptors

크로노박터 사카자키를 감염시키는 박테리오파지의 분리 및 특성분석과 서로 다른 수용체를 이용하는 파지 칵테일 구축

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ABSTRACT

Application of bacteriophage as novel antimicrobial agent has been given a positive assessment in the food safety aspect with their host cell lysis activity and specificity. Novel phages specific to *Cronobacter sakazakii*, notorious foodborne pathogen often contaminating powdered infant formulae, were isolated. To gain more effectiveness of phage treatment, phage cocktail consisting of two phages targeting different receptors was constructed. Phage CR8 and phage S13 utilize flagella and LPS as host receptors, respectively. Also they showed distinct characteristic in genome, replication cycle, and activity, but except for that they belong to the same *Myoviridae* family. The cocktail of these phages has synergistic activity compared to single phage. Phenotypic analysis the resistant mutant to the phage cocktail (CSR strain) revealed that the resistance came from the impairment of receptor for phages such as defects in motility and LPS structure. And consequentially, CSR strain couldn't normally invade into human epithelial cell, Caco-2. Newly isolated phages exploiting not LPS or flagella, YR1 and YR2, could infect CSR strain and the phage cocktail containing them also showed more effectivity than the single phages. These results suggest that the construction of phage cocktail based on receptor information has potentialities as practical method in phage treatment and that it needs many considerations such as

family, receptor, host range, and lytic activity of each phage to construct the best phage cocktail.

Keywords: *Cronobacter sakazakii,* bacteriophage, phage cocktail, phage receptor, phage resistant

CONTENTS

Ⅰ. INTRODUCTION

Cronobacter sakazakii is a gram-negative food-borne pathogen and classified as "Severe hazard for restricted populations, causing life threatening or substantial chronic sequelae or long duration" by International Commission on Microbiological Specification for Foods (Foods & Tompkin, 2002). Even though it is non-spore forming bacteria (Farmer, Asbury, Hickman, Brenner, & Group, 1980), it has resistance to dryness and it can form a biofilms on plastic surfaces (Iversen, Lane, & Forsythe, 2004; Oh, Chen, & Kang, 2007). So it can be spread through the powdered infant formulae easily, and it has consequently been the threat for bottle-fed neonates by causing necrotizing enterocolitis of which mortality extends to 55% and survivors often showed neurological sequelas (Dumen, 2010; Harris & Oriel, 1989; Lucas & Cole, 1990; Muytjens et al., 1983; Van Acker et al., 2001) While the production of cytotoxins or enterotoxins by C. sakazakii was studied (Pagotto, Nazarowec-White, Bidawid, & Farber, 2003), however the mechanism of its pathogenesis has been little known.

In spite of the attention of food safety and the improvement of technologies for food safety, still millions of people suffer from foodborne pathogens annually, so the development of these pathogens-biocontrol agent

is urgently needed. Among the many endeavors, the application of bacteriophages is highlighted and has been actively studied (Brovko, Anany, & Griffiths, 2012; Garcia, Martinez, Obeso, & Rodríguez, 2008; Greer, 2005; Hagens & Loessner, 2007; Sillankorva, Oliveira, & Azeredo, 2012; Strauch, Hammerl, & Hertwig, 2007). The advantage of phages is their ability to control specific bacteria, so they don't interfere with the natural microflora (Hagens & Loessner, 2007; Strauch et al., 2007). The application of phages could be available in various ways such as phage therapy, biosanitation, and biopreservation (Garcia et al., 2008). Also, phages are ubiquitous on earth and very diverse in biological characteristics including host range, morphology, genomic characteristic, lytic activity, etc (Alexander Sulakvelidze, 2011). That is, there are infinitely many resources that can be studied and developed, and it means cost reduction on development of novel therapeutic agents (Kelly, McAuliffe, Ross, O'Mahony, & Coffey, 2011).

However, the appearance of phage-resistant variants can be a barrier to application of phages. To overcome this problem, superabundance of phage species can be exploited (Gu et al., 2012). Phage cocktail consisting of different phages isolated by a "Step-by-Step" approach showed broader host range (Kelly et al., 2011), or reducing phage-resistant frequency (Gu et al., 2012). And it was forecasted that different receptor-using phages will be

more effective for biocontrol in some studies (Filippov et al., 2011; M. Kim & Ryu, 2011). But to our knowledge, such construction of phage cocktail based on receptor information was not put into actual practice.

Moreover antimicrobial-resistant *C. sakazakii* strains were emerged in hospital or domestic kitchens (Dennison & Morris, 2002; Kilonzo-Nthenge, Rotich, Godwin, Nahashon, & Chen, 2012; Pitout, Moland, Sanders, Thomson, & Fitzsimmons, 1997) and powdered infant formulae can be contaminated by pathogenic bacteria during manufacturing process after sterilization step (Agostoni et al., 2004), there are a few of studies dealing with *C. sakazakii*-targeting phages which have potentiality for biocontrol agents (K.-P. Kim, Klumpp, & Loessner, 2007; Zuber et al., 2008) and most of the reports are about analysis of full genome sequence (Abbasifar et al., 2013; J. H. Lee, Choi, Shin, Lee, & Ryu, 2012; Y.-D. Lee, Chang, & Park, 2011; Shin, Lee, Kim, & Ryu, 2012). In this study, novel *C. sakazakii*-specific phages from environmental feces and soil sample were isolated and characterized to formulate phage cocktail, especially consisting of phages whose host receptor is different, and the synergistic effect of that was examined.

Ⅱ. MATERIALS AND METHODS

2.1. Bacterial strains and growth condition

Cronobacter sakazakii ATCC 29544 (Farmer et al., 1980) was used for the isolation, propagation of phages and construction of mutant strains. All the bacterial strains were cultured aerobically in tryptic soy broth (TSB, BD Bacto, Franklin Lakes, NJ, USA) or tryptic soy agar (TSA, BD Difco, Franklin Lakes, NJ, USA) at 37°C for 12 h with 220 rpm agitation.

2.2. Isolation and propagation of bacteriophages

Feces samples from University animal farm college of agriculture and life sciences Seoul national university (Suwon, South Korea) and soil sample from a food garbage dump (Seoul, South Korea) were used to isolate *C. sakazakii*-targeting bacteriophages. The 25 g samples were homogenized in 225 ml sterile Butterfield's phosphate-buffered dilution water (0.25 M KH_2PO_4 adjusted to pH 7.2 with NaOH) using a blender (BacMixer 400, Interscience Laboratory Inc., St. Nom, France). The 25 ml of each homogenized sample was mixed with 25 ml 2X concentrated TSB and incubated at 37°C for 8 h with 220 rpm agitation. After incubation, the culture was centrifuged (9,000 ×*g*, 10 min, 4°C) and the supernatant was

filtered using 0.22-μm-pore-size filters (Millipore, Billerica, Massachusetts, USA). The 10 ml filtrate was mixed with 50 ml of TSB containing 5 mM $CaCl_2$ and 500 μl overnight culture of *C. sakazakii* ATCC 29544 and incubated at 37°C for 8 h with 220 rpm agitation, followed by centrifugation and filtration as described above. Tenfold serial dilutions of the last filtrate were dotted on the bacterial lawn (5 ml of 0.4% soft TSA containing 100 μl of overnight culture of *C. sakazakii* ATCC 29544 and being poured into TSA plate) and the plate was incubated overnight at 37°C to confirm the presence of bacteriophages via formation of plaques. Each single plaques on dotted or overlaid plate was picked with a sterile tip and dissolved in sterilized sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$). This process was repeated five times to isolate single bacteriophage.

To propagate and concentrate the isolated phages, the culture of *C. sakazakii* ATCC 29544 (optical density at 600 nm = 0.5) was infected with the lysate of a single plaque of phage and incubated at 37°C until the culture became clear. After centrifugation of the cleared culture (15,000 ×*g*, 10 min, 4°C), the supernatant was filtered (0.22-μm-pore-size filter) to remove cell debris and precipitated with polyethylene glycol (PEG) 6000 (Junsei chemical, Japan) in 1 M NaCl at 4°C for 12 h. Centrifuged and precipitated

phages were dissolved in SM buffer and the phage particles were separated by cesium chloride (CsCl) density gradient ultracentrifugation (25,000 rpm, 2 h, 4°C), followed by dialysis using SM buffer and storage at 4°C.

To isolate new phages which utilize different receptor from upper isolated phages, the same procedure was repeated using *C. sakazakii* ATCC 29544 LPS (rfaC-) defective mutant strain as host strain. Feces and water samples from a farm (Eumseong, South Korea) were used to source of bacteriophages.

2.3. Transmission electron microscopy

Phage stocks were diluted (about 1×10^{10} pfu/ml) with SM buffer and placed on carbon-coated copper grids. After removal of the excess phage suspension, 2% aqueous uranyl acetate (pH 4.0) was dropped for negatively staining. The excess solution was removed and the grids were examined by transmission electron microscope (TEM; LEO 912 AB; Carl Zeiss, Germany) at 80 kV and the phage particle images were obtained by device camera at the National Academy of Agricultural Science (Suwon, South Korea). Based on the examined morphologies, each phage was classified according to the guidelines of the International Committee on Taxonomy of Viruses (Fauquet, Mayo, Maniloff, Desselberger, & Ball, 2005).

2.4. Genome sequencing and bioinformatics analysis

Bacteriophage genomic DNA was extracted from the phage lysate according as Lambda DNA extraction protocol from the Phase lock gel (PLG; 5 PRIME, Hamburg, Germany). Whole-genome sequencing of the extracted phage DNA was carried out with a Genome Sequencer FLX (GS-FLX) titanium sequencer (Roche, Mannheim, Germany) and assembled with Newbler v2.3 (Roche) at Macrogen Inc., South Korea. Open reading frames (ORFs) were predicted using GeneMarkS (Besemer, Lomsadze, & Borodovsky, 2001), Glimmer v3.02 (Delcher, Bratke, Powers, & Salzberg, 2007), and FgenesB softwares (Softberry, Inc. Mount Kisco, NY, USA), and confirmation of ribosomal binding sites was through RBSfinder (J. Craig Venter Institute, Rockville, MD, USA). BLASTP (Altschul, Gish, Miller, Myers, & Lipman, 1990) and InterProScan (Zdobnov & Apweiler, 2001) programs are used to annotate predicted ORFs, and these sequencing and annotation data were handled by Artemis14 (Carver et al., 2008).

2.5. Construction of candidates for phage receptor deletion mutants and complementation

C. sakazakii ATCC 29544 strains with deletion of candidate gene for

phage receptor was constructed using the one-step gene inactivation method as described previously (Datsenko & Wanner, 2000). For briefly, in case of *flgK* gene encoding flagella hook-filament junction protein, the kanamycin resistance (Km^r) cassette from plasmid pKD13 was amplified by PCR using primers containing the sequence upstream or downstream of the *flgK* gene. The resulting PCR product was used to transform the ATCC 29544 strain containing pKD46, and *flgK*::Km^R transformant was selected on kanamycin plate. And then the Km^r cassette was removed by introducing plasmid pCP20.

For complementation of the *flgK* deletion mutant, the gene of *C. sakazakii* ATCC 29544 was amplified by PCR using the primers containing restriction site (HindIII, NheI respectively). The PCR product was digested with the restriction enzymes and ligated into the HindIII/NheI-digested pBAD18 vector harboring the ampicillin resistant gene (Guzman, Belin, Carson, & Beckwith, 1995). The sequence of the gene in the vector was confirmed by sequencing, and pBAD18::*flgK* was transformed into the *flgK* deletion mutant. Expression of the *flgK* gene was induced by arabinose (0.2%, final concentration) and susceptibility to phages was examined by dotting assay. The other candidate genes are deleted and complemented in the same way and primers used in this study are listed in Table 1.

2.6. Bacteriophage adsorption assay

C. sakazakii ATCC 29544 wild-type or mutant strain was incubated to exponential growth phase (OD_{600} = 1.0 to 1.5) and distributed into 6 microtubes (900 μl culture per 1.75-ml tube). Each of the culture was infected by phage (MOI = 0.01) with 5 min intervals and incubated until the first infected tube was incubated for 20 min. After incubation, all tubes were centrifuged (16,000 rpm, 1 min, 4°C) and the supernatant was filtrated (0.22-μm-pore-size filter) to remove bacterial cells. Overlay assay was carried out to as previously described (M. Kim & Ryu, 2011), to determine the number of unadsorbed phage particles. The control was the same concentration of phage-added fresh TSB without host bacteria.

2.7. Bacteriophage host range analysis; Spotting assay

A 100 μl overnight culture of each tested bacteria was inoculated into 5 ml of molten 0.4% soft TSA and mixed by gentle vortexing. The mixture was poured into the 1.5% TSA plate (with 50 μg/ml ampicillin or kanamycin, if necessary) and solidified at room temperature (RT) for 20 min to be bacterial lawns. And then 10 μl of tenfold serial dilutions of phage stock was dotted on the bacterial lawn. The plates were dried at RT for 20 min, followed by incubation overnight at 37°C. The sensitivity of tested

bacteria to phages was inspected on the next day.

2.8. Bacteriophage one-step growth curve

A 30 ml culture of the *C. sakazakii* ATCC 29544 was incubated at 37°C until the OD_{600} reached 1.5 and washed by centrifugation (6,000 ×*g*, 10 min, 4°C) and resuspension in 50 ml fresh TSB medium. Phage was added at a MOI of 0.001 with 10mM $CaCl_2$, $MgCl_2$ and static incubated for 5 min at room temperature to adsorption. The mixture was washed as described above to remove the excess phage and incubated at 37°C with 220 rpm agitation. Every 5 min two sets of sample was taken and immediately diluted, and 100 µl of the last diluted sample was mixed with molten 5 ml Soft TSA (0.4% agar) and 100 µl overnight culture of *C. sakazakii* ATCC 29544. And then the mixture was overlaid on the 1.5% TSA plate and incubated for 7 h at 37°C for phage titration. Before the dilution, 1% chloroform (final concentration) was added to the second set of sample to allow intracellular phages to be released so that the eclipse period can be determined. The latent period and bust size were calculated based on the one-step growth curve.

2.9. Bacterial growth inhibition assay

An overnight culture of *C. sakazakii* ATCC 29544 was 1%

subinoculated into 50 ml of fresh TSB medium and incubated at 37°C with agitation for 1.5 h (OD at 600 nm = 0.4~ 0.6). Phage dilutions were added to the culture at a MOI of 1. Samples were taken from the culture every hour (or 2 hours if OD_{600} is constantly increases) and diluted 5-fold. And then OD_{600} of the sample was measured to monitor bacterial growth (GE healthcare, Ultrospec 6300 pro, Little Chalfont, UK). In case of phage cocktail, phage CR8 and S13 were added at an each MOI of 1. To count viable cell, samples taken from the culture were centrifuged (15,000 rpm, 1 min) to remove free phage and the pellet was resuspended in same volume of phosphate-buffered saline (PBS, GeneDEPOT, Barker, TX, USA) (pH 7.4). Then, it was 10-fold diluted with PBS, plated onto TSA plate, and followed incubation at 37°C for 12 h.

2.10. Isolation of phage cocktail-resistant *C. sakazakii* mutant

Phage-resistant *C. sakazakii* strain was isolated via overlay assay as described early(Capparelli et al., 2010). Briefly, 100 μl of each phages (about 10^{10} pfu/ml, for each) and the same volume of overnight bacterial culture were added to molten 5-ml soft TSA (0.4% agar). The mixture was poured onto TSA plate and incubated at 37°C until resistant colonies appeared. Three colonies were picked and subcultured 20 times on fresh TSA plate in

the absence of phages. One of them was selected for further study and the maintenance of phage resistance was certified by spotting assay.

2.11. Phenotype analysis of phage cocktail-resistant *C. sakazakii* mutant

To examine whether there are phenotype change in the resistant strain, motility assay was carried out. One microliter of the overnight culture was stuck in the middle of a TSA plate which contains low concentration of agar (0.3%) to allow bacteria to swim in the plate. The plate was dried for 30 min at room temperature and incubated for 8h at 37°C. As a positive and negative control, *C. sakazakii* ATCC 29544 wild type strain and flagella (*flgK*-) defective mutant strain were used, respectively.

Extraction and analysis of bacterial lipopolysaccharide (LPS) were performed as previously described (M. Kim & Ryu, 2012). Briefly, LPS extraction was through hot phenol-water extraction methods and then the extracted LPS were analyzed by deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE). Fluorescent staining of the gels was achieved by the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes, Cat. No. P20495; Eugene, Oregon, USA), and the gel was visualized under 300 nm UV by the Gel doc™ EZ System (Bio-rad). As a positive and negative control, *C. sakazakii* ATCC 29544 wild type strain and LPS (*rfaC*-)

defective mutant strain were used, respectively.

2.12. Gentamicin protection assay

Invasive ability of the bacteria strains to mammalian Caco-2 cells was determined via gentamicin protection assay as described previously (K. Kim et al., 2010), with the following little modifications. Main culture containing 1% inoculum of overnight culture was incubated for 3 h at 37°C with 220 rpm agitation. Centrifuged cells were resuspended with 400 μl of EMEM for infection. Five hundred microliter of 1% Triton X-100 was added and incubated for 15 min for cell lysis. Relative invasion was obtained by calculating percent invasion [100 × (number of survived bacteria)/(number of inoculated bacteria)] as compared to the invasion of the *C. sakazakii* ATCC 29544 wild type strain.

2.13. Nucleotide sequence accession number

The complete genome sequences of *C. sakazakii*-targeting bacteriophages CR8 and S13 are available in GenBank under the accession numbers KC954774 and KC954775, respectively.

Table 1. Primers used in this study

Gene	Primer Name	Oligonucleotide sequence 5` - 3` [a,b]
btuB	btuB-red-F	ACC AGA ATG GTT GGG CGG GCC TCA GAA GGT GTA GCT GCC TGA CAA CGT GTA TTC TTG TAG GCT GGA GCT GCT TCG
	btuB-red-R	GCA TCT CGC GCG CTT ATC GCG TTC TAT TAA GTG CGC CTG CGG CAT CCT ATA CGT TAT TCC GGG GAT CCG TCG ACC
	btuB-confirm-F	TCA TAA ACA GAA AGC CCA CCC ACG
	btuB-confirm-R	AAG TCC TCA TCC GTA ACA CAC CTC
fhuA	fhuA-red-F	TCA AAC AGG TTA TTG ACG TTT AAG GCG ACA GAC GAG CCC GGC AGG CCT AAA CGC GTG TAG GCT GGA GCT GCT TCG
	fhuA-red-R	TAG CAT GGC GCG TTC CAC TCA CAC TCA GAT CAA TAC CAG GAT TTG CAG ACT GGC GAT TCC GGG GAT CCG TCG ACC
	fhuA-confirm-F	GCG TAG TCT TTG TAG CAG CTA GAG
	fhuA-confirm-R	CAA CCT TTC GCA TAT CAT CTC GGG
	fhuA-comple-F-SalI	AAA **GTC GAC** TTG TTG AAG GGC ACG CC
	fhuA-comple-R-EcoRI	AAA **GAA TTC** GCA TAT CAT CTC GGG CGC AT
flgK	flgK-red-F	CGC ATG TTC TGC TGA TAC ATC ATT TGT GTA CTA ATA CGC ATC GTT CGG TTC CCT GTG TAG GCT GGA GCT GCT TCG
	flgK-red-R	GCG CTG CCG ATA ATT ATC GTC AGG ACC CGC ATA TGA ATG TTC AAA AGG AAC CTC CAT TCC GGG GAT CCG TCG ACC
	flgK-confirm-F	TAT TTC AGC CAG GTA CTC TGC GAG TCG GTA
	flgK-confirm-R	CTC GAC GCT GTT CTG AAA CCA CTC AAG TTC
	flgK-comple-F-HindIII	GTG TAC TAA T**AA GCT T**CG TTC GGT TCC CTG
	flgK-comple-R-NheI	TAA GC**G CTA GC**G ATA ATT ATC GTC AGG ACC
lamB	lamB-red-F	GAG ATA GAA TGA TGA TAA CTC TGC GTA AAC TCC CTC TGG CTG TGG CCG TGA TGG CTG TAG GCT GGA GCT GCT TCG
	lamB-red-R	TAC CAC CAG ATT TCC ATC TGG GCA CCG AAG GTC CAC TCA TCA TTG TCG CCA CGG CAT TCC GGG GAT CCG TCG ACC
	lamB-confirm-F	CCC CGC TTA CAA AGA AAA GC
	lamB-confirm-R	CTT TCG CCC CTC TTG TTA CA

[a], Sequences of priming sites in pKD13 were underlined.
[b], Restriction sites are bold.

Table 1. Primers used in this study (continued)

Gene	Primer Name	Oligonucleotide sequence 5` - 3` [a,b]
ompC	ompC-red-F	TCG GAC AAT GGA TTT GCC CGC TAG TTC CCT GAA TTA GTG AGC AGT GGC AAT AAT ATG TAG GCT GGA GCT GCT TCG
	ompC-red-R	GGA GCC CGC AGG CTC CTT TTG CAC ATC AGG TCG GGG ATT AGA ACT GGT AAA CCA GAT TCC GGG GAT CCG TCG ACC
	ompC-confirm-F	CTG TTG GAT TAT TCG GCT CC
	ompC-confirm-R	CAC ACG TTT CTC CTC TGT AAC
	ompC-comple-F-NheI	AAA **GCT AGC** TAG TTC CCT GAA TTA GTG
	ompC-comple-R-HindIII	CAG G**AA GCT T**TT GCA CAT CAG GTC
rfaC	rfaC-red-F	AAC GGA TGT TTC CCC GCA AAG CCA GGG ACG CAG TTG TTC AAA AAC GGT AGC GGC GTG TAG GCT GGA GCT GCT TCG
	rfaC-red-R	TGC CCG CGT TCT GGA GAC GCT CAA CGA ACT GCT GCT GAA CGA GGA AGC CTG ACG GAT TCC GGG GAT CCG TCG ACC
	rfaC-confirm-F	AGC AGG GAG AGG TCA ACA ATA CGT
	rfaC-confirm-R	AGG CTA TCA CCA GAG TCT CAT CGA
	rfaC-comple-F-SalI	AAA C**GT CGA C**GT GGT GGC GCA GCT T
	rfaC-comple-R-EcoRI	AAA A**GA ATT C**TG GAG ACG CTC AAC GAA CTG
pBAD18	pBAD18-F	GTC CAC ATT GAT TAT TTG CAC G
	pBAD18-R	CAG GCT GAA AAT CTT CTC TCA T

[a], Sequences of priming sites in pKD13 were underlined.
[b], Restriction sites are bold.

Ⅲ. RESULTS

CHAPTER 1.

3.1.1. Isolation and morphology analysis of bacteriophages

A total of five phages were isolated from the environmental samples and phage CR8 and S13, isolated from feces sample and soil sample respectively, were selected for further study because of their superiority of bacteriolysis activity (data not shown). The selected phages were propagated and the resulting stock (about 3.0×1012 pfu/ml, for each) was diluted with SM buffer for experiments in this study.

Transmission electron microscopy showed that both phages have contractile tail which is a characteristic of the family Myoviridae (Fig. 1). Phage CR8 has isometric head, the mean diameter of which is 78.6±1.7 nm while phage S13 has longish head with the major axis of 103.7±4.1 nm and the minor axis of 74.7±6.0 nm. Their tail length is similar to each other – non-contracted tail and contracted tail length of phage CR8 are 122.1±2.3 nm and 58.1 nm, respectively, and those of phage S13 are 112.0±2.1 nm and 55.0±2.1 nm. Also, phage CR8 has thinner and longer tail fiber than that of phage S13 (Fig. 1).

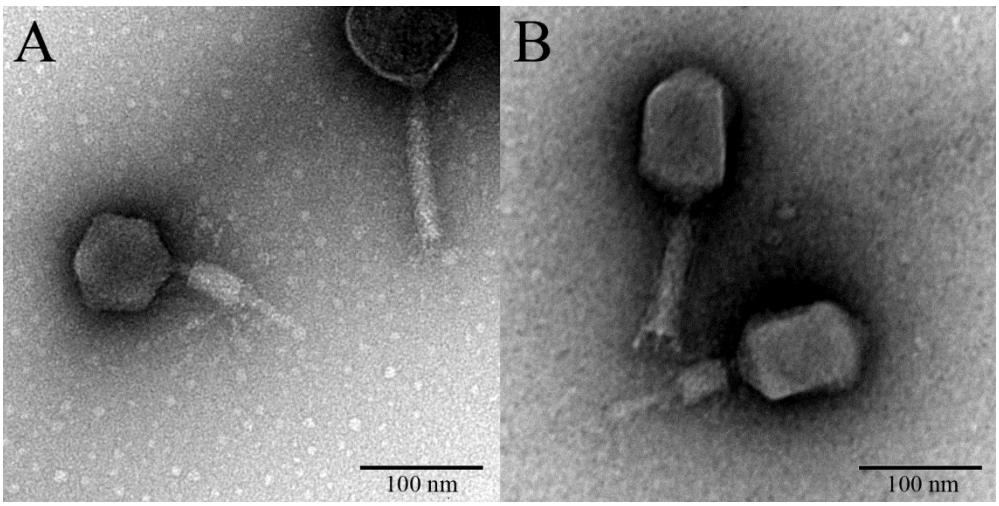

Figure 1. Morphology of phages. Transmission electron microscopy images of CR8 (A), S13 (B) negatively stained with 0.2% uranyl acetate. Scale bar is indicated at the bottom right-hand corner of the image.

3.1.2. Genome sequencing and bioinformatics analysis

Bacteriophage CR8 and S13 genomes contain 149,162-bp and 182,145-bp length DNAs with 269 ORFs, 17 tRNAs and 270 ORFs, 25 tRNAs, respectively (Table 2 and Fig. 2). The GC contents are 50.8% and 40.2% and the percentages of hypothetical proteins are 86.6% and 64.1%, respectively, indicating that the similarity of these two genomes is very low, even though they are categorized as same *Myoviridae* family. Further dot plotting analysis of these two genomes in DNA level supports this (Fig. 3A). While phage CR8 genome showed 97% identity in DNA level with reported phage CR3 genome (Shin et al., 2012), they did not have similarity with genomes in GenBank database, indicating that annotation information about *C. sakazakii* phages in the GenBank database may not be sufficient for the functional prediction of encoded proteins. However, phage S13 genome showed little similarity with well-know T4 phage genome and dot plotting analysis supports that similarity (Fig. 3B).

The functional ORFs of phage CR8 were categorized as only three functional groups (structure/packaging, DNA manipulation, and additional groups). This phage CR8 has two tail fiber proteins (CR8_028 and CR8_033) probably targeting flagella of *C. sakazakii* host as receptor binding protein (RBP). While host-lysis-related genes encoding holin and endolysin were not

detected in phage CR8 genome, its genome has a cell wall hydrolase (CR8_090), suggesting that this hydrolase may acts as host lysis protein.

On the other hand, the functional ORFs of phage S13 were classified as six functional groups (structure/packaging, DNA manipulation, RNA manipulation, regulation, host lysis and additional groups). This genome encodes many DNA manipulation proteins (DNA polymerases, helicases, methyltransferase, primases, topoisomerase, and nucleases) and RNA manipulation proteins (ribonucleases, RNA ligases, and RNA polymerase) and one host-lysis-related protein (lysozyme, S13_190). Furthermore, there are some regulation proteins (such as DNA binding protein, putative sigma factor, and putative anti-sigma factor) in this genome, suggesting that they are probably related to preferential transcription of the phage genes rather than the host genes. This phage S13 has several tail fiber subunits and putative RBP (S13_008) targeting LPS of *C. sakazakii* host.

Table 2. Genomic characteristics of phage CR8 and phage S13.

Characteristics	**Bacteriophages**	
	CR8	**S13**
Length (bp)	149,162	182,145
Overall G+C content (%)	50.8	40.2
No. of annotated genes	269	270
Percentage of hypothetical proteins (%)	86.6	64.1
Avg gene length (bp)	497	632
Gene coding content (%)	89.6	93.7
No. of tRNAs	17	25

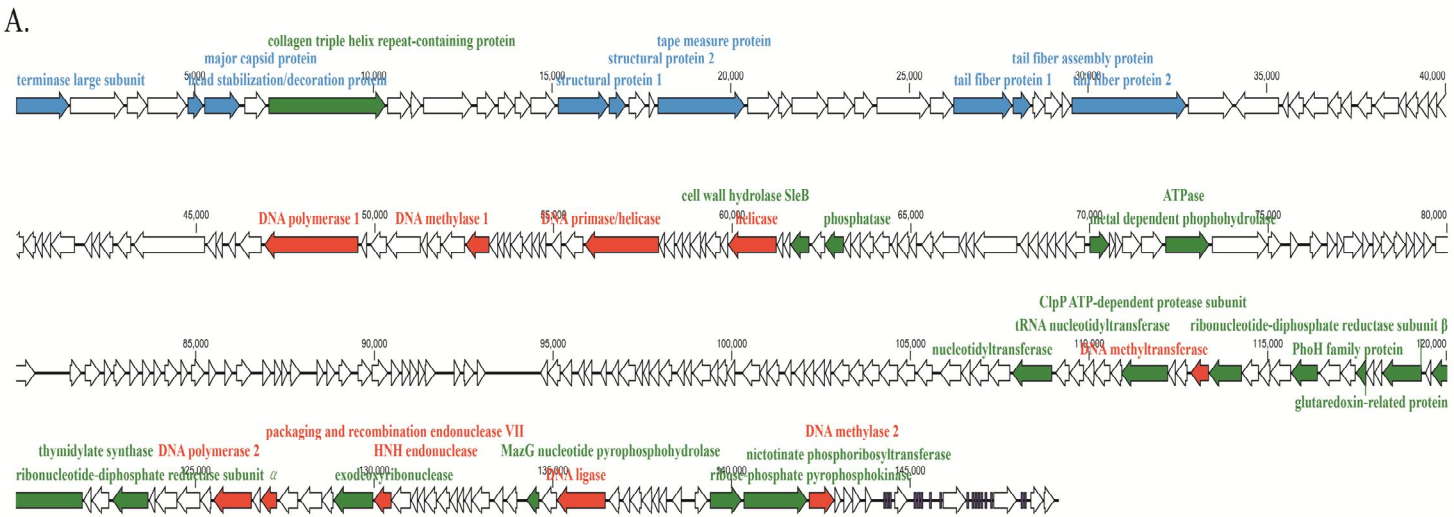

Figure 2. Genome maps of phage CR8 (A) and S13 (B). The arrows are the gene coding regions and the color of each gene indicates the functional categories; phage structure and packaging (blue), DNA manipulation (red), RNA manipulation (orange), regulation (yellow), cell lysis (black), and additional function (green). Scale unit is base pair.

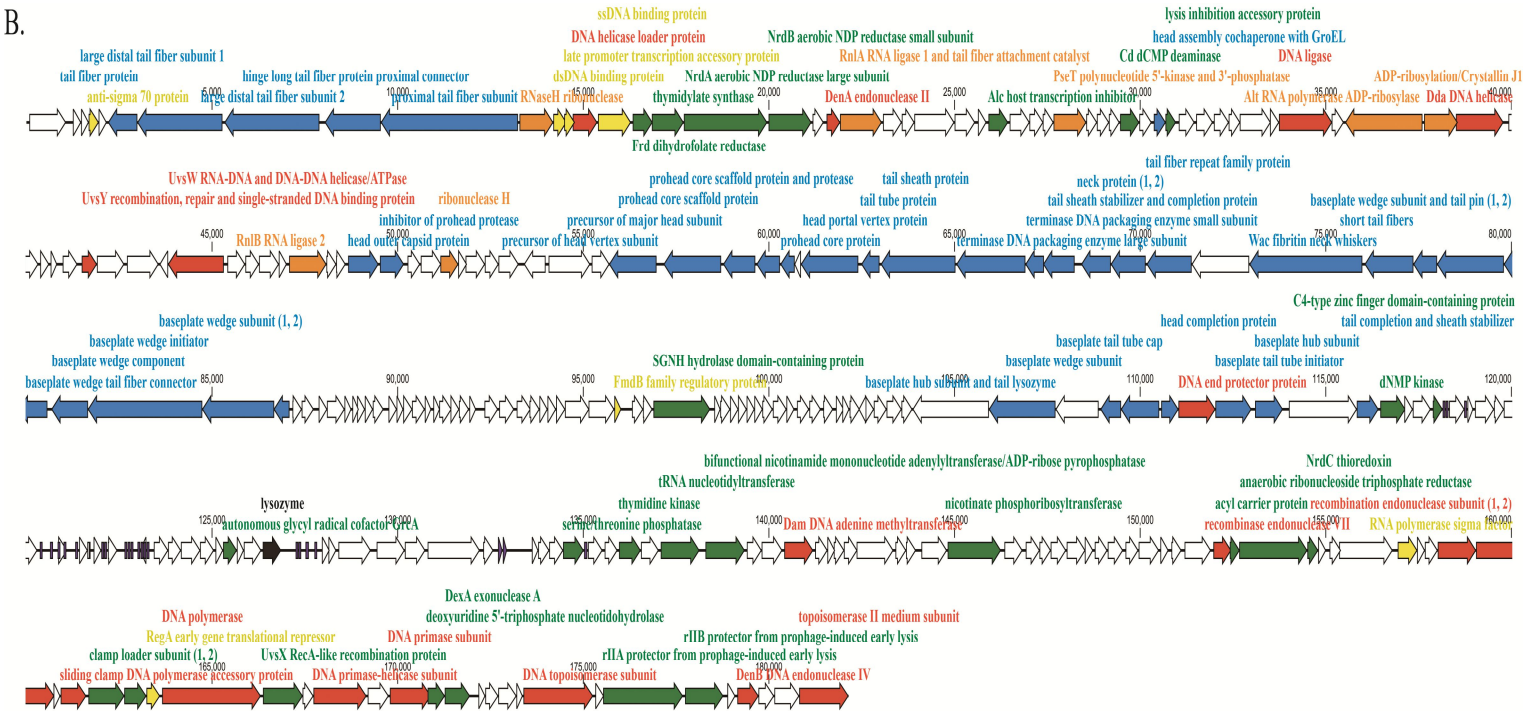

Figure 2. Genome maps of phage CR8 (A) and S13 (B) (Continued)

A.

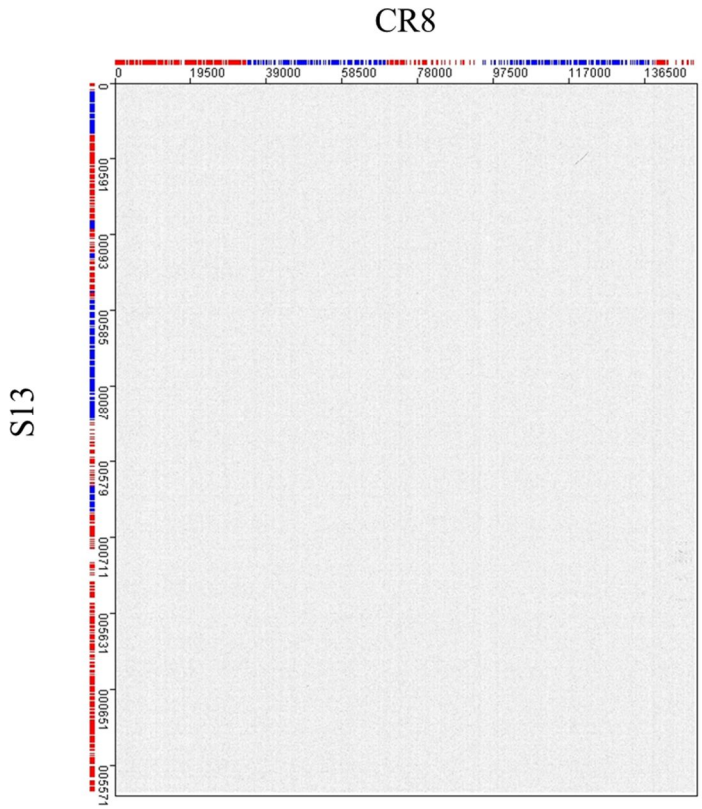

Figure 3. Comparative dot plot analysis of CR8 with S13 (A), and T4 with S13 (B).

B.

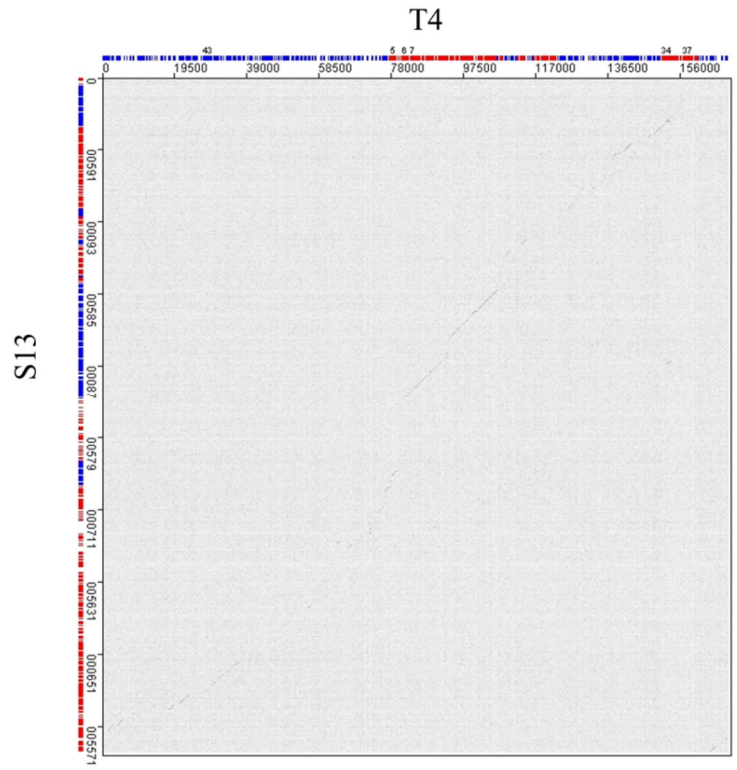

Figure 3. Comparative dot plot analysis of CR8 with S13 (A), and T4 with S13 (B).

3.1.3. Identification of phage receptor

To identify which bacterial component act as receptor for the phages, six candidate genes which are reported as receptor for phage to date in gram-negative bacteria (Rakhuba, Kolomiets, Szwajcer Dey, & Novik, 2010) were selected. The deletion mutants of *C. sakazakii* ATCC 29544, lacking flagella (Δ*flgK*-), LPS (Δ*rfaC*-), outer membrane transporter for ferrichrome (Δ*fhuA*-), vitamin B12 (Δ*btuB*-), maltose (Δ*lamB*-), and outer membrane porin OmpC (Δ*ompC*-) were constructed and tested for susceptibility to the phages. Phage CR8 could infect all of the mutants except the *flgK* deletion mutant, and phage S13 except the *rfaC* deletion mutant. The failure of infection was overcome by complementation of the gene (Fig. 4), indicating that flagella and LPS may play an important role in the infection of CR8 and S13, respectively. This expectation was confirmed by phage adsorption assay. As shown in Fig. 5, each phage couldn't adsorb to the receptor deletion mutant and thereby remained as free phage in supernatant.

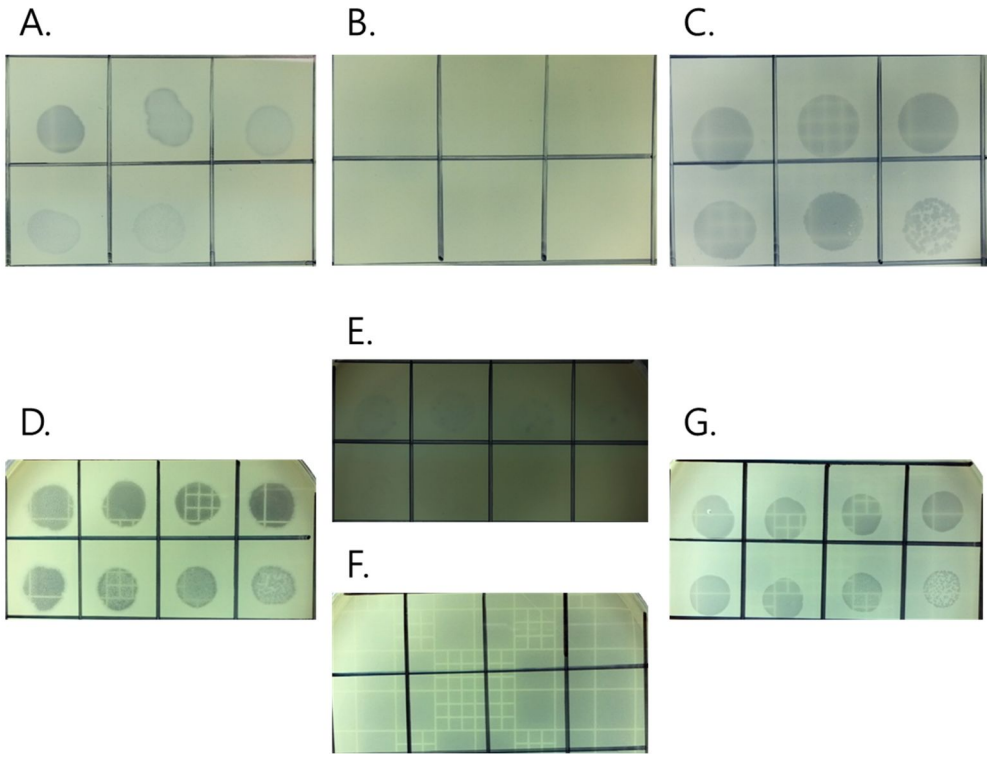

Figure 4. Flagella and LPS are receptor for CR3 and S13, respectively. The sensitivity of the bacteria to CR3 (A-C) and S13 (D-F) phages was determined by spotting assay. Wild type, deletion mutant, complemented mutant from the left turn.

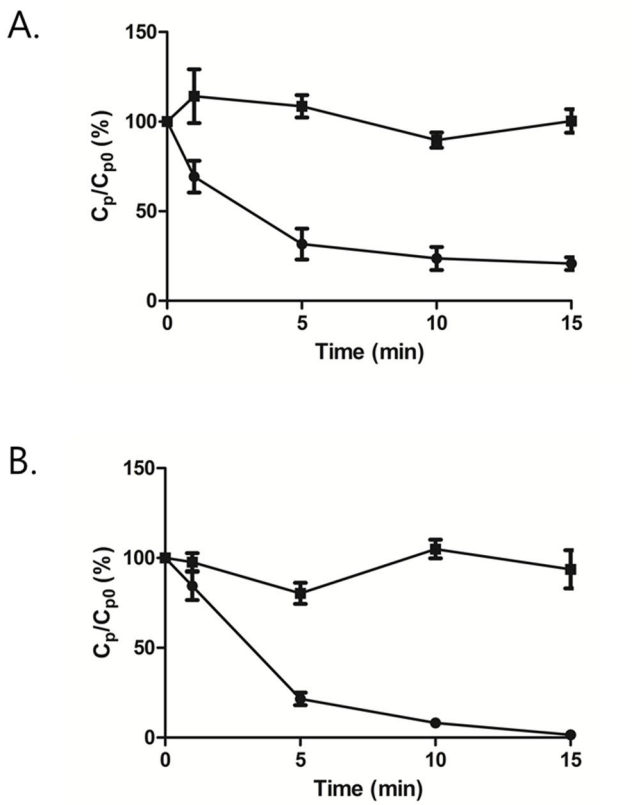

Figure 5. Adsorption kinetics of CR8 (A) and S13 (B) against *C. sakazakii* ATCC 29544 (circle) and receptor defective mutant (square). Each phage was added to host culture in exponential phase (MOI, 0.01). Y-axis indicates a percentage of PFU in supernatant to initial PFU. The data are shown as means and standard error of the mean of triplicate assays

3.1.4. Host range of phages

Four *C. sakazakii* strains and some of the other genuses were tested for sensitivity to phages CR8 and S13. These *C. sakazakii* –specific phages didn't form plaque on a lawn of the other genuses regardless of gram positive or negative bacteria. In particular, CR8 showed strict host specificity within the *C. sakazakii* species (Table 3).

Table 3. Host range of *C. sakazakii* bacteriophage CR8 and S13.

Bacterial strains	Plaque formation[a] of CR8	Plaque formation[a] of S13	Source[b]
Cronobacter sakazakii ATCC 29544	C	C	ATCC
Cronobacter sakazakii ATCC BAA-894	-	C	ATCC
Cronobacter sakazakii ES15	-	C	(J. H. Lee et al., 2012)
Cronobacter muytjensii ATCC 51329	C	C	ATCC
Salmonella Typhimurium ATCC 19586	-	-	ATCC
Escherichia coli K-12 MG1655	-	-	(Hayashi et al., 2006)
Listeria monocytogenes ATCC 19114	-	-	ATCC
Bacillus cereus ATCC 14579	-	-	ATCC
Cronobacter sakazakii ATCC 29544 Δ*flgK*	-	C	This study
Cronobacter sakazakii ATCC 29544 Δ*flgK* pBAD18::*flgK*	C	C	This study
Cronobacter sakazakii ATCC 29544 Δ*rfaC*	C	-	This study
Cronobacter sakazakii ATCC 29544 Δ*rfaC* pBAD18::*rfaC*	C	C	This study

[a], C, formation of clear plaque; -, not susceptible to phage.
[b], ATCC, American Type Culture Collection.

3.1.5. One-step growth curve

The replicative characteristics such as eclipse and latent periods, and burst size of the phages were analyzed. Two phages have certainly different growth pattern as shown in Fig. 6. Contrary to normal pattern of S13, CR8 showed unique growth curve with very short eclipse period (5 min) and ambiguous burst, with the burst size of 56 plaque forming unit (PFU) per infected cell and continuous increase pattern. The eclipse and latent periods of S13 were 15 min and 25 min, respectively, and the burst size was about 62 PFU per infected cell.

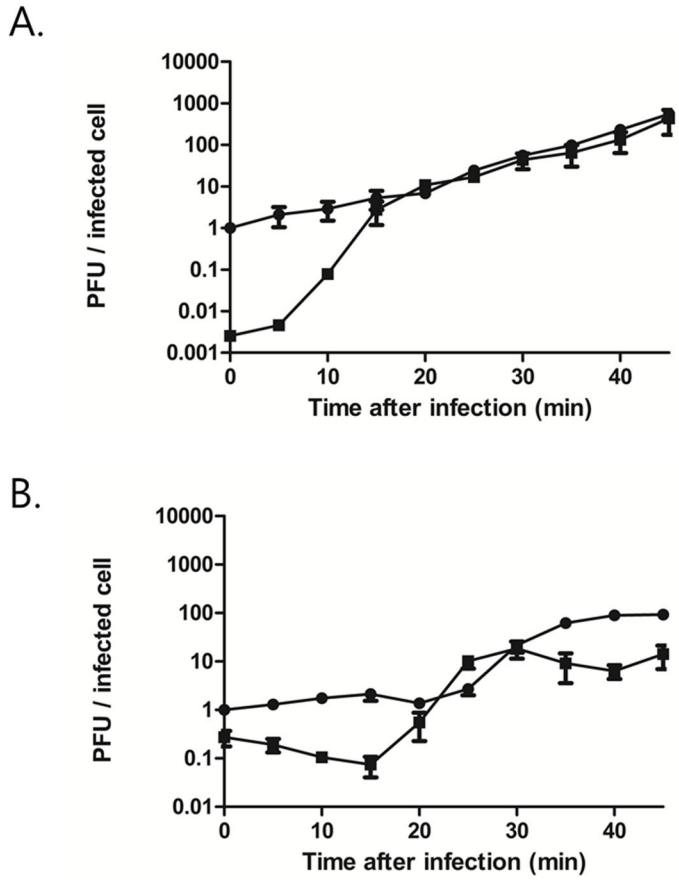

Figure 6. One-step growth curve of CR8 (A) and S13 (B). Each phages were added to host culture at a MOI of 0.001 and adsorbed to the host cell for 5 min. Samples were collected every 5 min and PFU were counted directly (circle), or after chloroform treatment (square) by overlay assay.

3.1.6. Synergistic effect of phage cocktail

The absorbance of the culture containing *C. sakazakii* ATCC 29544 and phage was measured to determine how effective the phage could be in inhibiting the growth of host. CR8 and S13 showed similar ability to lyse host bacteria with rapid early cell lysis, and the phage resistant appeared 5 hours after infection. On the other hand, phage cocktail consisting of CR8 and S13 had synergistic effect in growth inhibition, so emergence of the resistant mutant was delayed until 8 hours after infection (Fig. 7A). Also, phage cocktail can achieve 4-log reduction of host bacteria whereas single phage maximum 2-log reduction (Fig. 7B).

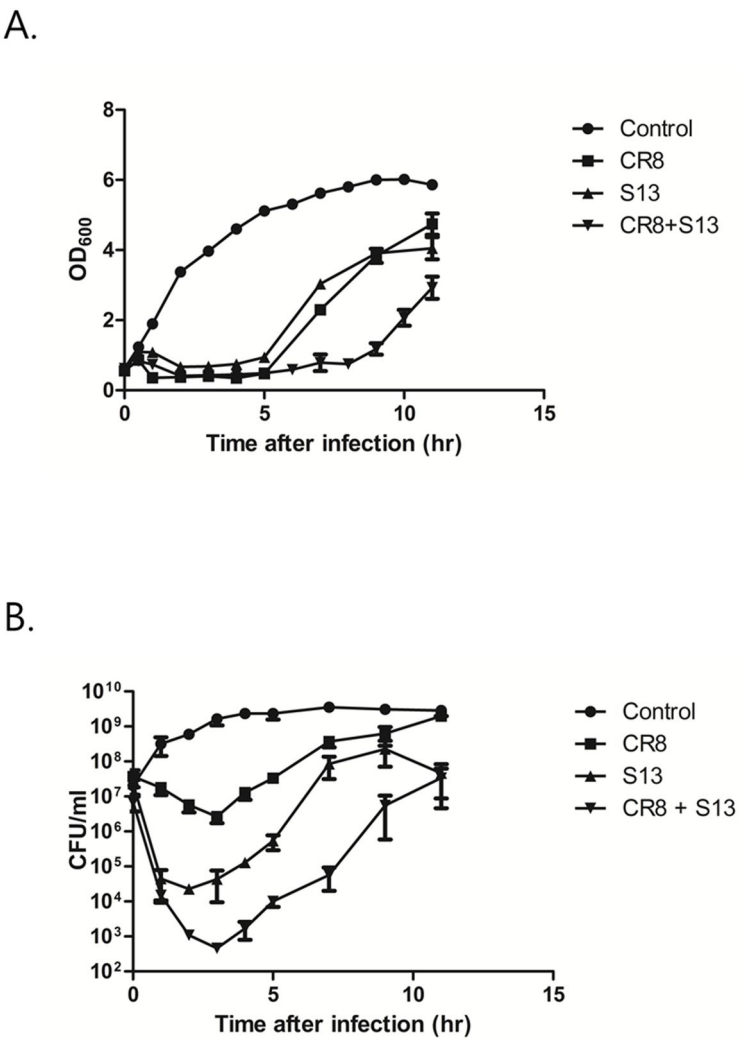

Figure 7. Growth inhibition assay of phages against *C. sakazakii* ATCC 29544. Each phages were added at a MOI of 1 to the bacterial culture after 1.5 h incubation. Bacterial growth was monitored by absorbance (A) or viable cell count (B).

3.1.7. Spontaneous mutagenesis of *C. sakazakii* against to phage cocktail

Spontaneous mutant which is resistant to both phage CR8 and S13, named CSR, was isolated from the high-titer phage cocktail overlay plate and the resistance was maintained throughout the sequential subculture on fresh TSA plates. To determine whether the resistance is caused by lysogen, resistant mutant was treated with mitomycin C, and there is no inducible prophage. And several phenotypic changes in resistant mutant were observed.

At first, CSR strain showed decreased motility as same as flagella defective (Δ*flgK*) mutant (Fig. 8A), Also it was observed that CSR cells quickly precipitated in comparison with wild-type cells when left in broth at room temperature. It was reported that these autoaggregation ability is related to the cleavage of LPS of *C. sakazakii* (Rahman, Kim, Kumura, & Shimazaki, 2008; Wang, Hu, Tao, & Wang, 2012), so LPS from wild-type and CSR strains were extracted and analyzed by DOC-PAGE. It was found that some parts of inner core and o-antigen regions of LPS were disappeared in CSR strain (Fig. 8B).

Also, it was expected that invasive ability of CSR strain is reduced because they has no motility and are defective in LPS which is crucial to the virulence of many gram-negative bacteria. Gentamicin protection assay demonstrated that CSR could invade mammalian Caco-2 cells one-tenth as

much as wild-type strain invaded, as expected (Fig. 8D).

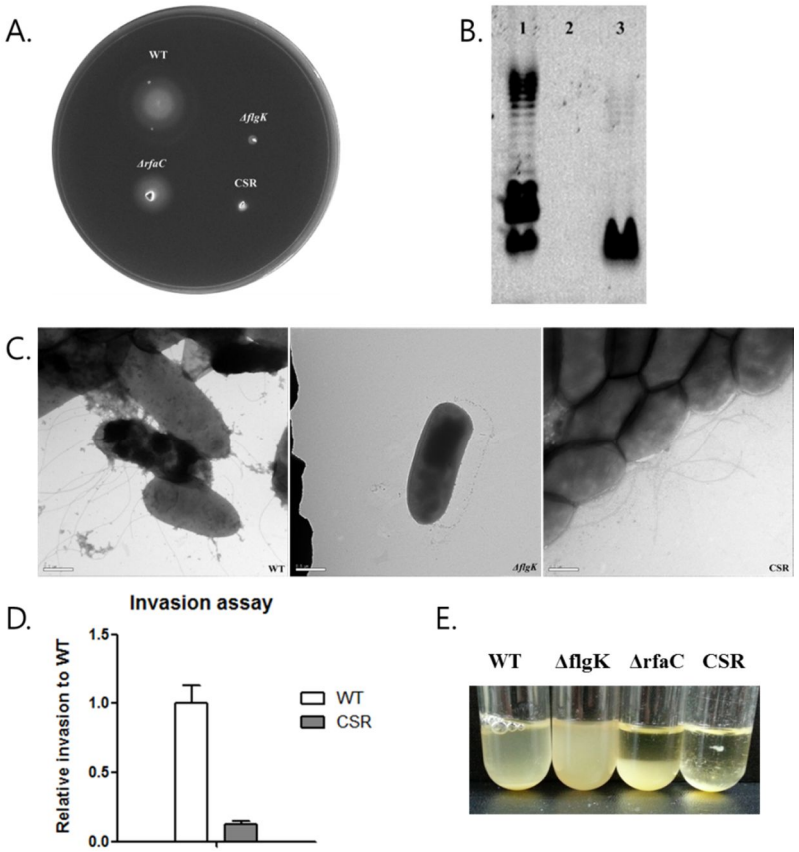

Figure 8. Characterization of resistant strain to both CR8 and S13.

(A) Motility assay, (B) DOC-PAGE of LPS. Lane 1, WT; lane 2, Δ*rfaC*; lane 3, CSR. (C) TEM analysis. Scale bars, 500 nm. (D) Invasive ability into human epithelial cells, Caco-2. (E) Autoaggregation ability.

CHAPTER 2.

3.2.1. Isolation and morphology analysis of bacteriophages

The aim of this chapter is isolation of phages utilizing different receptor from above studied phages to cope with the resistant mutant of host bacteria. Several phages which can infect LPS deletion (Δ*rfaC*) mutant were isolated and further tested if they can infect flagella deletion (Δ*flgK*) mutant or not. Finally, two phages were picked up and named YR1 and YR2, for each. After the propagation step, the resulting stocks (10^{10} to 10^{11} pfu/ml) were diluted with SM buffer and stored at refrigeration temperature for further study.

The morphology of the phages was observed via transmission electron microscopy. Both phages are members of the *Siphoviridae* family, with long and noncontractile tails (Fig. 9). Also YR1 and YR2 have smaller isometric head than the phages CR8 and S13, with the mean diameter of 53.7±3.0 nm and 67.5±2.5 nm, respectively. Tail length of YR2 (229.2±17.6 nm) is twice as long as that of YR1 (108.0±5.7 nm).

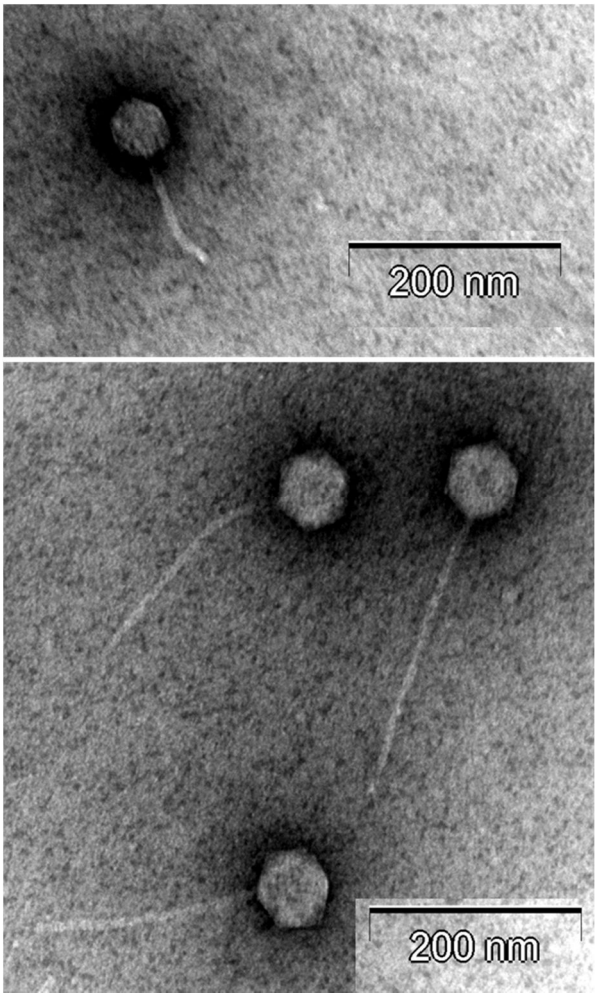

Figure 9. Morphology of phages. Transmission electron microscopy images of YR1 (above), YR2 (below) negatively stained with 0.2% uranyl acetate. Scale bar, 200 nm.

3.2.2. Genome sequencing and bioinformatics analysis

The genomes of phage YR1 and YR2 are smaller than those of phage CR8 and phage S13, with 51,815-bp and 108,528-bp length, respectively. The G+C composition of YR1 genome is 48.61% and is similar to that of YR2, 50.53%. It is predicted that total 95 ORFs are present in the genome of YR1, where total 134 ORFs including one tRNA for asparagine in YR2.

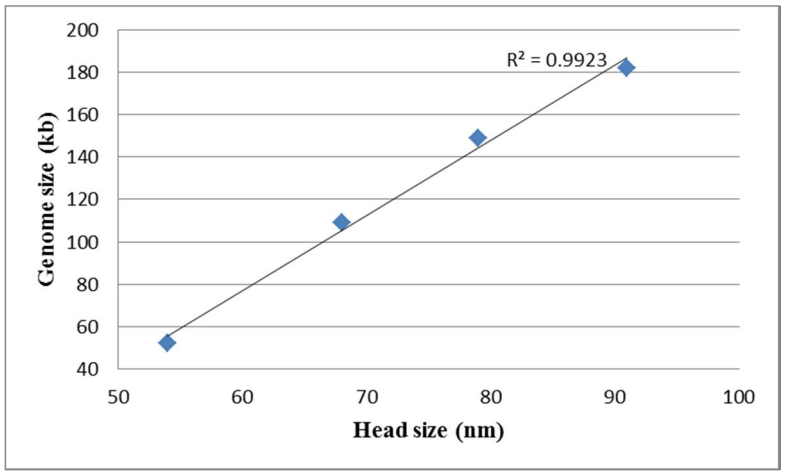

Figure 10. Correaltaion between head and genome size of phages. All data in this graph were rounded off below the decimal points. Head size of S13 is average value of major and minor axes.

3.2.3. Identification of phage receptor and host range of phages

Among the candidate mutants except flagella and LPS defective mutants, Δ*ompC* and Δ*fhuA* can evade the infection of YR1 and YR2, respectively. When the genes were complemented by plasmid vectors infectivity of YR2 was fully restored, whereas that of YR1 was partially restored as shown in Figure 11.

Adsorption of phages to each mutant strain was decreased compared with wild type (Fig. 12), supporting that outer membrane porin C is receptor for phage YR1 and outer membrane transporter for ferrichrome is for phage YR2. Also Phage YR2 showed the fastest adsorption than other phages in this study, with that over 90% of the phage adsorb to wild type strain within 5 minutes.

As expected, phage YR1 and YR2 could infect CSR strain. Also interestingly, phage YR2 has broad host range within gram negative bacteria, whereas phage YR1 exhibited specificity to *Cronobacter sakazakii* (Table 4). *Shigella flexneri* 2a strain 2457T and *Escherichia coli* MG1655 were infected by YR2 with clear plaques, while turbid plaque was formed on the lawn of *Salmonella* Typhimurium ATCC 19586.

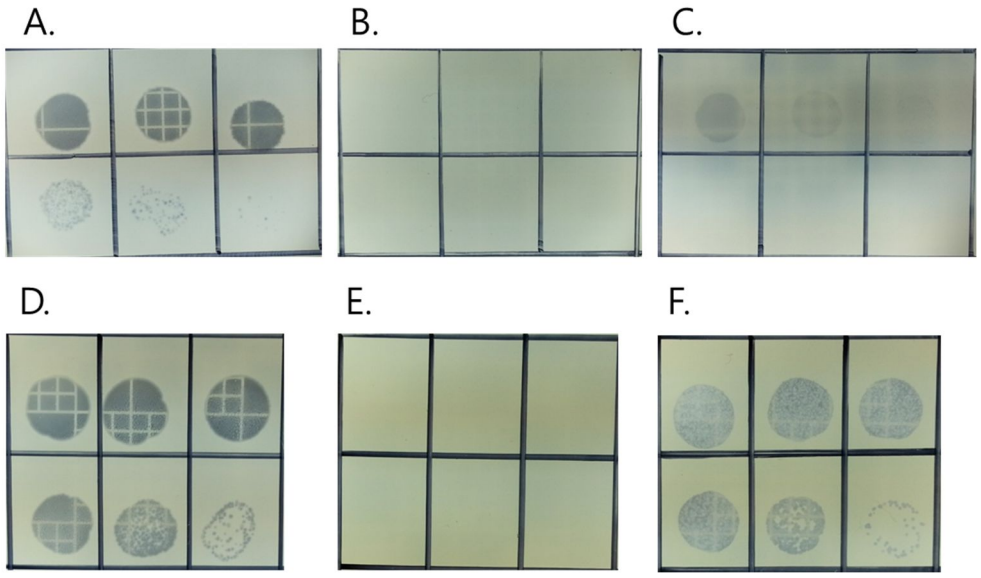

Figure 11. Outer membrane porin C and transporter for ferrichrome are receptor for phage YR1 and YR2, respectively. The sensitivity of the bacteria to YR1 (A-C) and YR2 (D-F) phages was determined by spotting assay. Wild type, deletion mutant, complemented mutant from the left turn.

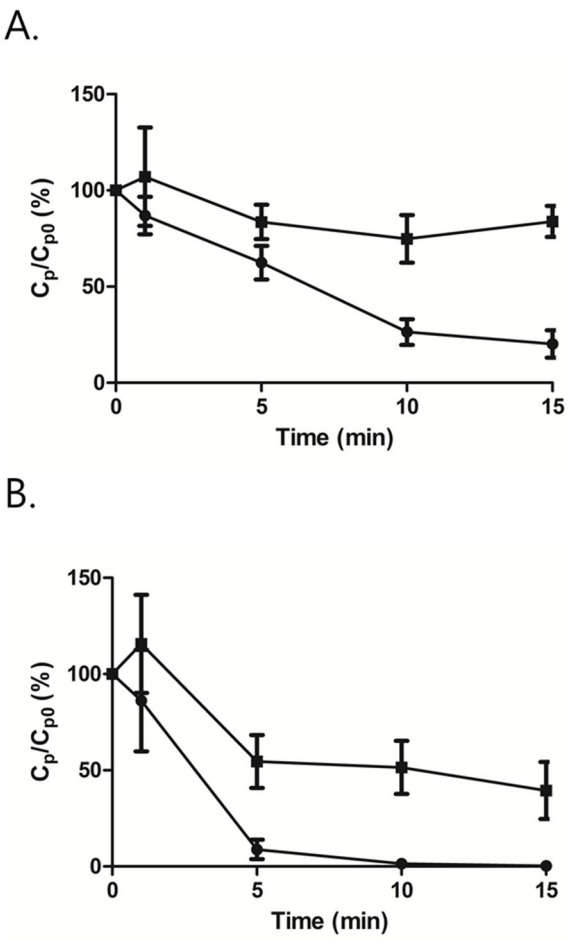

Figure 12. Adsorption kinetics of YR1 (A) and YR2 (B) against *C. sakazakii* ATCC 29544 (circle) and receptor defective mutant (square). Each phage was added to host culture in exponential phase (MOI, 0.01). Y-axis indicates a percentage of PFU in supernatant to initial PFU. The data are shown as means and standard error of the mean of triplicate assays

Table 4. Host range of *C. sakazakii* bacteriophage YR1 and YR2.

Bacterial strains	Plaque formation[a] of YR1	Plaque formation[a] of YR2	Source[b]
Cronobacter sakazakii ATCC 29544	C	C	ATCC
Cronobacter sakazakii ATCC BAA-894	-	-	ATCC
Escherichia coli K-12 MG1655	-	C	(Hayashi et al., 2006)
Shigella flexneri 2a strain 2457T	-	C	(Wei et al., 2003)
Salmonella Typhimurium ATCC 19586	-	I	ATCC
Vibrio fischeri ES-114 ATCC 700601	-	-	ATCC
Listeria monocytogenes ATCC 19114	-	-	ATCC
Bacillus cereus ATCC 14579	-	-	ATCC
Cronobacter sakazakii ATCC 29544 Δ*ompC*	-	C	This study
Cronobacter sakazakii ATCC 29544 Δ*ompC* pBAD18::*ompC*	C	C	This study
Cronobacter sakazakii ATCC 29544 Δ*fhuA*	C	-	This study
Cronobacter sakazakii ATCC 29544 Δ*fhuA* pBAD18::*fhuA*	C	C	This study
Cronobacter sakazakii ATCC 29544 CSR	C	C	This study

[a], C, formation of clear plaque; I, formation of inhibition zone; -, not susceptible to phage.
[b], ATCC, American Type Culture Collection.

3.2.4. Bacterial growth inhibition of phages

The same procedure as the former chapter was conducted to test to figure out the effectiveness of newly isolated phages in host growth inhibition. Phage YR1 showed similar pattern to that of former phages, CR8 and S13, with rapid lysis for 5 hours after infection (Fig. 14A). In the meantime, interesting phenomenon was discovered during the growth inhibition assay of phage YR2. When the cations such as Ca^{2+} and Mg^{2+} were not added to the culture, phage YR2 couldn't infect the host bacteria; resultingly, OD_{600} was not decreased. Also only when the overall concentration of the cations was 1 mM or more, lysis of bacteria was observed (Fig. 13). Therefore, adequate amounts of cations were put into following experiment associated with phage YR2. YR2 maintained the inhibition effect for 8 hours, the longest duration among the phages in this study.

Phage cocktail was also constructed to contain newly isolated phages, and tested for its effectivity. In addition to delaying the advent of phage-resistant mutant, the new cocktails showed faster and more durable lysis ability than that of the existing cocktail constructed in chapter 1 (Fig. 14B).

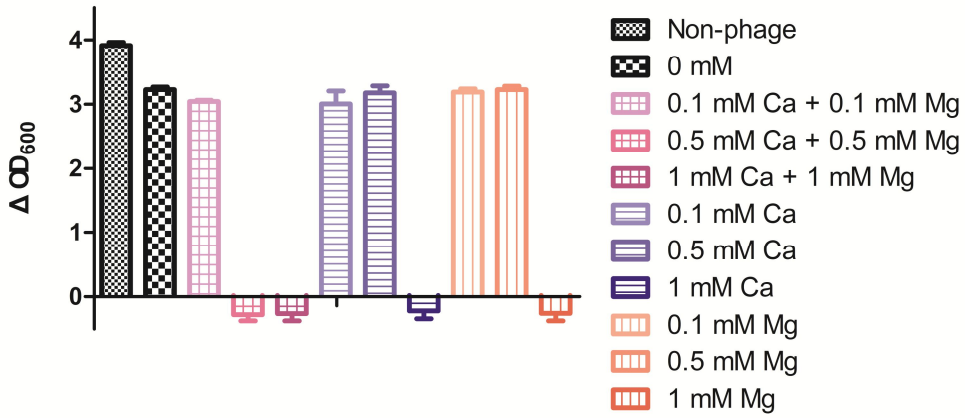

Figure 13. Phage YR2 requires more than 1 mM cations for successful infection. Phage was added to host culture (3 ml) in exponential phase (MOI, 1) and incubated 5 hours. Y-axis indicates an increment in OD_{600} value over 5 hours after the infection.

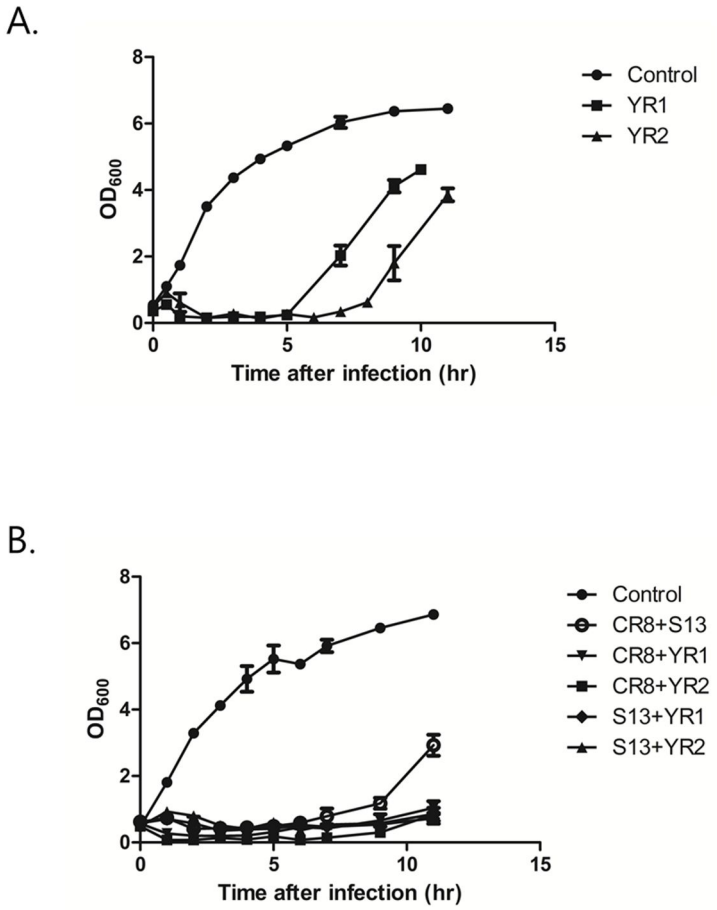

Figure 14. Growth inhibition assay of newly isolated phages against *C. sakazakii* ATCC 29544. Each phages were added at a MOI of 1 to the bacterial culture after 1.5 h incubation. Growth inhibition effect of phage cocktail (B) was more lasted than single phage (A).

Ⅳ. DISCUSSION

Cronobacter sakazakii has been detected in various foods and environmental sources (Kandhai, Reij, Gorris, Guillaume-Gentil, & van Schothorst, 2004), but it is difficult to sterilize the pathogen without affecting the quality of foods (Dumen, 2010). When the foods were stored improperly, the pathogen can propagate to be enough to cause the disease in immunocompromised human. Outbreaks of disease associated with *C. sakazakii* have been occurred in fits and starts, and the victims are mainly infants, suggesting that new measure to protect infants from the threat of the pathogen is needed. In the food safety aspect, bacteriophages have been studied and positively evaluated with their specificity (Garcia et al., 2008). The specificity of phage is related to their adsorption on bacterial cell surface, especially receptors (Rakhuba et al., 2010). In the present study, novel phages which utilize different receptor each other were isolated and characterized to construct effective phage cocktail.

It was revealed that phage CR8 and phage S13 belong to the family *Myoviridae*, and interestingly, S13 has not isometric head, but slightly elongated (Fig. 1). This prolate head is unusual because isometric heads are in a majority (85%) of the tailed phages (Calendar & Abedon, 2005). After

the genome sequencing and analysis, genome size of phage was found to be in proportion to its head size (Fig. 10), as previously reported (Pariza & Iandolo, 1974). Phage CR8 has more hypothetical proteins than phage S13, suggesting this phage is unique among the reported phages to date. Also there are a little of genetic information of *C. sakazakii* phages, so further study about that is needed.

Among the several sequential stages of phage infection, adsorption is first and crucial phase to define the specificity of phage. Phages recognize the receptors on bacterial cell surface and adsorb to that. In case of gram-negative bacteria, some constituents of the outer membrane were identified as receptor for phage, such as outer membrane proteins and lipopolysaccharides. In addition, many phages utilize bacterial flagella and pili as receptor (Rakhuba et al., 2010). In this study, flagella are used as receptor for CR8 and that LPS are for S13. Especially, the mutant lacking O-antigen ligase (*rfaL*) reduced S13 efficiency of plating (EOP) by 4 logs (Fig. 4E) whereas S13 couldn't infect the mutant defective in LPS inner core (*rfaC* gene). To put it concretely, therefore, S13 would adsorb to core of LPS. Host range analysis revealed that S13 can infect *C. muytjensii* strain, as well as *C. sakazakii* type strains (Table 3), and that is unexpected result because LPS-specific phage usually has narrow host range because of variability of O-

antigen structure (Rakhuba et al., 2010). This finding supports the inference that S13 utilize not O-antigen, but core region of LPS as receptor. At the same time, phage S13 can infect OmpC deletion mutant whereas phage T4 requires both LPS and OmpC in *E. coli* K-12 (Yu & Mizushima, 1982).

CR8 showed peculiar growth curve without clear cut replication cycle (Fig. 6A). Synthesis of the virion in the host cell seemed to fast because eclipse period is short, but release of the descendant phage is retarded.

This is first report demonstrating that these different characteristics between two phages, especially different receptor, can allow the phage cocktail to have synergistic effect. When the phages were added into host culture and bacterial growth was monitored, inhibition effect of phage cocktail was more lasted for several hours in both absorbance and cell viability aspects than each single phage (Fig. 7). If MOI was higher, that is, lower host concentration and higher phage concentration, it would resulted complete eliminate viable cells, as previously reported (K.-P. Kim et al., 2007). The effectivity of phage cocktail has been studied in the multifarious ways not only *in vitro*, but *in vivo* (Gu et al., 2012; Maura et al., 2012; Tanji et al., 2005; Zuber et al., 2008). Whereas in many studies phages belonging various family were used for cocktail, two myoviruses were used in the

present study. So when considering addition of new phage to the cocktail, like above studies, a phage belonging to the family *siphoviridae* or *podoviridae* while exploiting another receptor such as outer membrane protein will be proper.

Bacteria can evolve to gain resistance to phage through a range of mechanisms (Labrie, Samson, & Moineau, 2010), and loss or modification of receptor to prevent phage adsorption is notable one of those (Filippov et al., 2011). So it was expected that CSR has no flagella and LPS, and this expectation was supported by the loss of motility (Fig. 8A) and the autoaggregation (Fig. 8E). However, TEM analysis demonstrated that CSR still has flagella like wild-type strain (Fig. 8C). It is possible that resistance came from not strategy to prevent phage adsorption, but other phage replication step after adsorption. On the other hands, rotation of flagella in addition to the presence of flagella would determine whether the bacterium is infected by CR8 or not. Narrow host range of CR8 can be demonstrated in the latter case. In the meantime, DOC-PAGE analysis of LPS revealed that CSR has defect in LPS, both O-antigen and core region (Fig. 8B) Incomplete deletion of O-antigen suggest that LPS core domain of *C. sakazakii* contains branched oligosaccharide and that CSR was affected in some branch, as reported in *E. coli* K1 strain (Jiménez et al., 2012). But identification of gene

mutated in CSR was failed, and full genome sequencing will be helpful to achieve that, if necessary.

The virulence or mechanisms of *C. sakazakii* has been investigated in many parts of the world, and there are some study about enterotoxin-like compounds (Pagotto et al., 2003; Raghav & Aggarwal, 2007) and OmpA protein (K. Kim et al., 2010; Yan et al., 2012). And endotoxin and motility are commonly known as virulence factor in gram-negative bacteria (Ho Sui, Fedynak, Hsiao, Langille, & Brinkman, 2009), but these are not essential to bacterial life which is endangered by phage. The ultimate goal of bacteria is survival and maximum transmission (Mekalanos, 1992), so they would abolish the receptor for phage to protect itself against attack of phage. Consequently, attenuated bacteria would be conquered by human immune system and pushed out. In this study, CSR showed drastically decreased invasive ability into human epithelial cell (Fig. 8D). So this result indicates that the emergence of phage resistant pointed out as phage therapy's shortcoming can be overcome.

Also, emergence of spontaneous phage-resistant mutant can be dealt with new phage which can infect that bacteria (A. Sulakvelidze, Alavidze, & Morris, 2001). Unlike first isolation procedure of phage, Δ*rfaC* strain was used for isolation of new phage, because the pore proteins of the outer

membrane, prime candidates for new phage, might be shielded by LPS (van der Ley, de Graaff, & Tommassen, 1986). Then after second selection using Δ*flgK* strain, phages YR1 and YR2 were finally isolated. Fortunately, they are siphovirus expected to be suitable constituent for improved phage cocktail.

It seems that the cation-dependent activity of phage YR2 is due to the receptor for phage, FhuA (Fig. 13). This characteristic was discovered by chance because of disparity in composition of media; tryptic soy broth contains 0.22~0.24 mM calcium and tryptic soy agar 1.27~1.63 mM (Sasaki, Shintani, Itoh, Kamogawa, & Kajihara, 2000). It was reported that phage T1 and T5 whose receptor are FhuA, also requires 0.1 mM calcium to infect *E. coli* cells successfully (Bonhivers & Letellier, 1995; Puck, 1949).

Newly isolated phage YR1 and YR2 can infect CSR strain, and phage cocktail containing one of them showed better inhibition effect than combination of phage CR8 and S13 (Fig. 14). Therefore, various characteristics of phages should be considered such as family, receptor, host range, and lytic activity to construct the best phage cocktail.

In conclusion, four different phages were isolated from environmental samples and their receptors were identified. All of phage cocktails with various combinations of phages targeting different host

receptors have synergistic host growth inhibition effect, and emergence of phage-resistant mutants was consequently quite delayed. These results could be the basis for the development of phage treatment to control *C. sakazakii*.

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국문 초록

박테리오파지는 숙주 특이성을 갖는 바이러스로서, 식품 안전성 측면에서 새로운 항생제 대체제로 긍정적인 평가를 받고 있다. 크로노박터 사카자키는 분유 산업에서 악명 높은 식중독 원인균으로 이에 감염된 유아는 괴사성 장염으로 55%에 이르는 사망률을 보인다. 본 연구에서는 크로노박터 사카자키를 감염시키는 박테리오파지들을 분리하여 수용체를 확인하였고, 이에 따라 서로 다른 수용체를 이용하는 박테리오파지 두 가지를 섞는 파지 칵테일을 구축함으로써 파지 요법의 효율을 증대시키고자 하였다. 편모를 수용체로 하는 파지인 CR8과 LPS를 수용체로 하는 파지 S13은 수축성 꼬리를 갖는 미오비리대 과에 속한다는 공통점이 있지만 유전체, 복제 주기, 활성 등에서는 서로 다른 특성을 보였으며, 이 두 가지의 파지를 섞은 칵테일은 각각의 파지를 단독으로 처리했을 때에 비해 시너지 효과를 보였다. 이 파지 칵테일에 저항성을 갖게 된 돌연변이 균주 CSR에 대한 표현형 분석을 통해 파지 수용체에 생긴 결함으로부터 그 저항성이 나타났다는 것을 알 수 있었다. 이러한 운동성의 결여와 LPS 구조의 결함은 균주의 병원성을 저하시켜 CSR 균주가 사람의 상피세포인 Caco-2에 정상적으로 침투할 수 없음을 확인하였다. 또한 LPS와 편모를 수용체로 이용하지 않는 새로운 파지 YR1, YR2는 비수축성 꼬리

의 시포바이러스이며, 기대했던 바와 같이 CSR 균주를 감염시킬 수 있었다. 이들 파지들을 조합한 파지 칵테일은 사카자키 균을 제어하는 데 위의 칵테일보다 더 좋은 효과를 보였다. 이러한 결과들은 수용체를 기반으로 한 파지 칵테일의 실제적 적용에 대한 가능성을 제시함과 동시에 가장 좋은 파지 칵테일 조합을 위해서는 각 파지의 분류, 수용체, 숙주 범위, 용해능 등과 같은 다양한 특성들이 고려되어야 함을 시사한다.

주요어 : 크로노박터 사카자키, 박테리오파지, 파지 칵테일, 파지 수용체, 파지 저항성

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