J. Microbiol. Biotechnol. (2019), 29(5), 696–703
https://doi.org/10.4014/jmb.1812.12054

jmb

Characterization and Genomic Analysis of Novel Bacteriophage ΦCS01 Targeting *Cronobacter sakazakii*

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Received: December 26, 2018
Revised: April 8, 2019
Accepted: April 9, 2019

First published online
April 12, 2019

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pISSN 1017-7825, eISSN 1738-8872

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Cronobacter sakazakii is an opportunistic pathogen causing serious infections in neonates. In this study, a bacteriophage ΦCS01, which infects *C. sakazakii*, was isolated from swine feces and its morphology, growth parameters, and genomic analysis were investigated. Transmission electron microscopy revealed that ΦCS01 has a spherical head and is 65.74 nm in diameter with a 98.75 nm contracted tail, suggesting that it belongs to the family *Myoviridae*. The major viral proteins are approximately 71 kDa and 64 kDa in size. The latent period of ΦCS01 was shown to be 60 min, and the burst size was 90.7 pfu (plaque-forming units)/infected cell. Bacteriophage ΦCS01 was stable at 4–60°C for 1 h and lost infectivity after 1 h of heating at 70°C. Infectivity remained unaffected at pH 4–9 for 2 h, while the bacteriophage was inactivated at pH <3 or >10. The double-stranded ΦCS01 DNA genome consists of 48,195 base pairs, with 75 predicted open reading frames. Phylogenetic analysis is closely related to that of the previously reported *C. sakazakii* phage ESP2949-1. The newly isolated ΦCS01 shows infectivity in the host bacterium *C. sakazakii*, indicating that it may be a promising alternative to antibacterial agents for the removal of *C. sakazakii* from powdered infant formulas.

Keywords: Bacteriophages, *Cronobacter sakazakii*, *Myoviridae*, genomic analysis, food safety

Introduction

Cronobacter species, formerly known as *Enterobacter sakazakii* [1] were first isolated in 1980 [2] and are gram-negative, rod-shaped opportunistic pathogens of the family *Enterobacteriacea* [3]. These species are ubiquitous and have been isolated from food and environments, including water, soil, processed foods, and fresh produce [4].

Cronobacter sakazakii is associated with necrotizing enterocolitis and meningitis and is a contaminant in powdered infant formula (PIF) [5]. Contaminated PIF is a major cause of neonatal infection [6], and pathogenic bacterial contamination of PIF can be caused by improper product storage during the manufacture process [7]. Neonates and infants have weak immunity and *C. sakazakii* infection can cause more serious problems than in adults [8]. Therefore, pathogen-specific microbial control measures are required.

At present, antibiotics are widely used to prevent infection by pathogenic *C. sakazakii* [9]. Nevertheless, the emergence of antibiotic-resistant bacteria has caused serious problems for both biotechnological and medical applications [10]. In addition, *C. sakazakii* demonstrated antibiotic resistance in antibiotic susceptibility tests [11]. Therefore, a safe biocontrol agent that can control *C. sakazakii* and replace antibiotics should be developed.

In 2006, the US Food and Drug Administration (FDA) approved the use of purified bacteriophages as food additives [11]. In a previous study, the complete genomic sequence of a bacteriophage infecting *C. sakazakii* was reported, and phage genome analysis revealed no toxicity-related genes encoding toxic agents and toxins [11-18]. The use of bacteriophages as biological agents is a potential alternative to the use of antibiotics [19]. A bacteriophage is a virus that uses bacterial cells as hosts and selectively infects target bacteria to destroy bacterial cells [19]. This lytic activity makes bacteriophages a promising substitute for antibiotics [20].

In this study, ΦCS01, a bacteriophage that infects the pathogenic bacterium *C. sakazakii* was isolated from swine

feces. Morphological and growth characteristics of the phage were investigated, and genetic characteristics were assessed using next-generation sequencing. The identified phage has potential applications as an alternative to antibiotics for use as a biological control agent in the food industry.

Materials and Methods

Host Bacterial Strains and Growth Conditions

The host bacterial strain used in this study, *Cronobacter sakazakii* ATCC 29544, was provided by the American Type Culture Collection (ATCC). Brain heart infusion (BHI, Becton Dickinson, USA) broth and agar were used to culture the strain. Standard double-layer agar technique [21] was employed to generate a single plaque and calculate phage concentration.

Isolation and Propagation of the Phage

Bacteriophages were isolated from swine feces obtained from a pig farm located in Gangwon Province, Republic of Korea. Each sample was diluted 1:10 (w/v) in SM buffer (50 mmol/l Tris-HCl [pH 7.5], 0.1 mol/l NaCl, and 8 mmol/l $MgSO_4{\cdot}7H_2O$). The suspension was centrifuged at 3,000 ×*g* for 20 min. The supernatant was filtered using a 0.45 μm membrane filter (Dismic; Advantec, Japan). One hundred microliters of filtered supernatant were mixed with 100 μl of exponential phase *C. sakazakii* (optical density at 600 nm [OD_{600}] = 0.4) and 5 ml of top agar (0.7% agar in BHI broth). The mixture was poured into a petri dish containing 1.5% BHI agar and incubated at 37°C overnight. An individual clear plaque was picked from the overlaid agar with a sterile pipette tip, resuspended in 500 μl of SM buffer, and double-layer agar technique was repeated [22].

High-Titer Preparation of the Phage

Host single colonies were resuspended in 100 ml of BHI and incubated at 37°C with shaking at 160 rpm. When the culture reached OD_{600} = 0.4, it was centrifuged at 4,000 ×*g* for 10 min. Pelleted cells were collected and resuspended in 3 ml of SM buffer. Phages were added to the suspension at MOI of 1.0 and mixed rapidly. After incubation for 20 min at 37°C with intermittent shaking, the suspension was added to 500 ml of BHI and incubated overnight. Chloroform was added to a concentration of 2% (10 ml), and the culture was further incubated at 37°C with shaking for 30 min. Next, the culture was centrifuged at 11,000 ×*g* for 10 min at 4°C, and the supernatant was filtered using a syringe filter with a 0.45 μm pore size (Advantec). Solid polyethylene glycol (PEG 8000) was added to the suspension to a final concentration of 10% (w/v), and the mixture was cooled on ice for 2 h. The lysate was centrifuged at 110,000 ×*g* for 100 min, and the supernatant was discarded. The pellet was resuspended in 8 ml of SM buffer, and the same volume of chloroform was added. The mixture was vortexed for 30 s and then centrifuged at 3,000 ×*g* for 15 min at 4°C. The supernatant containing the bacteriophage particles was recovered and filtered using a syringe filter with 0.45 μm pore size. Phages were purified using the standard bacteriophage purification method [23].

Transmission Electron Microscopy (TEM)

Morphology was observed under a transmission electron microscope, JEOL JEM-2100F FE-TEM (KBSI, Korea), at 200 kV. A high-titer phage solution containing 10^8 plaque-forming units (pfu) was negatively stained with 2% (w/v) uranyl acetate. The phage particles were placed on a carbon coating grid and dipped into distilled water containing a drop of 2% uranyl acetate. The 200-mesh grids (Gatan, USA) were coated with a collodion film prepared from 2% collodion in amyl acetate and used to absorb carbon film fragments with phage particles. After air drying for 10 min, grids were subjected to TEM. Images of negatively stained phage particles were taken using a one-view camera (Gatan) at 100,000× and 150,000× magnification.

SDS-PAGE Analysis

For SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), 40 μl of a phage solution containing 10^8 pfu was added to 10 μl of 5× sample buffer (312.5 mmol/l Tris-HCl [pH 6.8], 50% glycerol, 5% SDS, 2% β-mercaptoethanol, and 0.05% bromophenol blue; Elpis Biotech, Korea). The mixture was heated at 95°C for 5 min, and 20 μl of the mixture was subjected to electrophoresis at 20–40 mV in a 15% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue G250 (Bio-Rad Laboratories, USA) for protein visualization [24].

One-Step Growth Curve Analysis

For one-step growth curve analysis, 5 ml of *C. sakazakii* culture in the exponential phase (OD_{600} = 0.4) was centrifuged at 4,000 ×*g* for 10 min. The pellet was resuspended in 1 ml of BHI broth. The phage solution was added to the suspension at a MOI of 0.1. The mixture was incubated at 37°C for 30 min and then centrifuged at 11,000 ×*g* for 10 min. The pellet was resuspended in 10 ml of BHI broth. The phage concentration in the solution was measured at 10 min intervals for 100 min using the double-layer agar technique [25].

Temperature and pH Stability of Phage Infectivity

One milliliter of phage suspension containing 10^7 pfu was incubated at 4, 10, 20, 30, 37, 50, 60, or 70°C for 1 h. The phage concentration was then measured using the double-layer agar technique. To assay the stability at various pH levels, the pH of the BHI broth was adjusted to pH 2–12. One hundred microliters of the phage suspension with a titer of 10^9 pfu was inoculated into 900 μl of the pH-adjusted media to obtain a final concentration of 10^8 pfu. After incubation at 37°C for 2 h, phage concentration was determined using the same double-layer agar technique. The experiment was conducted three times. The results are reported as the mean of three observations ± standard deviation [12].

Whole-Genome Sequencing

Phage DNA was purified using the Phage DNA Isolation Kit (Norgen Biotek, Canada) and subjected to next-generation sequencing using a HiSeq 4000 instrument (Illumina, Korea, Macrogen). A DNA fragment library was constructed with the TruSeq DNA PCR Free Library Preparation Kit (Illumina). A *de novo* assembly was generated in the SOAPdenovo software and MEGA 6 was used for ORF prediction for the whole genome. The genome sequence was compared with other genomes in GenBank via the BLAST program (http://blast.ncbi.nlm.nih.gov/) and Mauve analysis software (http://darlinglab.org). The whole-genome sequence of ΦCS01 was deposited in the NCBI database (GenBank accession number MH845412).

Phylogenetic Tree Construction

The evolutionary history of the bacteriophage was inferred using the neighbor-joining method, on the basis of the major capsid protein sequences of the isolated phage and 18 additional phages infecting *Cronobacter*. The percentage of replicate trees in which associated taxa clustered together in 500 bootstrap repetitions is shown next to the branches. Codon positions included were the first, second, and third positions and noncoding sites. All positions containing gaps and missing data were eliminated. There were 764 positions in the final dataset. Evolutionary analyses were conducted using MEGA 6 software (http://www.megasoftware.net).

Host-Range Test

To determine the host range of ΦCS01, plaque assays were performed. Each of the 12 different hosts were inoculated into 5 ml of soft agar (0.7%) and poured onto agar plates. Ten microliters of phage-titer solution were spotted onto the top agar plate and incubated at 37°C for overnight. After incubation, the appearance of lysis zones was examined. The lysis activity of the phage was classified as clear (+) and no reaction (-). For host-range testing, *Enterobacter* and other strains were obtained from KCTC (Korean Collection for Type Cultures) or ATCC (American Type Culture Collection) [12].

Results and Discussion

Morphological Analysis

Purified ΦCS01 was examined by transmission electron microscopy (TEM) (Fig. 1). The diameter of the quasi-spherical head with icosahedral symmetry was 65.74 nm, and the length of the rigid tail was 98.75 nm. In addition, ΦCS01 was observed to have a non-contracted tail and contracted tail, and with a sheath-like structure on the tail. The tail fibers are difficult to observe due to the resolution of TEM, but ORF annotation results indicate the existence of tail fibers (Table 1).

Another *Cronobacter sakazakii* bacteriophage, PBES02,

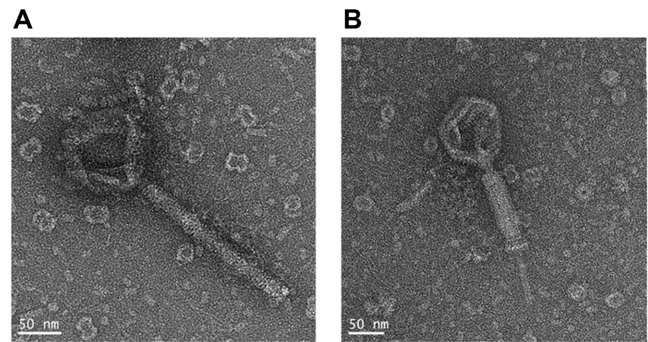

Fig. 1. Transmission electron micrographs (TEM) showing the morphology of ΦCS01.
Scale bars in the lower left corners represent 50 nm. (**A**) Non-contracted tail. (**B**) Contracted tail.

which belongs to the *Myoviridae* family, has a spherical head of 90 nm and a rigid tail of 130 nm [12]. Compared with PBES02, the head diameter of ΦCS01 is 24.26 nm smaller and the tail is 31.25 nm shorter, but the ratio of head to tail is similar for both phages. Based on its morphological characteristics, ΦCS01 was determined to also belong to the *Myoviridae* family of the order *Caudovirales*.

Characterization of ΦCS01

Purified ΦCS01 was stained with Coomassie blue and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). The results suggest that the bacteriophage has two major proteins and four minor proteins; and the 71 kDa protein is the most highly expressed. The 71 kDa protein is associated with ORF 71, at nucleotide positions 43,231 to 45,099, and putatively consists of 622 amino acid residues. The theoretical molecular weight of the ORF 71 product is 68.5 kDa, which is close to the 71 kDa major protein band observed by SDS-PAGE.

The one-step growth curve of ΦCS01 revealed that the latent period is 60 min, and that it takes 80 min after infection to complete the burst, with a burst size of 90.7 pfu (plaque-forming units)/infected cell (Fig. 2B). Compared with *C. sakazakii* phage PBES02, the latency period of ΦCS01 is 30 min longer, the time to complete the burst is 5 min longer, and the burst size is 159.3 pfu smaller [12].

To test the stability of ΦCS01 when exposed to various environmental conditions, phage stability was assessed at a range of temperatures and pH levels (Figs. 2C and 2D). When incubated at 4–37°C for 1 h, ΦCS01 was stable, with decreased infectivity at 50−60°C. In contrast, the samples lost infectivity after incubation for 1 h at 70°C. Therefore, a temperature range of 4−37°C was found to be the optimal

Table 1. Annotation of ORFs found in ΦCS01 genome.

ORF No.	Encoded phage protein	Function
CS01_01	Tail fiber protein [Cronobacter phage ESP2949-1]	Structure
CS01_02	Tail assembly protein [Cronobacter phage ESP2949-1]	Structure
CS01_03	Tail assembly protein [Salmonella phage phSE-5]	Structure
CS01_04	Minor tail protein [Cronobacter phage ESP2949-1]	Structure
CS01_05	Minor tail protein [Cronobacter phage ESP2949-1]	Structure
CS01_06	Tail tape-measure protein [Cronobacter phage ESP2949-1]	Structure
CS01_07	TfmS [Salmonella phage FSL SP-126]	Additional function
CS01_09	Major tail protein [Cronobacter phage ESP2949-1]	Structure
CS01_18	Major head subunit precursor [Cronobacter phage ESP2949-1]	Structure
CS01_19	Phage head morphogenesis protein [Cronobacter phage ESP2949-1]	Structure
CS01_20	Portal protein [Cronobacter phage ESP2949-1]	DNA packaging
CS01_21	Terminase large subunit [Cronobacter phage ESP2949-1]	DNA packaging
CS01_22	Terminase small subunit [Cronobacter phage ESP2949-1]	DNA packaging
CS01_34	ATP-binding protein [Cronobacter phage ESP2949-1]	Replication and regulation
CS01_43	Polynucleotide kinase [Cronobacter phage ESP2949-1]	Replication and regulation
CS01_49	gp76 [Escherichia phage Tls]	Additional function
CS01_53	Site-specific DNA methylase [Cronobacter phage ESP2949-1]	Replication and regulation
CS01_58	RzlA [Cronobacter phage ESP2949-1]	Additional function
CS01_59	Endolysin [Cronobacter phage ESP2949-1]	Host lysis
CS01_65	Dam methylase [Cronobacter phage ESP2949-1]	Replication and regulation
CS01_68	ATP-dependent helicase [Cronobacter phage ESP2949-1]	Replication and regulation
CS01_69	Transcriptional regulator [Cronobacter phage ESP2949-1]	Replication and regulation
CS01_70	DNA primase [Cronobacter phage ESP2949-1]	Replication and regulation
CS01_71	Tail fiber [Cronobacter phage ESP2949-1]	Structure
CS01_72	Single-stranded DNA binding protein [Cronobacter phage ESP2949-1]	Replication and regulation
CS01_73	RecT [Cronobacter phage ESP2949-1]	Additional function
CS01_75	Exodeoxyribonuclease [Cronobacter phage ESP2949-1]	Replication and regulation

condition for ΦCS01 storage. ΦCS01 would be a suitable antimicrobial agent for this industrial application because finished PIF products are not exposed to temperatures over 70°C after production or during storage at room temperature [12].

In the pH stability test, infectivity persisted after exposure to pH 4–11 for more than 1 h. On the other hand, when exposed to pH 1–3 or pH > 11, infectivity was lost. In general, phages are stable at a pH range of 5 to 9. Usually, no viable phage particles are detected after incubation at pH 11.8–14 or pH < 2 [26].

Genome Analysis

The double-stranded (ds)DNA ΦCS01 genome consists of 48,195 bases, with a G+C content of 50.11% (GenBank Accession No. MH845412) (Fig. 3A). Open reading frame (ORF) analysis revealed that this genome contains 75 genes (Table 1). Twenty-seven ORFs were annotated, including nine genes associated with replication and regulation, ten genes related to structural proteins, three genes associated with DNA packaging, one gene related to host lysis, and four genes associated with other functions. Analysis of 75 ORFs did not reveal allergenic or toxin-related proteins.

A Basic Local Alignment Search Tool (BLAST) search of GenBank revealed the most similar phage was ESP2949-1 (accession number JF912400.1) [27] and indicated that the two phages have 98% identity from 97% coverage of nucleotide sequence. The two phage genomes were compared by Mauve analysis (Fig. 3B).

The whole dsDNA genome of phage ESP2949-1 contains 49,116 bases and 43 ORFs. The genome of ΦCS01 is 921 base pairs shorter but contains 32 more ORFs than that of

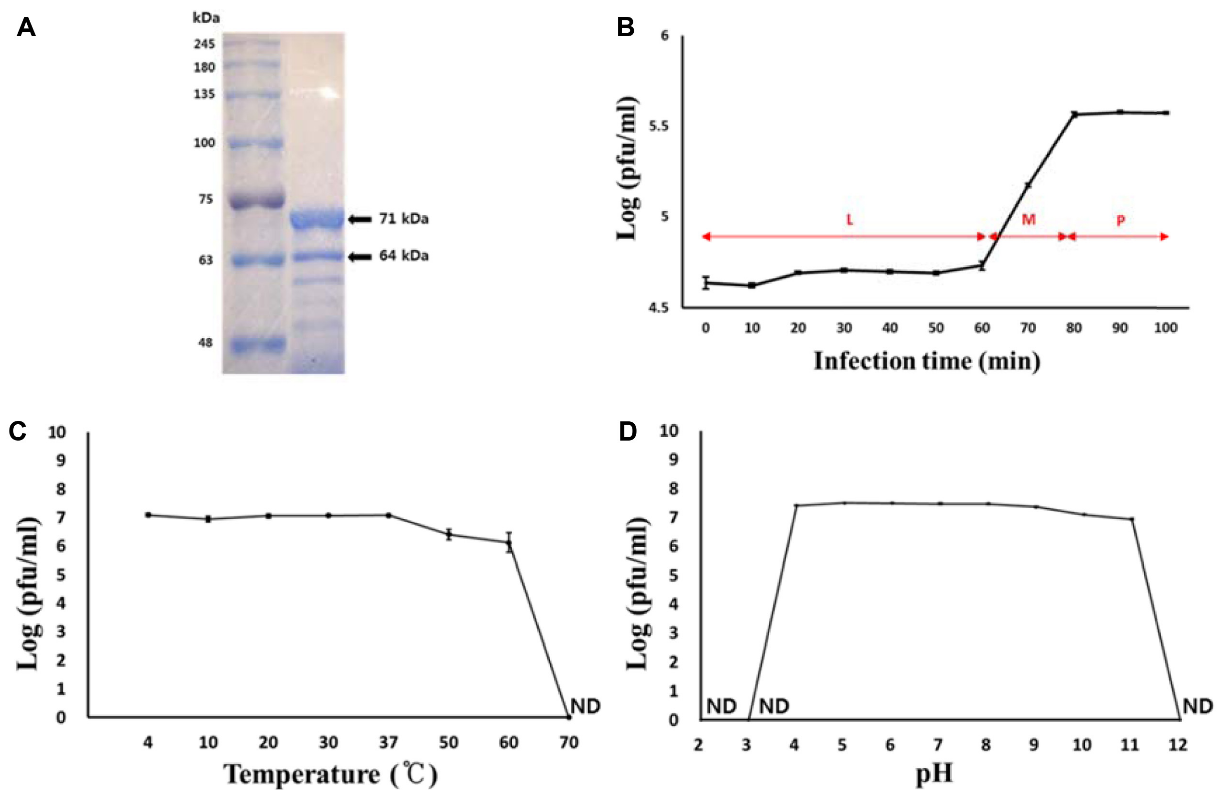

Fig. 2. Molecular characterization of ΦCS01.

(**A**) SDS-PAGE analysis of major ΦCS01 proteins. (**B**) One-step growth of ΦCS01 at 0.1 MOI, 37°C (L: latent phase, M: maturation phase, P: plateau phase. (**C**) Stability of ΦCS01 after exposure to various temperatures, and (**D**) stability of ΦCS01 after exposure to various pH levels. ND: not detected.

ESP2949-1. Because ESP2949-1 has not yet been biologically characterized, comparisons other than those based on genome analysis are currently impossible. The genome lengths, similarities, and the number of ORFs suggest that ESP2949-1 and ΦCS01 phages may have a common ancestor.

Phylogenetic Analysis

Phylogenetic analysis involving genomic DNA sequences (registered in GenBank) of 18 genes encoding major capsid proteins of *Cronobacter* phages revealed 100% agreement with the capsid protein of *C. sakazakii* phage ESP2949-1 (Fig. 4). The phylogenetic tree was built with 500 bootstrap repetitions in MEGA 6. This finding suggests that the two phages are evolutionarily closely related and indicates that the ΦCS01 phage may have an infection mechanism similar to that of ESP2949-1.

Host-Range Test

The host specificity of ΦCS01 was examined with 12 different species (Table 2). ΦCS01 infected only *C. sakazakii*.

Table 2. Host-range test for phage CS01.

Bacteria	Strain No.[a]	Susceptibility[b]
Cronobacter sakazakii (formerly Enterobacter sakazakii)	ATCC 29544	+
Enterobacter sp.	ATCC 21754	-
Enterobacter asburiae	ATCC 35956	-
Enterobacter pyrinus	KCTC 2590	-
Enterobacter aerogenes	KCTC 2190	-
Enterobacter cloacae	ATCC 13047	-
Bacillus subtilis	ATCC 9372	-
Bacillus cereus	ATCC 14579	-
Lactobacillus plantarum	ATCC 14917	-
Staphylococcus aureus	ATCC 25923	-
Escherichia coli	ATCC 25922	-
Shigella flexneri	KCTC 2998	-

[a]ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures.

[b]Susceptibility was determined by measuring plaque formation.

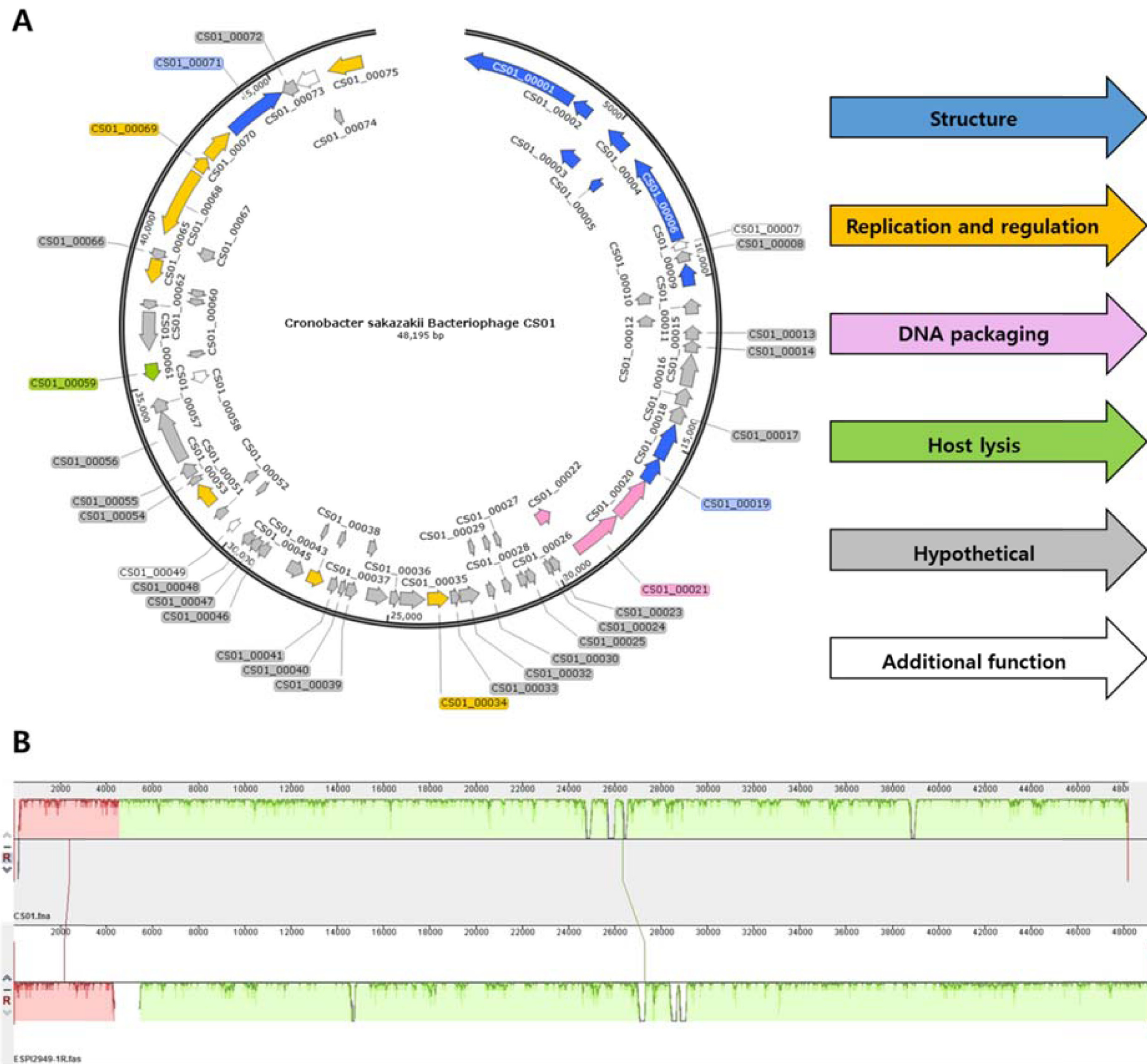

Fig. 3. Schematic representation of the whole ΦCS01 dsDNA genome.

(**A**) Putative ORFs are represented by arrows, with predicted functions when available. Proposed modules are based on predicted functions. Blue: structural protein; yellow: replication and regulation; pink: DNA packaging; green: host lysis; gray: hypothetical protein; white: additional function. (**B**) Mauve analysis of genomic DNA from ΦCS01 (upper) and ESP2949 (lower).

In contrast, ΦCS01 is an *Enterobacter* sp. (KCTC 2625), *Enterobacter asburiae* (KCTC 23920), *Enterobacter pyrinus* (KCTC 2590), *Enterobacter aerogenes* (ATCC 13048), *Enterobacter cloacae* (KCTC 2361), *Bacillus subtilis* (ATCC 9372), *Bacillus cereus* (ATCC 14579), *Lactobacillus plantarum* (ATCC 14917), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Shigella flexneri* (KCTC 2998) were not infected. These results suggest that the ΦCS01 has the ability to infect *C. sakazakii* from *Enterobacter* species, and is an expected biological control agent suitable for *C. sakazakii*.

Use of bacteriophages as biocontrol agents is a promising method for controlling pathogenic bacteria including antibiotic-resistant bacteria. Bacteriophages are applicable as safe bactericides for the elimination of pathogens. In this study, the stability of the bacteriophage ΦCS01 was evaluated at various temperatures and pH levels, and one-step growth behavior was assessed. Molecular and genetic characteristics of ΦCS01 were assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and genome analysis. Our results indicate that ΦCS01 has high potential for application as a biocontrol agent against food borne pathogens.

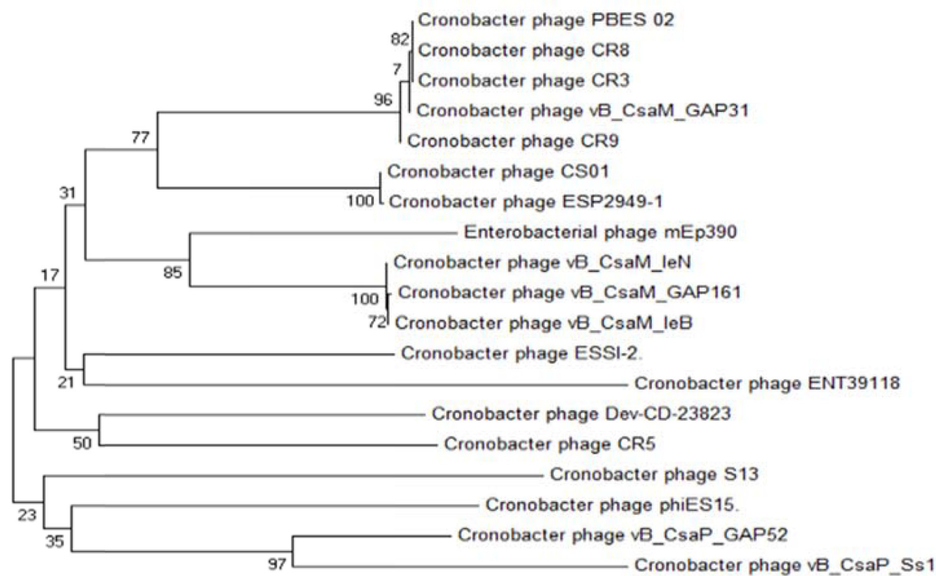

Fig. 4. Phylogenetic tree based on the major capsid protein sequences from 18 different phages that infect *Cronobacter*.

In this study, a bacteriophage capable of infecting *C. sakazakii*, ΦCS01, was isolated and characterized. Our findings suggest that ΦCS01 could be used as an alternative to antibiotics for use against antibiotic-resistant bacteria, and our stability test results suggest that ΦCS01 is suitable for industrial application in PIFs for controlling *C. sakazakii* contamination. Our results highlight the stability of ΦCS01 and its potential for applications to promote food safety in the food and PIF industries.

Acknowledgments

This study was funded by a grant of the National Research Foundation of Korea (2015R1D1A1A01058374).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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