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A Thesis for the Degree of Doctor of Philosophy

**Molecular and Genomic Study of Bacteriophages
Targeting *Salmonella enterica* serovar Typhimurium
and *Bacillus cereus***

Salmonella enterica serovar Typhimurium과

*Bacillus cereus*에 특이적인

박테리오파지에 대한 분자적/유전체적 연구

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Abstract

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Salmonella enterica subspecies *enterica* serovar Typhimurium is a Gram-negative pathogen causing salmonellosis. *Salmonella* Typhimurium-targeting bacteriophages have been proposed as alternative biocontrol agents to antibiotics. To further understand infection and interaction mechanisms between the host strains and the bacteriophages, the receptor diversity of these phages needs to be elucidated. Twenty-five *Salmonella* phages were isolated and their receptors were identified by screening several mutant strains of *S. Typhimurium* SL1344. Among them, only three types of receptors were identified: flagella, vitamin B₁₂ uptake outer membrane protein, BtuB and lipopolysaccharide-related O-antigen. TEM observation revealed that the phages using flagella (group F) or BtuB (group B) as a receptor belong to *Siphoviridae* family, and the phages using O-antigen of LPS as a receptor (group L) belong to *Podoviridae* family. Interestingly, while some of group F phages (F-I) target FliC host receptor, others (F-II) target both FliC and FljB receptors, suggesting that two subgroups are

present in group F phages. Cross-resistance assay of group B and L revealed that group L phages could not infect group B phage-resistant strains and reversely group B phages could not infect group L SPN9TCW-resistant strain. In addition, the host receptors of group B or group L SPN9TCW phages hinder other group phage infection, probably due to interaction between receptors of their groups. This study provides novel insights into phage-host receptor interaction for *Salmonella* phages and will inform development of optimal phage therapy for protection against *Salmonella*.

To understand phage infection and host lysis mechanisms with pathogenic *Salmonella*, a novel *Salmonella* Typhimurium-targeting bacteriophage SPN9CC, belonging to the *Podoviridae* family, was isolated and characterized. The phage infects *S. Typhimurium* via the O-antigen of lipopolysaccharide (LPS) and forms unique clear plaques with cloudy centers due to lysogen formation. Phylogenetic analysis of phage major capsid proteins (MCPs) revealed that this phage is a member of lysogen-forming P22-like phage group. However, comparative genomic analysis of SPN9CC with P22-like phages indicated that their lysogeny control regions and host lysis gene clusters share very low identities, suggesting that lysogen formation and host lysis mechanisms may be diverse among phages in this group. Analysis of the expression of SPN9CC host cell lysis genes encoding holin, endolysin, and Rz/Rz1-like proteins individually or in combinations in *S. Typhimurium* and *E. coli* hosts revealed that

collaboration of these lysis proteins is important for both host lysis, and holin is a key protein. To further investigate the role of the lysogeny control region in phage SPN9CC, a *ΔcI* mutant (SPN9CCM) of phage SPN9CC was constructed. The mutant does not produce a cloudy center in the plaques, suggesting that this mutant phage is virulent and no longer temperate. Subsequent comparative one-step growth analysis and challenge assays revealed that SPN9CCM has shorter eclipse/latent periods and a larger burst size as well as higher host lysis activity than SPN9CC. The present work indicates the possibility of engineering temperate phages as promising biocontrol agents similar to virulent phages.

The *Bacillus cereus* group phages infecting *B. cereus*, *B. anthracis*, and *B. thurigiensis* (Bt) have been studied at a molecular level and recently at a genomic level to control pathogens of *B. cereus* and *B. anthracis* and to prevent phage contamination of the natural insect pesticide Bt. A comparative phylogenetic analyses revealed three different phage groups with different morphologies (*Myoviridae* for group I, *Siphoviridae* for group II, and *Tectiviridae* for group III), genome size (group I > group II > group III), and lifestyle (virulent for group I and temperate for group II and III). A subsequent phage genome comparison using a dot plot analysis showed that phages in each group are highly homologous, substantiating the grouping of *B. cereus* phages. Endolysin is a host lysis protein that contains two conserved domains such as a cell wall binding domain (CBD) and an

enzymatic activity domain (EAD). In *B. cereus sensu lato* phage group I, four different endolysin groups were detected, according to combinations of two types of CBD and four types of EAD. Whereas group I phages share two copies of tail lysins and one copy of endolysin, the functions of the tail lysins are still unknown. In the *B. cereus sensu lato* phage group II, the *B. anthracis* phages have been studied and applied for typing and rapid detection of the pathogenic host strains. In the *B. cereus sensu lato* phage group III, the *B. thuringiensis* phages, such as Bam35 and GIL01, were studied to understand phage entry and lytic switch regulation mechanisms. In this study, I suggest that further study of the *B. cereus* group phages would be useful for various phage applications, such as biocontrol, typing, and rapid detection of the pathogens *B. cereus* and *B. anthracis* and for the prevention of phage contamination of the natural insect pesticide Bt.

Key words : Bacteriophage, *Salmonella* Typhimuirum, *Bacillus cereus*, phage-host interaction, phage genomics

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Chapter I. General Introduction

I-1. Bacteriophage

I-1-1. Introduction

In 1915 and 1917, Twort and d'Herelle independently discovered a microorganism that lyse and kill bacteria (25, 27, 75). d'Herelle described these microorganisms as “ultraviruses” and termed them as “bacteriophage” (25), which is derived from a Greek word meaning to devour/swallow bacteria. Bacteriophages are described as bacterial viruses of prokaryotes. They invade specific bacterial hosts, replicate using the host's DNA replication and protein biosynthesis systems, and lyse the hosts for propagation (48). In addition, they can selectively infect host bacteria without affecting other bacteria in the same habitat (14, 48). Bacteriophages are ubiquitous in the world. They are found in every part of the biosphere where their hosts live such as the oceans, soil, water, and even in food. They are the most abundant microorganism and their numbers in nature have been estimated to be over 10^{30} particles (77). It comes as surprise that research on the biology and diversity of phages is sparse and based on a few model phages.

I-1-2. Taxonomy of bacteriophage

Bacteriophages are classified by the International Committee on Taxonomy of Viruses (ICTV) according to phage morphology and nucleic acid type (dsDNA, ssDNA, dsRNA, and ssRNA) (Table 1.1) (44).

Approximately 96% of all bacteriophages belong to the order *Caudovirales*, and a minority belongs to the order *Ligamenvirales*. Some phages that have been classified into families (*Tectiviridae*, *Inoviridae*, *Cystoviridae*, *Leviviridae*, and so on) are yet to be classified into orders (53). Within the order *Caudovirales*, those bacteriophages with tails can be subtyped into three different families; *Myoviridae* with a contractile tail consisting of a sheath and a central tube, *Siphoviridae* with a long non-contractile tail, and *Podoviridae* with a short and non-contractile tail (44). In this order, phage lambda, T4, T5, and T7 are best-well studied. The order *Ligamenvirales* consists of two sub-families; *Lipothrrixviridae*, lipoprotein enveloped rod shaped phages, and *Rudiviridae*, non-enveloped straight and rigid rod shaped phages. Phages in this order are specific to *Archaea* only. Lastly, those that are not assigned to an order are classified according to their morphologies such as cubic symmetry (*Microviridae*, *Corticoviridae*, *Tectiviridae*, *Leviviridae*, *Cystoviridae*), helical symmetry (*Inoviridae*), and pleomorphic morphologies (*Plasmaviridae*, *Fuselloviridae*, *Guttaviridae*).

Table 1.1. Taxonomy of bacteriophages

Order	Nucleic acid^a	Family	Morphology	Examples
<i>Caudovirales</i>	dsDNA (linear)	<i>Myoviridae</i>	Nonenveloped, contractile tail	T4
	dsDNA (linear)	<i>Siphoviridae</i>	Nonenveloped, non-contractile tail (long)	Lambda, T5
	dsDNA (linear)	<i>Podoviridae</i>	Nonenveloped, non-contractile tail (short)	T7
<i>Ligamenvirales</i>	dsDNA (linear)	<i>Lipothrixviridae</i>	Enveloped, rod-shaped, lipids	TTV1
	dsDNA (linear)	<i>Rudiviridae</i>	Nonenveloped, rod-shaped	SIRV-1
Unassigned	dsDNA (linear)	<i>Ampullaviridae</i>	Enveloped, bottle-shaped	ABV
	dsDNA (circular)	<i>Bicaudaviridae</i>	Nonenveloped, lemon-shaped	ATV
	dsDNA (circular)	<i>Clavaviridae</i>	Nonenveloped, rod-shaped	APBV1
	dsDNA (circular, supercoiled)	<i>Corticoviridae</i>	Nonenveloped, double capsid, lipids	PM2
	dsRNA (linear, multipartite)	<i>Cystoviridae</i>	Enveloped, spherical, lipids	Phi6
	dsDNA (circular, supercoiled)	<i>Fuselloviridae</i>	Nonenveloped, lemon-shaped, lipids	SSV1

Table 1.1. Taxonomy of bacteriophages (continued)

Order	Nucleic acid^a	Family	Morphology	Examples
Unassigned	dsDNA (linear)	<i>Globuloviridae</i>	Enveloped, isometric	PSV
	dsDNA (circular, supercoiled)	<i>Guttaviridae</i>	Nonenveloped, droplet-shaped, ovoid	SNDV
	ssDNA (circular)	<i>Inoviridae</i>	Nonenveloped, long filamentous	fd, MV-L51
	ssRNA (linear)	<i>Leviviridae</i>	Nonenveloped, isometric	MS2
	ssDNA (circular)	<i>Microviridae</i>	Nonenveloped, conspicuous capsomers	phiX174
	dsDNA (circular, supercoiled)	<i>Plasmaviridae</i>	Enveloped, no capsid, pleomorphic, lipids	L2
	dsDNA (linear)	<i>Tectiviridae</i>	Nonenveloped, double capsid, pseudo-tail, lipids	PRD1, Bam35

^a, dsDNA, double stranded DNA; ssDNA, single stranded DNA; dsRNA, double stranded RNA; ssRNA, single stranded RNA.

I-1-3. Early history

In 1915, the first report on bacteriophages and the beginnings of the phage study was made by Frederick W. Twort (75). His study of phages started from an observation of glassy micrococci colonies that he found could be transferred to other fresh colonies by inoculation with a bit of substance from the glassy colony. By microscopy, he observed that the bacterium was lysed and formed small granules. And in 1917, Felix d'Herelle independently reported a microorganism that has lysis activity in broth culture as well as on the surface of agar plates overlaid with bacteria (25). Further, Herelle found that the titer of phage in patients with infectious disease rose during the recovery progress, and hence, was the first to establish the concept of phage therapy for the control of infectious diseases. Later, he reported the use of phages for control of the avian typhosis (gastrointestinal disease) and bovine hemorrhagic septicemia (23).

The first therapeutic use of bacteriophage was reported by Bruynoghe and Maisin in 1921 (12). They prepared staphylococcal phage that could be used for the reduction in swelling, pain and even fever. In parallel, d'Herelle worked to expand the use of phage treatment to human in the 1920s (24). In the view of current standard for human trials, these phage treatments for human are rough and

rudimentary in scientific and ethical respects. However, d'Herelle's approaches in phage application were highly acclaimed for, and he was accredited as a pioneer in the field of phage research.

I-1-4. General overview of the infection process

The lifecycle of a bacteriophage has two major parts; the lysogenic cycle for phage genome integration into the host chromosome, and the lytic cycle for lysis of the bacterial host due to bactericidal activity (14, 48, 53). While virulent phages have only the lytic cycle, temperate phages have both lytic and lysogenic cycles. The general infection process of tailed phages consists of host recognition/adsorption, DNA transfer, DNA replication, morphogenesis/DNA packaging, and host lysis. The initial interaction between the phage's receptor binding proteins (such as tail fiber or tail spike) and the host's surface molecules (host receptors) is specific allowing phages to discriminate their hosts. In Gram-negative bacteria, various lipopolysaccharide (LPS) components and outer membrane proteins were reported as host receptors for phage infection. On the other hand, in Gram-positive bacteria, peptidoglycan elements, embedded teichoic acids and lipoteichoic acids, and associated proteins act as host receptors. Then, the DNA transfer step requires correct positioning of the phage tail on the host cell surface to penetrate and transfer phage DNA into the host. After DNA ejection into the host, phage DNA replication and head/tail morphogenesis is conducted by exploiting the host's transcription/translation system or phage's own system. Subsequently, phage DNA molecules are packaged into the prohead

structure and progeny phage particles are constructed by combining the head and tail components. Lastly, host cell is lysed by the lysis system containing holin and endolysin. Holin makes holes in the cytoplasmic membrane. This hole is used as a transport channel for endolysin, which digests the peptidoglycan layer in the membrane. In addition, Rz/Rz1-like proteins often help endolysin activity as accessory proteins.

Some phages integrate their nucleic acid into the host genome or exist as episomes such as a linear plasmid. In the lysogenic cycle, in which the host cells are not lysed or phage progenies are not produced within host. Furthermore, some of these temperate phages can alter the phenotype of the host bacteria such as the chemical properties of the LPS. They can also be harbored in the host bacteria for several generations as prophages. In general, the induction of these prophages is triggered when the host bacterium is under harmful conditions.

I-1-5. Bacteriophage applications as biocontrol/therapeutic agents

Host lysis activity and host specificity have made bacteriophages research targets for various applications such as the removal of selective host strains. In the early history of phage application, d'Herelle used phages to treat several infectious diseases. One of the ways he evaluated the safety of phage therapy was to inject his family and colleagues with the phages. However, with the development of antibiotics that were able to effectively kill bacteria, further research on phage and phage therapy were essentially stopped. Recently, as a result of increasing outbreaks of food poisoning and emergence of antibiotic-resistant strains, bacteriophage biology has been revisited in search for a novel biocontrol/therapeutic agent against food-borne pathogens and antibiotic-resistant strains, respectively (13, 22, 59). Phages are particularly good candidates as they are able to target specific pathogens without affecting other beneficial bacteria in foods and even in humans. Further, they infect only bacterial hosts and not human cells, allowing safe applications in humans (62). As an example, a phage treatment on rats using *Listeria monocytogenes*-specific phage at a dose of 10^{12} PFU/kg body weight/day showed that there were no side effects. And clinical trials in which healthy volunteers were given oral doses of T4 phage, and another in which volunteers with HIV and other immunodeficiency diseases were

intravenously injected with phiX174 phage, indicated that phage treatment in humans is also safe (11). In addition, already thousands of people have undergone phage therapy in the former Soviet Union and Poland. Another safeness of the use of phage can be demonstrated by the fact that phages are ubiquitous by nature, and are isolated from various foods such as pork, mushrooms, chicken, cheese, yogurt, beef, and lettuce, suggesting that they are readily ingested by people. Moreover, a cocktail (ListShield™, Intralytix, Inc., Baltimore, MD, USA) of *Listeria*-infecting bacteriophages was approved by the US Food and Drug Administration in 2006, gaining the status of “Generally Recognized as Safe (GRAS)” (13, 59). Consequently, this FDA approval of phage preparations for preservation of food has triggered the researches and applications of phages. Therefore, development of novel biocontrol/therapeutic agents using bacteriophages has been recently spotlighted (13, 22, 59, 62).

I-1-6. Bacteriophage resistance mechanisms

When bacteriophages infect bacterial cells, they encounter the antiviral host defense mechanisms (49, 53, 74). These bacteriophage resistance mechanisms could be classified into at least three categories: the blocking of phage adsorption, the degradation of phage DNA, and the abortive infection systems.

The blocking of phage adsorption. In the general infection process, the adsorption of phages to host cell surface receptors is the initial step for host recognition. To obtain resistance to phages, bacteria have evolved adsorption-blocking mechanisms such as the blocking of host receptors and the production of extracellular matrix. For example, *Escherichia coli*-specific phage T5 encodes lipoprotein (Lp) that binds to host receptor protein, FhuA (ferrichrome-iron receptor) to prevent superinfection (63). Some bacteria produce TraT protein to screen host receptor (OmpA) for T-even phages (67). Furthermore, the production of extracellular polymers can form a physical barrier between host receptors and phages. The extracellular polysaccharide capsules produced by *E.coli* and *Staphylococcus aureus* showed resistances to T7 (71) and M^{Sa} (15) , respectively.

The degradation of phage DNA. Restriction-modification (RM) systems are well known as the protection system from foreign DNA (65). When phage injects its unmethylated DNA to host cells that have a RM system, the host detects phage DNA as of a foreign source and degrades this DNA using the RM system. Recently, the function of clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated (*cas*) genes were reported in the prokaryotic immune system (7, 73). CRISPRs are loci within the bacterial genome that contain multiple short direct repeats with short segments of foreign DNA (spacers) that serve as a memory of past exposures. This CRISPR-Cas system targets foreign nucleic acids such as phage genomes and plasmids. Although the detailed mechanisms of this system are still unknown, the molecular method behind this type of phage resistance by bacteria is beginning to be characterized (41). The RM system provides a form of innate immunity whereas the CRISPR system provides a form of acquired immunity in microorganisms.

The abortive infection systems. The abortive infection (Abi) systems lead to cell death and limit phage replication and reproduction among the bacterial population (58). While Abi systems differ from the antiviral systems described above, the systems provide population-wide protection by promoting

suicide of the phage-infected cell. For example, the Rex system of phage lambda-lysogenic *E. coli* strain is the best well known Abi system (61). This system is a two-component system containing RexA (intracellular sensor) and RexB (ion channel). When phage infects *E. coli* strains harboring the Rex system, RexA is activated by the production of a phage protein-DNA complex. In turn, RexA activates anchored RexB, leading to a decline of the cellular ATP level for cell multiplication. Furthermore, toxin-antitoxin (TA) systems have been recently reported as a type of Abi systems (29). The ToxIN system in *Erwinia carotovora* subspecies *atroseptica* acts as phage defense mechanisms by aborting phage-infected cell.

One of the notable obstacles in the field of phage application/therapy is the risk of the emergence of phage-resistant bacterial strains. While some studies of phage resistance mechanisms using a single phage-host model have been done, the emergence of phage-resistant strains may arise from a combination of different host defense mechanisms for phages. To overcome this hindrance for successful phage application/therapy, various phage-host interactions should be studied and elucidated.

I-2. *Salmonella enterica* serovar Typhimurium and specific bacteriophages

Salmonella infection ranks second among causes of food-borne illnesses (more than 30% of all bacterial foodborne poisoning) (6). *Salmonella* causes about 1.4 million cases of salmonellosis including 17,000 hospitalization and 600 deaths every year in US and these numbers have increased by 10% in recent years (18, 56). *Salmonella* is a Gram-negative, motile, rod-shaped, and non-spore forming bacterium belonging to the *Enterobacteriaceae* family. Based on DNA-DNA hybridization, this genus is classified as two species (*Salmonella enterica* and *Salmonella bongori*). And *S. enterica* consists of six subspecies such as *arizonae*, *diarizonae*, *enterica*, *houtenae*, *indica*, and *salamae* (10).

Salmonella causes typhoid fever that normally can be treated with broad-spectrum antibiotics, including tetracycline, chlorotetracycline, oxytetracycline, demeclocycline, methacycline, doxycycline, minocycline and a number of other semisynthetic derivatives. However, *Salmonella* resistance to antibiotics has become a problem in recent years. Moreover, emergence of multi-drug resistant *Salmonella* such as *S. Typhimurium* phage type DT104 has become more

problematic (30, 57). Therefore, use of *S. Typhimurium* bacteriophages is now receiving more attention than ever as an alternative approach for the treatment of antibiotic-resistant pathogens.

Recent reports have described the isolation of new *Salmonella* bacteriophages and evaluated their bactericidal effects (35, 54, 55, 60). For example, *Salmonella*-specific phage st104a or st104b reduced the bacterial cell number by up to 2 logs within 1 h of each phage infection (60). Φ 25 phage reduced the *S. Typhimurium* viable cell numbers up to 2.19 logs within 24 h (4, 40). However, the rapid emergence of phage-resistant *Salmonella* is an obstacle to use phages as effective biocontrol agents (1, 4, 13, 16, 33). As a preventative measure against phage-resistance, phage cocktails have been developed and found to be effective for the control of phage-resistant *Salmonella* (1, 20, 38, 78). For example, a phage cocktail containing 45 different phages reduced *Salmonella* cell numbers up to 5 log in 2 h (1). The phage cocktail approach can also broaden the host range, for example, one phage cocktail designed for serovar Typhimurium increased the host range to include *S. enterica* serovar Enteritidis and serovar Kentucky (79).

Because the attachment of bacteriophage to a specific receptor of the host bacteria is the critical first step of phage infection (52) , mutation of the receptor is the most frequent route to phage-resistance made by the host. To date, several *Salmonella* host receptors for phage infection were experimentally determined and characterized, such as the flagella (40, 69), Vi capsular antigen (64), LPS (68) , and host outer membrane proteins (OmpC (36), BtuB (37, 42), TolC (66), and FhuA (17)).

I-3. *Bacillus cereus* group and specific bacteriophages

Bacillus is a Gram-positive, rod-shaped, and spore forming bacterium belonging to the Firmicutes phylum. And *Bacillus* species are ubiquitous in nature and classified as obligate aerobes or facultative anaerobes. The *Bacillus cereus* group consists of *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* (39, 76). Among them, *B. cereus*, *B. anthracis*, and *B. thuringiensis* have been suggested as a single species of *B. cereus sensu lato* (26, 34). These species are important pathogens infecting humans, animals, and even insects (9, 31, 32).

Bacillus cereus is a food-borne pathogen producing enterotoxins such as hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K (47). Due to these toxins, the uptake of contaminated foods can cause vomiting, diarrhea, and nausea (9, 31). Generally, a high number of *B. cereus* cells (10^4 to 10^9 CFU per gram of contaminated food) is required for disease outbreak (51). However, responses to infection in humans such as diarrheal syndrome (8 to 16 h) and emetic syndrome (0.5-6 h) are relatively quick, due to toxins produced and released by *B. cereus* (19, 51). The outbreaks of *Bacillus* species in the European Union were reported to contribute up to 1.4% of all food-borne pathogenic

outbreaks in 2005 (2) , and the number of *B. cereus* outbreaks has been increasing in developed countries, highlighting the importance of controlling *B. cereus* levels in foods (21, 43, 47). In addition, *B. anthracis* is a category A biothreat agent causing fatal anthrax disease in humans and animals. Its endospore has been used as a biological weapon in 2001, resulting in more than 45% mortality (8). To generally control pathogens, various antibiotics have been widely used. However, *B. cereus* is generally insusceptible to penicillin-related antibiotics due to its production of β -lactamase and even to other antibiotics such as erythromycin and tetracycline (45, 70). In addition, long-term antibiotic treatment with various antibiotics against *B. anthracis* showed a rapid acquisition of antibiotic resistance activities (3). Therefore, due to such emergence of antibiotic resistant strains, alternative biocontrol approach against these pathogens needs to be developed. Treatment of bacteriophages infecting *B. cereus* or *B. anthracis* could be an effective method to control these pathogens. And already some studies on bacteriophages infecting *B. cereus* have been studied and reported (5, 28, 46, 50, 72). As an example, two *B. cereus* phages, FWLBc1 and FWLBc2 were isolated from a soil sample and treated with mashed potatoes, resulting in more than 5 log reduction, suggesting that phage application to foods may be useful for control of food-borne pathogens such as *B. cereus* (50). Bandara *et al.*

(5) reported that divalent cations such as Ca^{2+} , Mg^{2+} or Mn^{2+} are required to enhance the host lysis activity of the bacteriophage in fermented foods. In addition, *B. cereus* phage BCP1-1 showed high host specificity and inhibited only *B. cereus*, not fermentative bacteria such as *B. subtilis* in Korean fermented soybean food, suggesting selective growth inhibition of only the target bacterium. Interestingly, about 40% of the fermented foods contained *B. cereus*-infecting phages, suggesting that *B. cereus* is prevalent in fermented foods where bacteriophages probably inhibit its growth and consequently, limit contamination (72).

However, *B. thuringiensis* (Bt) has been well known and used as a biological pesticide for biocontrol of insect pests. It produces the insecticidal crystal proteins (ICPs) which is highly toxic to the pest larvae, but not to humans and animals. While Bt has been widely used for insect control, bacteriophage contamination causes great damage to Bt during fermentation. To overcome this problem, bacteriophages infecting *B. thuringiensis* should be studied to understand their infection mechanism as well as their lytic/lysogenic determination mechanism.

I-4. Objectives of this study

Bacteriophages are good candidates as novel biocontrol/therapeutic agents against food-borne pathogens such as *Salmonella* Typhimurium and *Bacillus cereus*. There were several reports about the applications of *Salmonella*-specific bacteriophages in beef/poultry products, cheeses, and plant products. However, these phage treatments and applications have limitations in that there is fast recovery of phage-resistant *Salmonella* strains. To solve this limitation against the use of *Salmonella*-specific phages, more insight into the molecular and genomic aspects about phage-host interaction and phage infection mechanisms should be gained. On the other hand, there exist only a few reports about *B. cereus*-specific phages. In this respect, there is pressing need to isolate new virulent phages specific to *B. cereus* and to characterize about their lysis activity at the molecular and genomic level. Therefore, these studies were performed with the objectives below.

1. Understanding the infection, interaction, and host lysis mechanisms between *Salmonella* Typhimurium host and its bacteriophages through molecular and genomic study.

2. Evaluation of the possibility of engineered temperate bacteriophage as promising biocontrol agents similar to the virulent bacteriophages.

3. Investigation of the *Bacillus cereus*-targeting bacteriophage genomes for further application.

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**Chapter II. Receptor Diversity and Host Interaction
of Bacteriophages Infecting *Salmonella enterica*
serovar Typhimurium**

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II-1. Introduction

Emergence of antibiotic-resistant pathogens due to abuse of various antibiotics is driving the development of alternative approaches to pathogen control. Bacteriophages are considered a possible biocontrol agent for bacterial pathogens (23, 50). This approach has advantages including narrow species specificity and safety for human applications (8). As an example, a clinical trial in which volunteers were given oral doses of T4 phage indicated that it was safe (6). In addition, a cocktail (ListShield™, Intralytix, Inc., Baltimore, MD, USA) of *Listeria*-infecting bacteriophages was approved by the US Food and Drug Administration in 2006, gaining the status of “Generally Recognized as Safe” (7, 50), supporting that bacteriophage may be a good candidate as a biocontrol agent for human applications.

Salmonella is a Gram-negative foodborne pathogen causing 1.4 million cases of salmonellosis including 17,000 hospitalization and 600 deaths every year in US (11, 49). *S. enterica* serovar Typhimurium is common serotype in human infection and is frequently isolated from clinical and non-clinical samples from chicken sources. A large proportion of *S. Typhimurium* strains are resistant to several antimicrobial drugs, for

example the multi-drug resistant *S. Typhimurium* phage type DT104 (12). Therefore, use of *S. Typhimurium* bacteriophages is now getting more attractive as an alternative approach in the treatment for antibiotic-resistant pathogens.

Recent reports have described the isolation of new *Salmonella* bacteriophages and evaluation of their bactericidal effects (28, 47, 48, 51). For example, *Salmonella*-specific phage st104a or st104b reduced the bacterial cell number by up to 2 logs within 1 h of each phage infection (51). Φ 25 phage reduced *S. Typhimurium* viable cell number up to 2.19 logs within 24 h (4, 38). However, the rapid emergence of phage-resistant *Salmonella* is an obstacle to effective biocontrol using phages (3, 4, 7, 9, 24). To reduce the impact of phage-resistance, phage cocktails have been developed and found to be effective in control of phage-resistant *Salmonella* (3, 13, 33, 67). For example, a phage cocktail containing 45 different phages reduced *Salmonella* cell numbers up to 5 log in 2 h (3). The phage cocktail approach can also broaden the host range, for example, one phage cocktail designed for serovar Typhimurium increased the host range to include *S. enterica* serovar Enteritidis and serovar Kentucky (68).

Because attachment of bacteriophage to the specific receptor of the host bacteria is the critical first step of phage infection (45), mutation of the

receptor is the most frequent route to phage-resistance of the host. To date, several *Salmonella* phage receptors are known including FhuA (10), TolC (57), BtuB (32, 39), OmpC (30), Vi capsular antigen (55), lipopolysaccharide (LPS) (58), and flagella (38). The study of phage receptors is expected to provide insight into the emergence of phage-resistance in *Salmonella* and guide optimization of phage cocktails for *Salmonella* control.

In this study, host receptors for 25 new *Salmonella* phages were determined using several mutants of *S. Typhimurium*. Cross infection studies with these phages and resistant strains revealed novel phage-host interactions and infection mechanisms. Further investigation of diversity of host receptors in *Salmonella* phages will increase our ability to circumvent phage resistance using phage cocktails and thus prevent food spoilage due to *S. Typhimurium*.

II-2. Materials and Methods

II-2-1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 2.1. All strains were grown in Luria-Bertani (LB) broth medium (Difco, Detroit, MI, USA) at 37°C with shaking for 12 h. *Salmonella enterica* serovar Typhimurium strain SL1344 was used for isolation of *Salmonella*-infecting phages from the collected samples, and prophage-free *S. Typhimurium* strain LT2C (20) (Cancer Research Center, Colombia, MO, USA) was used for purification of phages.

Table 2.1. The bacterial strains and plasmids used in this chapter

Strains [Description]	References ^a	Strains	References ^a
<i>Salmonella enterica</i>		<i>Salmonella</i> Typhimurium SL1344 mutants	
serovar Typhimurium		$\Delta btuB$	(39)
SL1344	NCTC	$\Delta fhuA$	(32)
UK1	(69)	$\Delta flgK$	(16)
LT2	(46)	$\Delta fliC$	(16)
LT2C	(20)	$\Delta fliR$	(16)
[Prophage-cured LT2 strain]		$\Delta fljB$	(16)
ATCC 14028	ATCC	$\Delta fliC \Delta fljB$	(16)
ATCC 19586	ATCC	$\Delta lamB$	This study
ATCC 43147	ATCC	$\Delta ompC$	Laboratory collection
DT104	(56)	$\Delta rfaL$	(53)
serovar Enteritidis ATCC 13078	ATCC	$\Delta rfbG$	(53)
serovar Typhi Ty 2-b	IVI		
serovar Paratyphi		Gram-negative bacteria	
A IB 211	IVI	<i>Shigella flexineri</i> 2a strain 2457T	IVI
B IB 231	IVI	<i>Shigella boydii</i> IB 2474	IVI
C IB 216	IVI	<i>Vibrio fischeri</i> ES-114 ATCC 700601	ATCC
serovar Dublin IB 2973	IVI	<i>Pseudomonas aeruginosa</i> ATCC 27853	ATCC
subsp. <i>arizonae</i> ATCC 13314	ATCC	<i>Cronobacter sakazakii</i> ATCC 29544	ATCC
subsp. <i>arizonae</i> ATCC 12324	ATCC		
subsp. <i>salamae</i> ATCC 15793	ATCC	Gram-positive bacteria	
subsp. <i>salamae</i> ATCC 43972	ATCC	<i>Enterococcus faecalis</i> ATCC 29212	ATCC
subsp. <i>indica</i> ATCC 43976	ATCC	<i>Staphylococcus aureus</i> ATCC 29213	ATCC
subsp. <i>houtenae</i> ATCC 43974	ATCC	<i>Bacillus cereus</i> ATCC 14579	ATCC
subsp. <i>diarizonae</i> ATCC 43973	ATCC	<i>Listeria monocytogenes</i> ATCC 19114	ATCC

Table 2.1. The bacterial strains and plasmids used in this chapter (continued)

Strains	References ^a	Plasmid [Description]	References ^a
<i>E. coli</i>		Plasmid	
K-12	(26)	pKD13	(17)
DH5 α	ATCC	[R6K γ <i>ori</i> , Amp ^R -FRT, and Km ^R -FRT]	
DH10B	(19)	pKD46	(17)
		[pSC101(Ts) <i>ori</i> , Amp ^R , and P _{araBAD} γ β <i>exo</i>]	
<i>E. coli</i> O157:H7		pCP20	(15)
ATCC 43888	ATCC	[pSC101(Ts) <i>ori</i> , Amp ^R , Cm ^R , <i>cI857</i> , and λ P _R <i>flp</i>]	
ATCC 43895	ATCC		

^aNCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; IVI, International Vaccine Institute.

II-2-2. Bacteriophage isolation and propagation

Bacteriophages isolated in this study were listed in Table 2.2. No live chickens were used in this study. Seventy-six samples obtained from chicken feces collected from farms and commercially processed broiler skins obtained from markets were used as sources for isolation of *Salmonella*-specific bacteriophages. The chicken feces were collected with permissions from the farm owners and the samples were collected for the purpose of this research only. Twenty-five grams of each sample were mixed with 225 ml of sodium chloride–magnesium sulfate (SM) buffer (100 mM NaCl, 10 mM MgSO₄·7H₂O and 50 mM Tris·HCl, pH 7.5) without gelatin in sterile bags. Twenty-five milliliters of each homogenized sample were mixed with 25 ml of 2× concentrated LB broth and incubated with shaking at 37°C for 12 h. After centrifugation (5,000 × g for 10 min), the supernatant was filtered using 0.22-µm pore size filters (Millipore, Billerica, MA, USA). Ten milliliters of each filtered sample were mixed with 50 ml LB broth with 10⁷ CFU/ml of overnight cultured *S. Typhimurium* SL1344. The mixture was incubated with shaking at 37°C for 12 h. After centrifugation (5,000 × g for 10 min), the supernatant was filtrated using 0.22 µm pore size filters. The presence of phages was assessed using a plaque forming assay with molten 0.4% LB soft agar containing 10⁷ CFU/ml

of *S. Typhimurium* SL1344. After incubation at 37°C for 12 h, individual plaques were picked and eluted with 1 ml of SM buffer without gelatin. Plaque isolation and elution were repeated more than five times for pure isolation of individual phages in *S. Typhimurium* LT2C. One liter of exponentially growing *S. Typhimurium* LT2C ($OD_{600\text{ nm}} = 1.0$) was infected with each SPN phage at a multiplicity of infection (MOI) of approximately 1 and incubated with shaking at 37°C for 4 h. Cell debris was removed by centrifugation at $5,000 \times g$ for 10 min and filtered using 0.22- μm pore size filters. Phage particles were precipitated from the filtrate by addition of 10% polyethylene glycol 6,000 (Sigma, St. Louis, MO, USA). Finally, a stepped CsCl density ultracentrifugation (himac CP 100 β , Hitachi, Japan) with step densities of 1.3, 1.45, 1.5, and 1.7 g/ml at $78,500 \times g$ for 2 h was conducted at 4°C. The bands of viral particles were withdrawn from the tube with a syringe and dialyzed using 1 L of SM buffer for 1 h and stored at 4°C.

Table 2.2. Characteristics of the isolated *S. Typhimurium*-specific bacteriophages and their identified receptors

Group ^a (Family)	Phage	Source	Mutated genes of the phage-resistant strains
F-I (<i>Siphoviridae</i>)	SPN2T	Chicken feces1	<i>flgK</i> , <i>fliR</i> ^b or <i>fliC</i>
	SPN3C	Chicken feces2	<i>flgK</i> , <i>fliR</i> or <i>fliC</i>
	SPN8T	Processed broiler skin1	<i>flgK</i> , <i>fliR</i> or <i>fliC</i>
	SPN9T	Processed broiler skin2	<i>flgK</i> , <i>fliR</i> or <i>fliC</i>
	SPN11T	Soil1	<i>flgK</i> , <i>fliR</i> or <i>fliC</i>
	SPN13B	Water1	<i>flgK</i> , <i>fliR</i> or <i>fliC</i>
	SPN16C	Chicken feces3	<i>flgK</i> , <i>fliR</i> or <i>fliC</i>
F-II (<i>Siphoviridae</i>)	SPN4S	Processed broiler skin3	<i>flgK</i> or <i>fliR</i>
	SPN5T	Processed broiler skin4	<i>flgK</i> or <i>fliR</i>
	SPN6T	Processed broiler skin5	<i>flgK</i> or <i>fliR</i>
	SPN19	Processed broiler skin6	<i>flgK</i> or <i>fliR</i>
B (<i>Siphoviridae</i>)	SPN7C	Processed broiler skin7	<i>btuB</i> ^c
	SPN9C	Processed broiler skin2	<i>btuB</i>
	SPN10H	Soil2	<i>btuB</i>
	SPN12C	Soil3	<i>btuB</i>
	SPN14	Soil4	<i>btuB</i>
	SPN17T	Silky fowl feces	<i>btuB</i>
	SPN18	Processed broiler skin8	<i>btuB</i>
L (<i>Podoviridae</i>)	SPN1S	Water2	<i>rfaL</i> ^d or <i>rfbG</i>
	SPN2TCW	Chicken feces1	<i>rfaL</i> or <i>rfbG</i>
	SPN4B	Processed broiler skin3	<i>rfaL</i> or <i>rfbG</i>
	SPN6TCW	Processed broiler skin5	<i>rfaL</i> or <i>rfbG</i>
	SPN8TCW	Processed broiler skin1	<i>rfaL</i> or <i>rfbG</i>
	SPN13U	Water3	<i>rfaL</i> or <i>rfbG</i>
	SPN9TCW	Processed broiler skin2	<i>rfaL</i> or <i>rfbG</i>

^aF-I and F-II, flagella-specific phage group; B, BtuB-specific phage group; L, LPS-specific phage group.

^b*flgK*, *fliR* mutations were complemented using pACYC184 vector expressing the *flgK*⁺ or *fliR*⁺ gene.

^c*btuB* mutation was complemented using pACYC184 vector expressing the *btuB*⁺ gene.

^d*rfaL* mutation was complemented using pUHE21-*lacI*^d vector expressing the *rfaL*⁺ gene.

II-2-3. Receptor screening and host range determination by spotting assay

To screen mutant strains resistant to each SPN phage and to determine host range of each SPN phage, double layer spotting assay was used. A 100- μ l aliquot of bacterial culture was added to 6 ml molten 0.4% LB agar and then poured on a 1.5% LB agar plate. After solidification of the top agar, 10 μ l serially diluted phage suspension ranging from 10^2 to 10^5 plaque forming unit/ml was spotted on the top agar and the plates were incubated at 37°C for 12 h. After incubation, the sensitivity of indicator strains to the tested phages was determined by degrees of clearing in the spots or plaques. The plaque assay was performed in triplicate.

II-2-4. Construction of deletion mutant

S. Typhimurium SL1344 derivatives with deletions of *lamB* gene was constructed using the lambda red recombination method (17). The kanamycin resistance (Kan^R) cassette from plasmid pKD13 was amplified using primers specific for *lamB* gene, *lamB*-*lamb*-F1 and *lamB*-*lamb*-R1. Sequences of all primers for this construct are provided in Table 2.3. The polymerase chain reaction (PCR) products were transformed into *S. Typhimurium* SL1344 harboring pKD46 and integrated into the

chromosomal *lamB* gene. Finally, the Kan^R cassette was removed using pCP20 plasmid following the procedure of Cherepanov *et al.* (15).

Table 2.3. Primers used in this chapter

Target	Primer	Sequence	Reference
<i>lamB</i>			
deletion-F	<i>lamB</i> -lamb-F1	5'-CGCAGTTTTAGAAAGGTGGC AGCGTTTAAAGAAAAGCAAT GATCTCAGGAGATAGATGTAG GCTGGAGCTGGAGCTGCTTC G-3'	This study
deletion-R	<i>lamB</i> -lamb-R1	5'-AGACCTGATGTTTCCGAGG GGCTTGCGCCCCTCGTTACGT CAGATGACCATCGTAATTCCG GGGATCCGTCGACC -3'	This study
Real-time PCR			
<i>btuB</i> forward	RT_ <i>btuB</i> _F	5'-AGGACACTAGCCCGGATAACC-3'	This study
<i>btuB</i> reverse	RT_ <i>btuB</i> _R	5'-CAGTACATGGCTGGAGTTGG-3'	This study
<i>fliC</i> forward	RT_ <i>fliC</i> _F	5'-CTCGGCTACTGGTCTTGGTG-3'	This study
<i>fliC</i> reverse	RT_ <i>fliC</i> _R	5'-AGTTGCAAATGCTGATTTGA-3'	This study
Control forward	RT_ <i>rrsH</i> _F	5'-CGGACGGGTGAGTAATGTCT-3'	This study
Control reverse	RT_ <i>rrsH</i> _R	5'-CTCAGACCAGCTAGGGATCG-3'	This study

II-2-5. Electron microscopy

The morphology of CsCl-purified SPN phages was determined using transmission electron microscopy (TEM). Concentrated viral samples were diluted with SM buffer without gelatin and 5 µl of each phage sample was applied to the surface of carbon coated copper grids. Excess volume was removed by carefully touching the side of grid with filter paper and 5 µl 2% uranyl acetate (pH 4.0) was spotted on the grid for negative staining and removed after a short interval. The prepared samples were observed using TEM (LIBRA 120, Carl Zeiss, Switzerland) at 80 kV. Taxonomy of the SPN phages was determined according to the guidelines of the International Committee on Taxonomy of Viruses (22).

II-2-6. Isolation of phage-resistant strains and determination of cross resistance

To investigate influence of resistance against one type of receptor to infection by phages using different receptors, resistance strains were developed against phages and then they were used for the infections by phages using different host receptors. Group L phage-resistant strains showing resistance to re-infection were isolated using high-titer overlay assay following modified Kim and Ryu's protocol (39). In this modified

protocol, separate colonies of group L phage-resistant strains were obtained by additional streaking on LB agar plate. Because high-titer overlay assay did not work for isolation of transiently phage-resistant strains by group F, group B, and group L SPN9TCW phage infections, they were isolated using high-titer broth assay to increase the yield of the phage-resistant strains and to maintain resistance in the presence of phages. For the high-titer broth assay, phages were added to an LB broth culture of *S. Typhimurium* LT2C ($OD_{600\text{ nm}} = 1.0$) at $MOI=100$, and the culture was incubated with shaking at 37°C until the $OD_{600\text{ nm}}$ reached 1.0, again. To remove excessive phages, the phage-infected cells were then harvested by centrifugation at $5,000 \times g$ for 10 min, resuspended in 200 μl ice-cold molecular grade water and used as a host for the second infection of SPN phages using different host receptors. The host resistance to the second infection was monitored using plaquing assays as described above.

II-2-7. Lysogen induction

All phage-resistant strains of *S. Typhimurium* LT2C were cultivated at 37°C until $OD_{600\text{ nm}}$ reached to 1.0 and 0.5 $\mu\text{g/ml}$ of mitomycin C (Sigma) was added to the cultures. Then, these cultures were additionally incubated at 37°C for 2 h. After incubation, the cells were removed by centrifugation

and filtration and the supernatant was collected. The spotting assay of this supernatant with *S. Typhimurium* LT2C was conducted to confirm the lysogen formation. To confirm the unstable lysogen formation of group F phage-resistant strains, the group F, B, L-SPN9TCW phage-resistant strains were plate in green plate (Evans blue uranine agar plate, 0.5% NaCl, 1% Tryptone, 0.5% Yeast extract, 0.5% K₂HPO₄, 0.04 M Glucose, 0.04% Evans blue, 0.04% Uranine, and 1.5% Micro agar, final concentration) as the procedure developed by Chan *et al.* (13).

II-2-8. Real-time reverse transcription (RT)-PCR

Total RNA was isolated from *S. Typhimurium* using the RNeasy Mini Kit (Qiagen) and converted to cDNA using the Omniscript RT Kit (Qiagen) and random hexamers (Invitrogen, Carlsbad, CA, USA) using manufacturer's instructions. Quantitative real-time RT-PCR was performed as previously described (34) with primers listed in Table 2.3.

II-2-9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Flagellin of *S. Typhimurium* was isolated as previously described (52), and suspended in loading buffer (0.05 M Tris ·HCl pH 8.0, 1.6% SDS,

25% glycerol, 5% 2-mercaptoethanol, 0.003% bromophenol blue, final concentration). Samples were heated in boiling water for 3 min, and then loaded in a well of a 12% acrylamide gel in 1X Tank Buffer (0.025 M Tris, 0.192 M glycine, and 0.1% SDS, final concentration). Gel electrophoresis was performed using Mini-gel kit (Bio-Rad). The gel was stained with colloidal blue.

II-3. Results

II-3-1. Bacteriophage isolation

Between September and December 2009, 25 bacteriophages were newly isolated from 18 of 76 samples (23.7% phage recovery frequency). All phages in Table 2.2 were designated as *Salmonella* Phage Number (SPN) and specific numbers were used to indicate the sample sources and sometimes characters were used to differentiate the isolated phages from the same samples, respectively.

II-3-2. Grouping of bacteriophages based on their receptors

To determine the host receptors for 25 phages, previously constructed mutants of *S. Typhimurium* SL1344 (listed in Table 2.1) were used. Interestingly, receptor screening using several specific deletion mutants revealed that only three different types of receptors were detected: flagellar production genes, the gene encoding the vitamin B₁₂ uptake outer membrane protein, and genes involved in LPS-related O-antigen production. The mutant screening results were confirmed by complementation experiments. The host receptor genes deleted by the lambda red recombination method are indicated in the receptor gene clusters presented

in Fig. 1. Based on the deleted specific genes for formation of phage receptors, 25 phages were grouped into group F, group B, and group L phages, respectively (Table 2.2 and Fig. 2A).

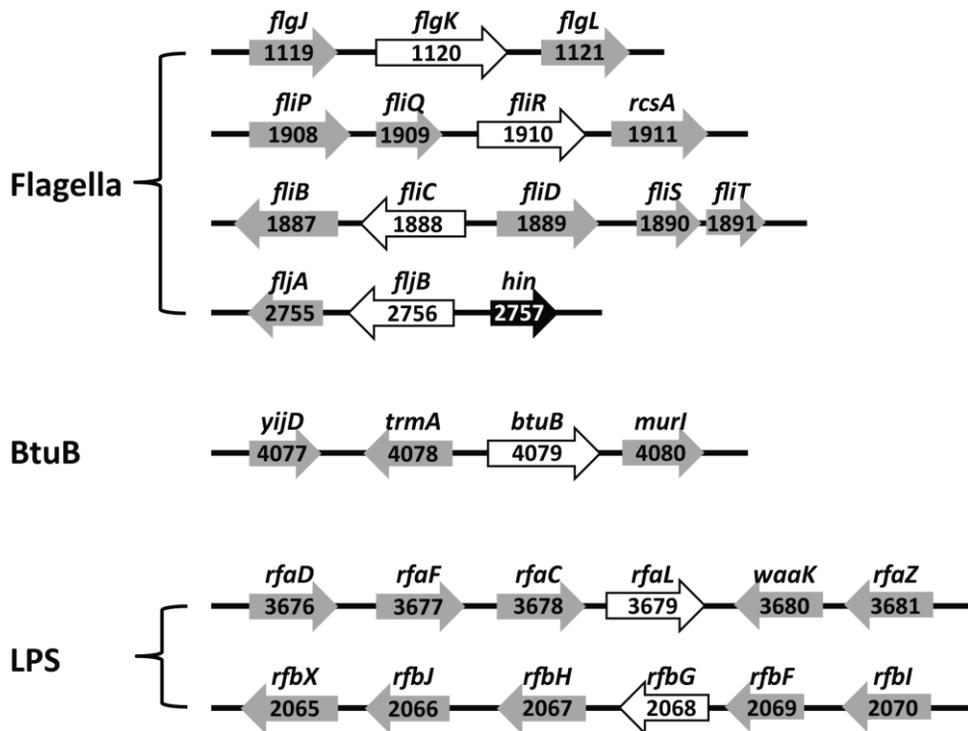


Figure 2.1. Genetic map of the receptor gene clusters and the mutated genes of resistant strains. Genes involved in the synthesis of flagella (*flgK*, *fliQ*, *fliC* and *fljB*), BtuB (*btuB*), and LPS (*rfaL* and *rfbG*) inactivated by transposon insertion were indicated by open arrows. Black arrow marked with *hin* is a promoter that transcribes the *fljB* gene. The numbers are locus-tag numbers indicating the locations of the genes in the genome sequence.

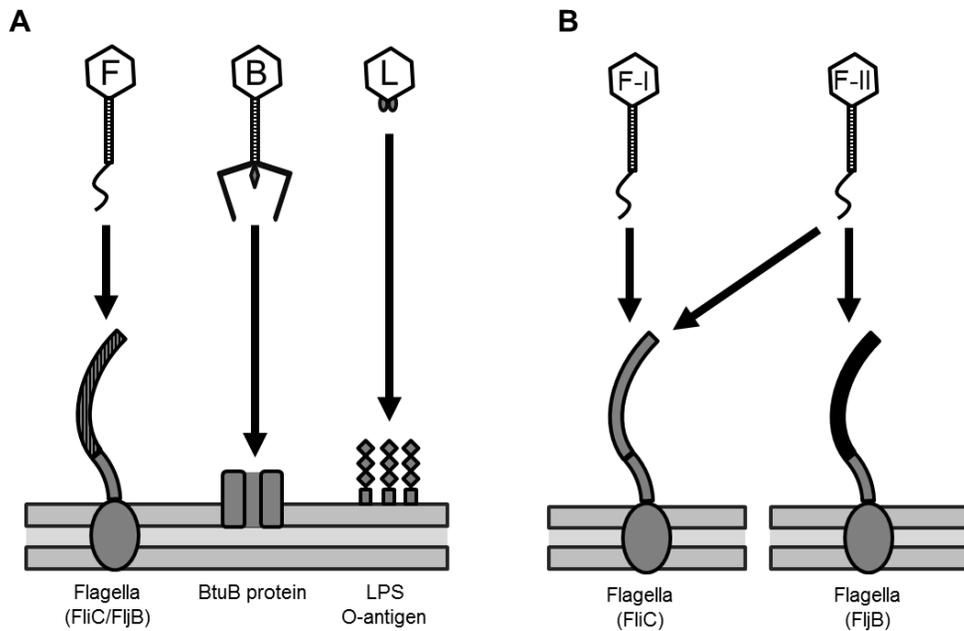


Figure 2.2. Host receptors of SPN phages. F, F-I, F-II, B, and L marked in the phage heads indicate group F, group F-I, group F-II, group B, and group L phages, respectively. (A) Group F, group B, and group L phages use flagella (FliC/FliJ), BtuB, and O-antigen of LPS as host receptors, respectively. (B) Group F-I and group F-II phages use FliC (grey-colored) and FliC/FliJ (black-colored) in the host flagella.

The deleted genes involved in flagella production included genes encoding the flagella hook-filament junction protein (*flgK*), a putative component of the type III flagella export apparatus (*fliR*), and the gene encoding flagellin (*fliC*). Because *S. Typhimurium* expresses either one of two flagellin genes, *fliC* or *fljB* (42), whether the group F phages can use both flagellins was tested by phage infection analysis of *fliC* and *fljB* deletion mutants. The group F phages can be categorized into two groups, F-I and F-II, as shown on Table 2.4 and Fig. 2B. The group F-I phage could not infect the *fliC* mutant or the *fliC/fljB* double mutant, but could infect the *fljB* mutant, suggesting that the group F-I phage can only use FliC as a receptor. Group F-II phage could not infect the *fliC/fljB* double mutant but could infect the *fliC* and the *fljB* single mutants, suggesting that the group F-II phage can use either FliC or FljB as a receptor (Table 2.4 and Fig. 2B).

One group of resistant mutants has deletion mutations in the *btuB* gene encoding the membrane transporter for vitamin B₁₂, suggesting that BtuB is a group B phage receptor. The mutated genes in the O-antigen biosynthesis are O-antigen ligase (*rfaL*) and CDP glucose 4,6-dehydratase (*rfbG*). Complementation of the deleted genes with pACYC184 and pUHE21-*lac*^q expression vectors containing the wild-type genes restored susceptibility, supporting that O-antigen is a receptor for group L phage

infection. Overall, 11 out of 25 phages use flagella (group F phage), seven out of 25 use BtuB (group B phage), and another seven out of 25 use LPS-related O-antigen (group L phage) as receptors. Although the *Salmonella* outer membrane proteins (OMPs) such as TolC (57), FhuA (10) and OmpC (30) are known receptors for some phages, no phages using those receptors was present in this set of 25 phages. It is not clear why BtuB was the only OMP detected as a receptor in this study.

Table 2.4. Flagellin-targeting phages: receptor and sensitivity patterns based on specific gene mutation

Strain genotype	Receptor present	Resistance profiles ^{a,b}	
		F-I phage	F-II phage
<i>ΔfliC</i>	FljB only	R	S
<i>ΔfljB</i>	FliC only	S	S
<i>ΔfliC ΔfljB</i>	neither	R	R

^aF-I, flagella-targeting phage group I; F-II, flagella-targeting phage group II.

^bS, sensitive to infection; R, resistant to infection.

II-3-3. Morphology

All 25 phages could be categorized into three morphological groups (Fig. 2.3). Interestingly, this morphological grouping is correlated with the grouping of *Salmonella*-specific phages based on their receptors (Table 2.2). All of the group F and B phages have isometric heads and non-contractile, cross-banded tails that are longer than the tails of the group L phages (Fig. 2.3). These phages can be classified into the B1 morphotype of the *Siphoviridae* family, although the group F phages have a single, long, kinky tail fiber structure and the group B phages have four or five L-shaped fibers (Fig. 2.3A and 2.3B). The group L phages are classified as members of the *Podoviridae* family. They have isometric heads with very short tails that are distinct from other groups.

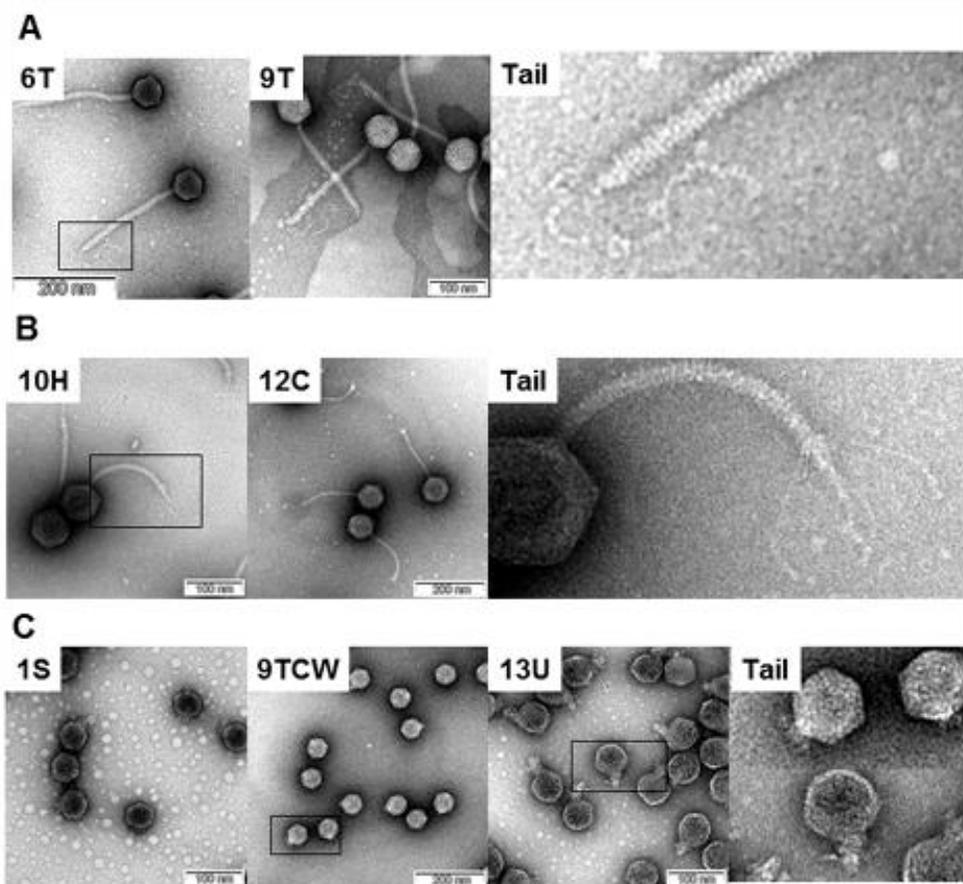


Figure 2.3. TEM morphology of representative SPN phages. Each phage name is indicated in the upper left corner of the picture. The representative tails of each group of phage were marked with boxes and the enlarged pictures were shown on the right. (A) Phages using flagella as a receptor. (B) Phages using BtuB as a receptor. (C) Phages using LPS as a receptor.

II-3-4. Host range of the isolated bacteriophages

Infection analysis of 25 *Salmonella*-specific bacteriophages was conducted with 21 *Salmonella* strains, five *E. coli* strains and nine other species of Gram-negative and Gram-positive bacteria (summarized in Table 2.5). In general, SPN bacteriophages infect *S. Typhimurium* strains but not other Gram-negative and Gram-positive bacteria, suggesting that they are specific to *S. Typhimurium*. The group F phages also infect *S. enterica* subsp. *arizonae* and subsp. *salamae*, and the group B phages can infect several strains of *E. coli*, indicating that these bacteriophages have a host range that extends to other *Salmonella* subspecies or *E. coli* strains. In addition, group L phages and some group F phages infect *S. Paratyphi*. The group B phages have much broader host range than the group F and L phages. They can infect *E. coli* and *Shigella flexneri* strains as well as *Salmonella* (Table 2.5), suggesting that the BtuB proteins of *S. Typhimurium*, *E. coli*, and *Shigella flexneri* are similar. Comparative sequence analysis of BtuB proteins in the group B phage susceptible strains *S. Typhimurium* LT2, *E. coli* MG1655 and *Shigella flexneri* 2a strain 2457T revealed >87% identity at the amino acid level between *S. Typhimurium* and the other two species, whereas *S. Typhimurium* and *Vibrio fischeri* ES-114, which is not susceptible to group B phage, share <35% identity at the amino

acid level. These findings support the hypothesis that the group B phage receptor motifs are shared among *Salmonella*, *E. coli* and *Shigella*.

Comparative host range analysis of group F-I and F-II phages revealed that F-II phages infect a larger number of *S. Typhimurium* isolates (data not shown), probably because the F-II phages can use either the FliC or the FljB flagellin as a receptor while the F-I phages can only use FliC (Table 2.4 and Fig. 2.2B).

Table 2.5. Host range of isolated bacteriophages

Host	Lytic spectrums ^{a,b}				
	Group F-I			Group F-II	
	I	II	III	IV	V
<i>Salmonella enterica</i>					
serovar Typhimurium					
SL1344	T	T	T	T	T
UK1, LT2, LT2C ^{c*}	T	T	T	T	T
ATCC 14028	-	-	-	T	I
ATCC 19586	C	T	C	T	T
ATCC 43147	T	T	T	T	T
DT104	T	T	T	T	T
serovar Enteritidis ATCC 13078	-	-	-	-	-
serovar Typhi Ty 2-b	-	-	-	-	-
serovar Paratyphi					
A IB 211	T	-	-	-	T
B IB 231	-	-	-	-	-
C IB 216	-	-	-	-	-
serovar Dublin IB 2973	-	-	-	-	-
subsp. <i>arizonae</i> ATCC 13314	-	T	T	T	T
subsp. <i>arizonae</i> ATCC 12324	-	T	T	T	T
subsp. <i>salamae</i> ATCC 15793	T	T	T	T	T
subsp. <i>salamae</i> ATCC 43972	-	-	-	-	-
subsp. <i>indica</i> ATCC 43976	-	-	-	-	-
subsp. <i>houtenae</i> ATCC 43974	-	-	-	-	-
subsp. <i>diarizonae</i> ATCC 43973	-	-	-	-	-
<i>E. coli</i>					
K-12, DH5 α , DH10B	-	-	-	-	-
<i>E. coli</i> O157:H7					
ATCC 43888	-	-	-	-	-
ATCC 43895	-	-	-	-	-
Gram-negative bacteria					
<i>Shigella flexneri</i> 2a strain 2457T	-	-	-	-	-
<i>Shigella boydii</i> IB 2474	-	-	-	-	-
<i>Vibrio fischeri</i> ES-114 ATCC 700601	-	-	-	-	-
<i>Pseudomonas aeruginosa</i> ATCC 27853	-	-	-	-	-
<i>Cronobacter sakazakii</i> ATCC 29544	-	-	-	-	-
Gram-positive bacteria					
<i>Enterococcus faecalis</i> ATCC 29212	-	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 29213	-	-	-	-	-
<i>Bacillus cereus</i> ATCC 14579	-	-	-	-	-
<i>Listeria monocytogenes</i> ATCC 19114	-	-	-	-	-

Table 2.5. Host range of isolated bacteriophages (continued)

Host	Lytic spectrums ^{a,b}						
	Group B					Group L	
	VI	VII	VIII	IX	X	XI	XII
<i>Salmonella enterica</i>							
serovar Typhimurium							
SL1344	C	C	C	C	C	T	C
UK1, LT2, LT2C ^{c*}	C	C	C	C	C	T	T
ATCC 14028	T	T	T	T	T	T	T
ATCC 19586	C	C	C	C	T	T	C
ATCC 43147	-	I	-	-	I	T	C
DT104	-	-	-	-	-	T	C
serovar Enteritidis ATCC 13078	C	C	T	T	C	T	C
serovar Typhi Ty 2-b	-	T	-	-	T	-	-
serovar Paratyphi							
A IB 211	C	C	C	C	C	-	-
B IB 231	T	T	T	T	T	T	C
C IB 216	T	T	T	T	T	T	-
serovar Dublin IB 2973	-	T	T	T	C	-	-
subsp. <i>arizonae</i> ATCC 13314	-	-	-	-	-	-	-
subsp. <i>arizonae</i> ATCC 12324	C	C	C	C	C	-	-
subsp. <i>salamae</i> ATCC 15793	-	-	-	-	-	-	-
subsp. <i>salamae</i> ATCC 43972	-	-	-	-	C	-	-
subsp. <i>indica</i> ATCC 43976	T	C	T	T	C	-	-
subsp. <i>houtenae</i> ATCC 43974	-	-	-	-	-	-	-
subsp. <i>diarizonae</i> ATCC 43973	T	T	T	C	T	-	-
<i>E. coli</i>							
K-12, DH5 α , DH10B	C	C	C	C	C	-	-
<i>E. coli</i> O157:H7							
ATCC 43888	-	-	C	-	-	-	-
ATCC 43895	-	-	T	-	-	-	-
Gram-negative bacteria							
<i>Shigella flexneri</i> 2a strain 2457T	C	-	C	C	-	-	-
<i>Shigella boydii</i> IB 2474	-	-	-	-	-	-	-
<i>Vibrio fischeri</i> ES-114 ATCC 700601	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i> ATCC 27853	-	-	-	-	-	-	-
<i>Cronobacter sakazakii</i> ATCC 29544	-	-	-	-	-	-	-
Gram-positive bacteria							
<i>Enterococcus faecalis</i> ATCC 29212	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 29213	-	-	-	-	-	-	-
<i>Bacillus cereus</i> ATCC 14579	-	-	-	-	-	-	-
<i>Listeria monocytogenes</i> ATCC 19114	-	-	-	-	-	-	-

^aLytic spectrum: I contains SPN2T, SPN3C, and SPN13B; II contains SPN8T and SPN9T; III contains SPN11T, SPN16C; IV, SPN4S, and SPN19; V contains SPN5T and SPN6T; VI contains SPN7C and SPN9C, VII, SPN10H; VIII contains SPN12C and SPN17T; IX, SPN14; X, SPN18; XI contains SPN1S, SPN2TCW, SPN4B, SPN6TCW, SPN8TCW, and SPN13U; XII, SPN9TCW.

^bC, clear plaque; T, turbid plaque; I, inhibition zone; -, no infection.

^cProphage-cured strain of *S. Typhimurium* LT2.

II-3-5. Lysogenization

It is intriguing that while the group F and L phages generally make turbid plaques, and thus may be temperate phages, the group B phages make clear plaques and may be virulent phages (36). Induction experiments in which mitomycin C was used to induce lytic growth indicate that the group L phage-resistant *S. Typhimurium* LT2C strains carry an inducible prophage. In contrast, mitomycin C treatment of the group L SPN9TCW phage-resistant and group B phage-resistant *Salmonella* strains did not yield phage, indicating that these phages do not make lysogens in the LT2C strain consistent with the clear plaque morphology (Table 2.5). Most of the group F phage-resistant *Salmonella* strains did not yield phage after treatment with mitomycin C even though they make turbid plaques. Five percent of the group F phage-resistant *Salmonella* made phage in response to mitomycin C, but they also lose resistance easily upon subculturing in the absence of the phages, suggesting the possibility of formation of unstable lysogens or pseudolysogens (1) (Table 2.6). To confirm if transient resistance is due to unstable lysogeny or pseudolysogeny, the green plate (Evans blue uranine agar plate) experiment was conducted (Fig. 2.4). In the green plate experiment, only the cells lysed by phage induction make blue colonies due to pH change. It revealed that while the resistant strains to the virulent

phages in group B and group L-SPN9TCW did not show any blue colony, the resistant strains to group F phages did show small number of blue colonies (approximately 5% of all colonies) in the green plates. These results indicate that a few of the strains resistant to group F phages were lysed, suggesting that group F phages do not form stable lysogens.

Table 2.6. Cross resistance of phage-resistant strains

Resistant strain	Phage sensitivity pattern ^a			Mitomycin C induction ^b
	Group F (Flagella)	Group B (BtuB)	Group L (LPS)	
Group F	R1	S	S	N ^c
Group B	S	R1	R1	N
Group L (SPN9TCW)	S	R1	R1	N
Group L (Other)	S	S	R2	I

^aR1, transiently resistant; R2, stably resistant; S, sensitive.

^bN, not induced; I, induced.

^cAlthough most of the group F phage-resistant *Salmonella* were not induced, <5% of the resistant *Salmonella* were induced by mitomycin C.

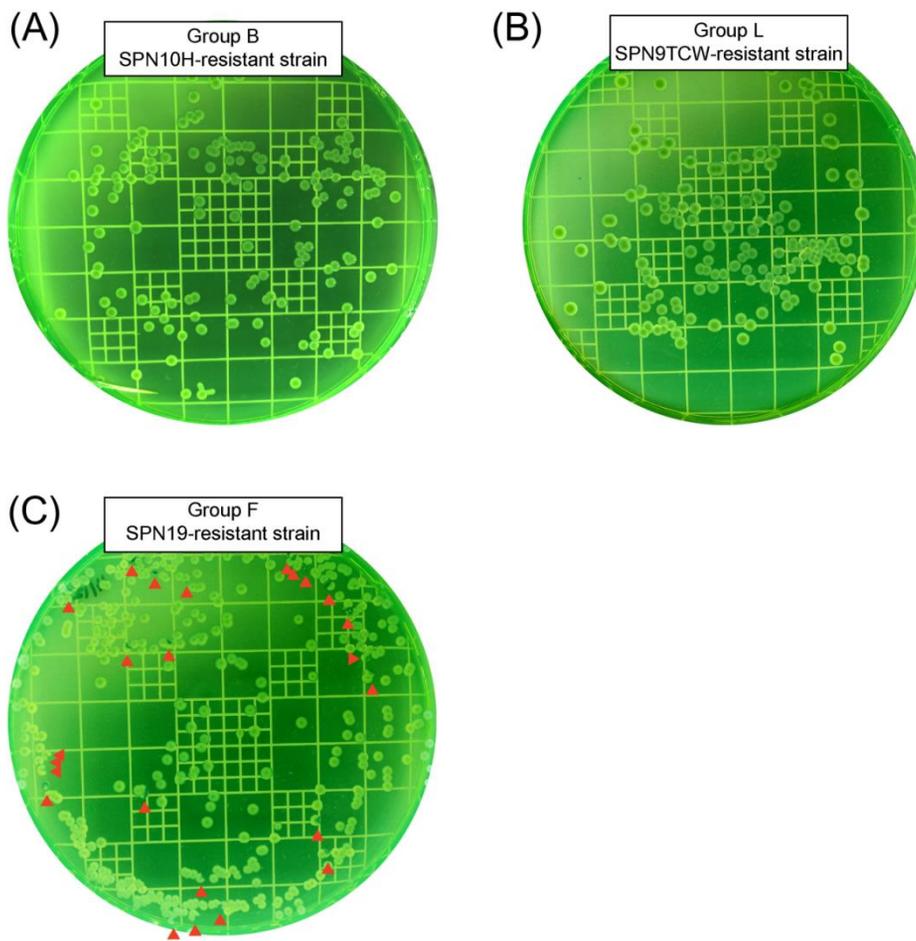


Figure 2.4. Green plate experiment of representative phages in three phage groups. (A) Group B SPN10H-resistant strain (B) Group L SPN9TCW-resistant strain (C) Group F SPN19-resistant strain. Red triangles indicate blue colonies on green plate.

II-3-6. Cross-resistance of phage-resistant *Salmonella* to the different receptor group phages

To understand the interaction of phage with the specific host receptors, derivatives of *S. Typhimurium* LT2C that are resistant to the group F, group B and group L phages described here were isolated and characterized. Group B phage-resistant *Salmonella* (39) were transiently resistant to re-infection with group B phages, and most group F phage-resistant and group L SPN9TCW phage-resistant strains also showed transient resistance to re-infection with phages from their own group. All group L phage-resistant strains, except for the strain resistant to phage SPN9TCW, showed stable phage resistance to group L phages. Interestingly, all group L phage-resistant strains except those resistant to SPN9TCW were lysogens, suggesting that the resistance for the group L phages is due to prevention of superinfection by a stable prophage (Table 2.6 and Fig. 2.5B) (5, 31). Cross-infection of group F phages on other phage-resistant strains showed sensitivity to these phages, suggesting no mutual influence between flagellin and other phage receptors on the sensitivity to the phages (Table 2.6 and Fig. 2.5). However, cross-infection of group B phages on group L phage-resistant strains yielded two different patterns. While the group L phage-resistant strains are sensitive, the SPN9TCW phage-resistant strain is

resistant to group B phage infection, depending on the formation of lysogen (Table 2.6 and Fig. 2.5BD). Furthermore, group L phages were not able to infect group B phage-resistant strains, suggesting a possible influence between the BtuB and LPS receptors on the sensitivity to the phages (Table 2.6 and Fig. 2.5C).

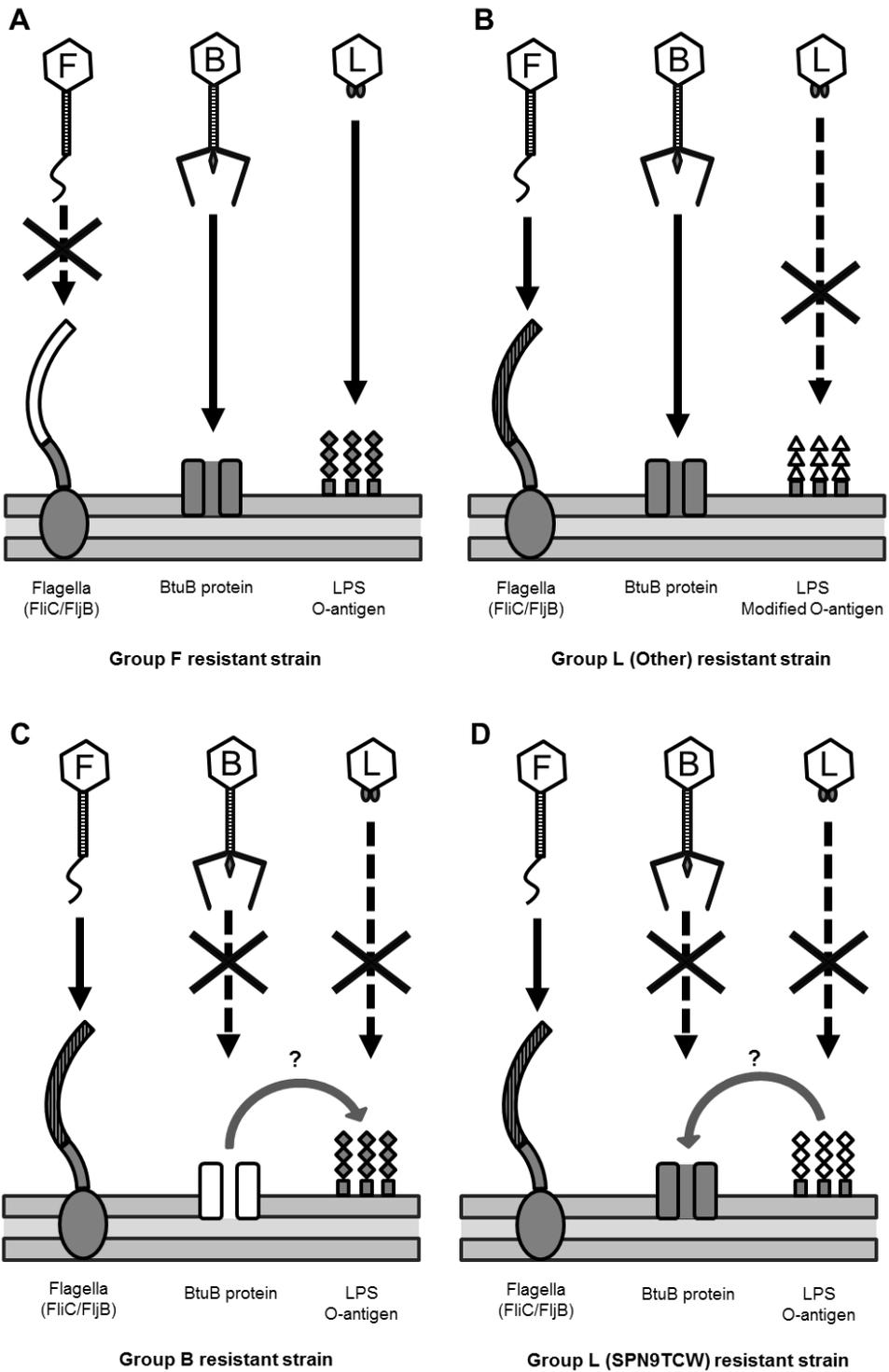


Figure 2.5. Cross-resistance of phage-resistant *Salmonella* to the different receptor group phages. F, B, and L marked in the phage heads indicate group F, group B, and group L phages, respectively. Each receptor in the phage-resistant strains is white-colored. (A) Group F phage-resistant strain is sensitive to group B and group L phages. (B) Group L phage-resistant strain is sensitive to group F and group B phages, but resistant to group L phages, due to modification of O-antigen of LPS. Modified O-antigen is indicated by white triangles. (C) Group B phage-resistant strain is sensitive to group F, but resistant to group B as well as group L phages, probably due to putative interaction between BtuB and O-antigen of LPS. (D) Group L (SPN9TCW phage)-resistant strain is sensitive to group F, but resistant to group B as well as group L phages, probably due to putative interaction between BtuB and O-antigen of LPS.

II-4. Discussion

The details of the molecular interactions between phages and their host receptors that determine host specificity are not fully understood yet. Here, I isolated 25 *Salmonella*-specific phages, identified their receptors, studied their host specificity, and examined cross-resistance among the phages and phage resistant hosts. Although this study on *Salmonella* phage receptors using 25 phages does not represent the complete range of infection mechanisms used by *Salmonella*-infecting bacteriophages, the results provide novel insights into general host-phage interactions of typical *Salmonella*-infecting bacteriophages.

Bacteriophages tend to use structures exposed on the outer membrane of the host bacteria as a receptor because they are easily accessible. Unexpectedly, only three kinds of receptors were identified in this study, flagella, O-antigen, and the outer membrane protein BtuB, although several other outer membrane proteins, including FhuA, TolC, and OmpC, are characterized as phage receptors of *Salmonella*. One possible reason may be the complex nature of *Salmonella* Typhimurium LPS (21, 25), which may block access of phage to some outer membrane proteins, making it more challenging to isolate those phages. In a seeming contradiction, LPS

may help attachment of group B phage to BtuB on a *S. Typhimurium* host. T5 phage, for example, has both a receptor-binding tail protein Pb5 and a L-shaped tail fiber protein on the phage tail that targets the host outer membrane protein and LPS, respectively (27, 65). The Pb5 as a major host specificity protein is reported to mediate irreversible binding to a specific outer membrane protein and the L-shaped tail fiber protein as a helper protein provides reversible binding to LPS. Therefore, the L-shaped tail fiber protein increases the infection rate of T5 phage by stabilization of binding between phage and an outer membrane receptor via Pb5. Group B phages are in the same family as T5 phage, *Siphoviridae*, and have a similar tail structure containing an Pb5-like protein possibly targeting BtuB and an L-shaped tail fiber protein possibly targeting LPS (Fig. 2.3). Due to this similarity between T5 and group B phages, it is suggested that this tail fiber protein may help the binding of Pb5-like protein to BtuB to increase the infection rate of group B phages, but this proposed binding mechanism needs further study.

Morphological characterization of the group F and group L bacteriophages also provided insight into the interaction of these phages with host receptors. The group F phages have relatively long, non-contractile and cross-banded tails with a single and twisted tail fiber

structure. This structure is very similar to that of chi phage that infects *E. coli*, *Salmonella* and *Serratia* through flagella filament receptors (59, 61), suggesting possible interaction between the group F twisted tail fibers and bacterial flagella. Group L phages are morphologically belong to *Podoviridae* and use LPS as a main receptor like other phages including ϵ 15 (14, 37), P22 (35) and T7 (41), which all interact with LPS via major host specificity proteins. However, *Salmonella* phages of *Myoviridae* family were not isolated in this study. It is not clear why I failed to isolate phages of *Myoviridae* family. The standard phage isolation protocol employed in our study may not be suitable for isolation of *Salmonella* phages in *Myoviridae* family because the genome sizes of *Salmonella* phages of *Myoviridae* family except for *Pedovirinae* subfamily are generally bigger than those of other family phages (2, 44, 53, 60, 62). Otherwise, there might be unknown bias in the phage isolation.

Host range analysis of group F-I phages showed that they successfully infected all *S. Typhimurium* strains tested except strain ATCC 14028. It is not clear why the group F-I phages that use only FliC as a receptor could not infect ATCC 14028 while the group F-II phages could. The complete genome sequence of strain ATCC 14028 (GenBank accession number CP001363) showed that it has a *fliC* gene and multiple sequence

alignment with the *fliC* genes of susceptible *Salmonella* strains revealed no sequence differences. Real-time RT-PCR and SDS-PAGE analysis confirmed that the *fliC* gene is expressed and translated for flagella formation, indicating that FliC in the strain ATCC 14028 is functional (data not shown). The group B phages also infected all *S. Typhimurium* strains except one, strain DT104. Sequencing and real-time RT-PCR analyses of the *btuB* gene in strain DT104 showed that it is expressed (data not shown), so it is not clear why the strain is resistant. These two examples imply that there may be an additional unknown factor(s) that makes the phage and host receptor interaction more specific and complicated. However, only *S. Typhimurium* ATCC 14028 has Gifsy-3 prophage, so this prophage could cause superinfection exclusion to group F-I phages.

The phages that can make lysogen normally make turbid plaques but many other factors are involved in turbid plaque formation that it is not simple to distinguish lysogen formation based on plaque turbidity. While the lysogen generally resists superinfection by expression of the phage genes, acquisition of phage resistance by host mutation has been hardly found in lysogen. Therefore, the aim of this lysogenization experiment was to test whether the resistance against phage infection was due to lysogenization or other factors related with a host receptor. To confirm their lysogen formation,

mitomycin C was treated to induce the prophages. Group L phages except for SPN9TCW did yield phage after mitomycin C induction, substantiating the lysogen formation by most of group L phage infection. However, most of group F phage-resistant strains did not yield phage by mitomycin C induction, even though these phages make turbid plaques, suggesting that these phages may make unstable lysogen, resulting in very low frequency of mitomycin C induction (approximately 5%) in the resistant strains. It has been known that Mu-like prophages were generally not induced by mitomycin C treatment (54), suggesting the possibility that group F phage may be mitomycin C-insensitive phage. To verify this, I performed PCR detection of group F phage genomes in the resistant hosts and the green plate experiment. Recently, the genomes of four phages in group F were completely sequenced and phage-specific primers were designed. Using these phage-specific primers, PCR was conducted to detect group F phage genomes in the genomes of group F phage-resistant *S. Typhimurium* LT2C strains. Interestingly, very low number of group F phage-resistant strains (approximately 5%) showed the presence of group F phage genomes in the host genomes, suggesting the formation of very unstable lysogens, supporting our observation of low mitomycin C induction with group F phage-resistant strains (data not shown). And green plate experiments also

showed that most of group F phage infection does not make stable lysogens. This very distinct feature of group F phages for unstable lysogen formation was not still understood and it needs to be elucidated soon.

Analysis of cross resistance among phage-resistant strains revealed that group F phage-resistant strains are sensitive to group L or B phages (Table 2.6 and Fig. 2.5A). This sensitivity indicates that the host resistance of group F phages does not disrupt the interactions between these other phages and the cell surface receptors. In contrast, group B phage-resistant strains are resistant to their phages as well as group L phages (Table 2.6 and Fig. 2.5C). The concurrent resistance to both group B and group L phages imply that BtuB may influence the interaction between LPS and phage, as in the case of *E. coli* phage T5 (27, 65). The interaction between LPS and phage has been reported to accelerate adsorption of phage T5 to *E. coli* even though an outer membrane protein is the cell surface receptor.

All but one group L phage-resistant strain was sensitive to infection by group B phages (Table 2.6 and Fig. 2.5B). The group L SPN9TCW phage-resistant strain was resistant to group B phages (Table 2.6 and Fig. 2.5D), even though phage SPN9TCW uses LPS as a receptor and the resistant strain does not appear to be a lysogen. Therefore stable lysogen formation of group L phages may be a key to determine the host resistance

against group B phages (Table 2.6). Further analyses of group B phage-resistant *Salmonella* strains are required to elucidate the mechanism of cross-resistance observed against the group B and L phages.

Many *Salmonella*-specific phages that use LPS as a receptor modify LPS as a mechanism to protect from superinfection when they lysogenize a host (40, 64). The recent complete genome sequence analysis of a lysogenic SPN1S phage in group L revealed that the phage genome encodes a GtrA and two copies of lipopolysaccharide modification acyltransferase, supporting this (63). Even though the LPS modification protects the lysogen from a superinfection by other group L phages, the lysogen is still sensitive to phages that target other receptors such as flagella and BtuB (Table 2.6 and Fig. 4B). Furthermore, group F, group B and group L SPN9TCW phage-resistant strains showed transient resistance to re-infection of the same phages (Table 2.6). These resistant strains were collected for the cross resistant experiment after the bacterial growth resumed in the presence of the phage. In this case, these collected phage-resistant strains are probably not lysogens, suggesting that host defense mechanisms such as CRISPR (18, 43) or restriction-modification systems (29, 66) or still unknown host defense mechanisms are probably activated during growth recovery.

II-5. References

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II-6. Appendix :
Genomic Analysis of Bacteriophages Targeting *Salmonella*
Typhimurium

**II-6-1. Complete Genome Sequence Analysis of Bacterial Flagellum-
Targeting Bacteriophage Chi**

(Accepted in Archives of Virology, 2013, In Press)

II-6-1-1. Abstract

Bacteriophage Chi is a well-known phage infecting pathogens such as *E. coli*, *Salmonella*, and *Serratia* via bacterial flagella. To further understand its host-phage interaction and infection mechanism via host flagella, the genome was completely sequenced and analyzed. The phage genome contains 59,407-bp length DNA with GC content of 56.51% containing 75 open reading frames (ORFs) with no tRNA genes. Its annotation and functional analysis revealed that Chi is evolutionarily very close to *Enterobacter* phage Enc34 and *Providencia* phage Redjac. However, most of the annotated genes encode hypothetical proteins, indicating that further genomic study of phage Chi is required to elucidate bacterial flagellum-targeting infection mechanism of phage Chi.

II-6-1-2. Main text

Intake of food-borne pathogens such as *E. coli* and *Salmonella* via contaminated foods causes food poisoning accompanied by high fever, diarrhea and vomiting (2, 14, 19). Although many food preservatives have been developed and used to control these food-borne pathogens, the number of food poisoning outbreaks is increasing every year (10, 11, 20). Therefore, effective and safe novel biocontrol agents should be developed to control food-borne pathogens.

Bacteriophages are bacterial viruses to infect and lyse specific bacterial host cells suggesting their bactericidal activity (6). In addition, they infect only specific host bacteria without affecting other bacteria in the same habitat (7). Recently human feeding trials showed efficient inhibition of specific bacterial host without side effects, suggesting that phage treatment should be safe for human applications (4). Therefore, phage applications have been reconsidered and tested as alternative approaches to inhibit food-borne pathogens in foods (5, 12, 22).

Bacteriophage Chi infecting major food-borne pathogens such as *E. coli*, *Salmonella*, and *Serratia* was first isolated and characterized in 1930's (25). While other bacteriophages generally infect host strains via extracellular membrane receptors such as lipopolysaccharide (LPS) and

outer membrane proteins (like BtuB, FhuA, and OmpC) (17), phage Chi is the first reported bacteriophage to infect host strains via flagella (21).

However, the infection mechanism of Chi phage via the host flagella is not understood fully in genomic level yet. In this study, to further understand this receptor specificity and host-phage interaction, the genome of phage Chi was completely sequenced and analyzed.

The Chi phage (ATCC 9842-B1TM) was obtained from American Type Culture Collection (ATCC). For propagation of phage Chi, it was added to the culture of *Salmonella enterica* serovar Typhimurium SJW1103 (27) at a multiplicity of infection (MOI) of 1 when optical density (OD) of the culture reaches to 1.0 at 600 nm wavelength. The mixture was incubated at 37°C for 4 h with vigorous shaking and phage particles were recovered by centrifugation at 6,000 × g for 10 min and subsequent filtration using 0.22 µm pore size filters (Millipore, Billerica, MA, USA). To purify the phage particles, precipitation with polyethylene glycol (PEG) 6,000 (Sigma, St. Louis, MO, USA) and ultracentrifugation (Himac CP 100β, Hitachi, Japan) with gradient CsCl₂ from 1.3 to 1.7 g/ml densities at 25,000 × g, 4°C for 2 h.

The genomic DNA of phage Chi was isolated as previously described by Wilcox *et al.* (26). Prior to isolation of phage genomic DNA, phage particles were treated with DNase I and RNase A at 37°C for 1 h to

remove bacterial host DNA and RNA, respectively. And then phage particles were lysed with standard lysis buffer (50 µg/ml of proteinase K, 0.5% of sodium dodecyl sulfate (SDS), and 20 mM of EDTA) for 2 h at 56°C. In the final step, phenol-chloroform treatment and ethanol precipitation of genomic DNA were conducted as described by Sambrook *et al.* (23).

Purified genomic DNA of phage Chi was sheared and randomly sequenced using the Genome Sequencer FLX (GS-FLX) (Roche, Mannheim, Germany) and the qualified filtered reads were assembled using the Newbler 2.3 program (Roche) at Macrogen, Inc. (Seoul, South Korea). Open reading frames (ORFs) were predicted using gene prediction programs such as Glimmer3 (13), GeneMarkS (3), and FgenesB (Softberry, Inc. Mount Kisco, NY, USA) programs and confirmed using RBSFinder program (J. Craig Venter Institute, Rockville, MD, USA). Their annotation and functional analysis were performed using BLASTP (1) and InterProScan (28) databases. Genomic DNA and annotation data were handled and edited using Artemis14 (8). Phylogenetic analysis of major capsid proteins (MCPs) of bacteriophages including phage Chi was conducted using MEGA5 based on the neighbor-joining method with *P* distance values (15). The lifestyle of phage Chi was predicted using PHACTS program (18).

Bacteriophage Chi genome contains 59,407-bp length DNA with

GC content of 56.51% containing 75 ORFs with no tRNA genes (Fig. 2.6). Annotated functions of all predicted ORFs in phage Chi were listed in Table 2.7. The average gene length is 748-bp and the gene coding percentage is 94.5%. The predicted functions of ORFs in phage Chi were classified into five functional groups; structure (head-tail joining protein (chi_053), decorator protein (chi_0056), major capsid protein (chi_057), tape measure protein (chi_065), tail assembly protein 1 and 2 (chi_067 and chi_068), tail fiber protein (chi_071), and prohead protease (chi_055)), packaging (terminase small and large subunits (chi_051 and 052, respectively), phage portal protein (chi_054)), host lysis (lysis protein A (chi_003) and B (chi_002), endolysin-like protein (chi_004), and Rz1 protein (chi_005)), DNA manipulation (recombination associated protein (chi_023), primase (chi_042), DNA polymerase I (chi_048), and helicase (chi_050)), and additional function (N-6-adenine-methyltransferase (chi_017)).

BLASTP analysis of the functional ORFs showed that this phage genome is very similar to those of *Enterobacter* phage Enc34 and *Providencia* phage Redjac (Table 2.8). Interestingly, phage head proteins are very similar to those of *Enterobacter* phage Enc34 with 66 to 92% protein sequence identity. Furthermore, host lysis proteins are also similar to those of *Enterobacter* phage Enc34 with 57 to 76% protein sequence identity.

However, phage tail proteins are very similar to those of *Providencia* phage Redjac with 70 to 90% protein sequence identity, suggesting that phage Chi structural genes may be derived from the common ancestor. BLASTP best matches of DNA manipulation genes are mixed with those of two different bacteriophages, supporting this hypothesis (Table 2.8). In addition, further phylogenetic analysis of phage Chi and other bacteriophages based on major capsid proteins (MCPs) revealed that phage Chi is evolutionarily very close to these phages, *Enterobacter* phage Enc34 and *Providencia* phage Redjac, substantiating their close evolutionary relationship (Fig. 2.7). To further elucidate type of phage Chi, additional phylogenetic analysis of phage Chi was performed based on terminase large subunit following Casjens and Gilcrease's method (9) and the analysis result showed that phage Chi belongs to λ -like 5'-extended COS ends group (Fig. 2.8). To predict the lifestyle of phage Chi, PHACTS analysis was conducted with amino acid sequences of all predicted ORFs. However, clear lifestyle prediction was not possible for phage Chi probably due to extremely low amino acid sequence identities of predicted ORFs in Chi phage to those of other phages (data not shown).

While bacterial flagellum infection model of phage Chi was suggested to follow the “nut and bolt” model infecting through counter

clock-wise rotating flagella (24), the infection mechanism of phage Chi via host flagella based on its complete genome sequence analysis is not clearly understood yet, probably due to insufficient database information on the bacterial flagellum-targeting bacteriophages. Genome annotation result showed that 52 of 75 predicted ORFs encode hypothetical proteins, supporting this. Therefore, further functional genome study of phage Chi would explain the infection mechanism via host flagella in the near future.

Nucleotide sequence accession number. The complete genome sequence of bacteriophage Chi is available in GenBank database under accession number JX094499.

Table 2.7. The annotated sequence records of Chi phage

Locug_tag	Start	End	Strand	Product
chi_001	29	1921	+	hypothetical protein
chi_002	1986	2324	+	lysis protein B
chi_003	2328	3041	+	lysis protein A
chi_004	3038	3292	+	endolysin like protein
chi_005	3219	3422	+	possible Rz1 protein
chi_006	3423	3884	-	hypothetical protein
chi_007	3881	4780	-	hypothetical protein
chi_008	4770	5483	-	hypothetical protein
chi_009	5485	5937	-	hypothetical protein
chi_010	5934	6245	-	hypothetical protein
chi_011	6242	6523	-	hypothetical protein
chi_012	6604	7116	-	hypothetical protein
chi_013	7098	7352	-	hypothetical protein
chi_014	7342	7563	-	hypothetical protein
chi_015	7560	7754	-	hypothetical protein
chi_016	7765	8583	-	hypothetical protein
chi_017	8661	9347	-	possible N-6-adenine-methyltransferase
chi_018	9347	10447	-	hypothetical protein
chi_019	10444	11118	-	hypothetical protein
chi_020	11115	11855	-	hypothetical protein
chi_021	12051	12326	-	hypothetical protein
chi_022	12331	12564	-	hypothetical protein
chi_023	12567	13640	-	possible recombination associated protein RdgC
chi_024	13621	13962	-	hypothetical protein
chi_025	13949	14284	-	hypothetical protein
chi_026	14271	14711	-	hypothetical protein
chi_027	14783	15154	-	hypothetical protein
chi_028	15627	15800	+	hypothetical protein
chi_029	15915	16175	+	hypothetical protein
chi_030	16189	16728	+	hypothetical protein
chi_032	16743	16943	+	hypothetical protein
chi_031	16946	17428	+	hypothetical protein
chi_033	17440	17643	+	hypothetical protein
chi_034	17656	18204	+	hypothetical protein
chi_035	18201	18422	+	hypothetical protein
chi_036	18548	19702	+	hypothetical protein
chi_037	19714	19986	+	hypothetical protein
chi_038	19989	20195	+	hypothetical protein
chi_039	20188	20442	+	hypothetical protein
chi_040	20435	20686	+	hypothetical protein

Table 2.7. The annotated sequence records of Chi phage (continued)

Locug_tag	Start	End	Strand	Product
chi_041	21843	22130	+	helix-turn-helix domain-containing protein
chi_042	22171	24756	-	possible primase
chi_043	24753	25034	-	hypothetical protein
chi_044	25276	25692	+	hypothetical protein
chi_045	25755	26084	+	hypothetical protein
chi_046	26077	27417	+	conserved hypothetical protein
chi_047	27472	28068	+	conserved hypothetical protein
chi_048	28135	30174	+	putative DNA polymerase I
chi_049	30176	30463	+	VRR-NUC domain-containing protein
chi_050	30510	31985	+	possible helicase
chi_051	31972	32541	+	possible terminase small subunit
chi_052	32531	34606	+	putative terminase large subunit
chi_053	34617	34871	+	possible head-tail joining protein Lambda W
chi_054	34868	36550	+	phage portal protein, lambda family
chi_055	36577	37863	+	putative prohead protease ClpP
chi_056	37878	38297	+	possible decorator protein
chi_057	38310	39374	+	putative major capsid protein
chi_058	39436	39729	+	hypothetical protein
chi_059	39732	40097	+	hypothetical protein
chi_060	40184	40723	+	hypothetical protein
chi_061	40720	41223	+	hypothetical protein
chi_062	41237	42382	+	hypothetical protein
chi_063	42479	42940	+	hypothetical protein
chi_064	42985	43182	+	hypothetical protein
chi_065	43175	47470	+	putative tape measure protein
chi_066	47476	49164	+	hypothetical protein
chi_067	49174	49992	+	putative conserved tail assembly protein 1
chi_068	50004	50234	+	putative conserved tail assembly protein 2
chi_069	50234	50473	+	hypothetical protein
chi_070	50463	54353	+	hypothetical protein
chi_071	54428	55093	+	possible tail fiber protein
chi_072	55103	56110	+	hypothetical protein
chi_073	56121	57083	+	hypothetical protein
chi_074	57097	58116	+	hypothetical protein
chi_075	58131	59360	+	hypothetical protein

Table 2.8. Comparative analysis of predicted ORFs using BLASTP

Locus_tag	Predicted function	Length^a	BLASTP best match	Identity (%)^b
chi_002	lysis protein B	112	holin [Enterobacter phage Enc34]	64/112 (57.1)
chi_003	lysis protein A	237	endolysin [Enterobacter phage Enc34]	181/237 (76.4)
chi_004	endolysin like protein	84	putative Rz protein [Enterobacter phage Enc34]	56/84 (66.7)
chi_005	possible Rz1 protein	67	putative Rz1 protein [Enterobacter phage Enc34]	51/67 (76.1)
chi_017	possible N-6-adenine-methyltransferase	228	DNA methyltransferase [Enterobacter phage Enc34]	173/228 (75.9)
chi_023	possible recombination associated protein RdgC	357	recombination-associated protein [Enterobacter phage Enc34]	208/357 (58.3)
chi_041	helix-turn-helix domain-containing protein	95	hypothetical protein PaP1_gp024 [Pseudomonas phage PaP1]	23/95 (24.2)
chi_042	possible primase	861	DNA primase [Enterobacter phage Enc34]	624/861 (72.5)
chi_048	putative DNA polymerase I	679	DNA polymerase I [Providencia phage Redjac]	529/679 (77.9)
chi_049	VRR-NUC domain-containing protein	95	VRR-NUC domain protein [Enterobacter phage Enc34]	62/95 (65.3)
chi_050	possible helicase	491	DNA helicase [Providencia phage Redjac]	382/491 (77.8)
chi_051	possible terminase small subunit	189	terminase small subunit [Enterobacter phage Enc34]	161/189 (85.2)
chi_052	putative terminase large subunit	691	terminase large subunit [Providencia phage Redjac]	624/691 (90.3)
chi_053	possible head-tail joining protein Lambda W	84	head-to-tail joining protein [Enterobacter phage Enc34]	56/84 (66.7)
chi_054	phage portal protein, lambda family	560	phage portal protein [Providencia phage Redjac]	509/560 (90.9)
chi_055	putative prohead protease ClpP	428	ClpP [Providencia phage Redjac]	297/428 (69.4)

Table 2.8. Comparative analysis of predicted ORFs using BLASTP (continued)

Locus_tag	Predicted function	Length ^a	BLASTP best match	Identity (%) ^b
chi_056	possible decorator protein	139	phage structural protein [Enterobacter phage Enc34]	109/139 (78.4)
chi_057	putative major capsid protein	354	major capsid protein E [Enterobacter phage Enc34]	326/354 (92.1)
chi_062	bacterial Ig-like domain-containing protein	381	phage structural protein [Providencia phage Redjac]	304/381 (79.8)
chi_065	putative tape measure protein	1431	tape measure protein [Providencia phage Redjac]	1002/1431 (70.0)
chi_067	putative conserved tail assembly protein 1	272	conserved tail assembly protein [Providencia phage Redjac]	247/272 (90.8)
chi_068	putative conserved tail assembly protein 2	76	tail assembly protein [Enterobacter phage Enc34]	60/76 (78.9)
chi_071	possible tail fiber protein	221	tail fiber protein [Providencia phage Redjac]	162/221 (73.3)

^a, base pairs (bp)

^b, amino acid sequence identity

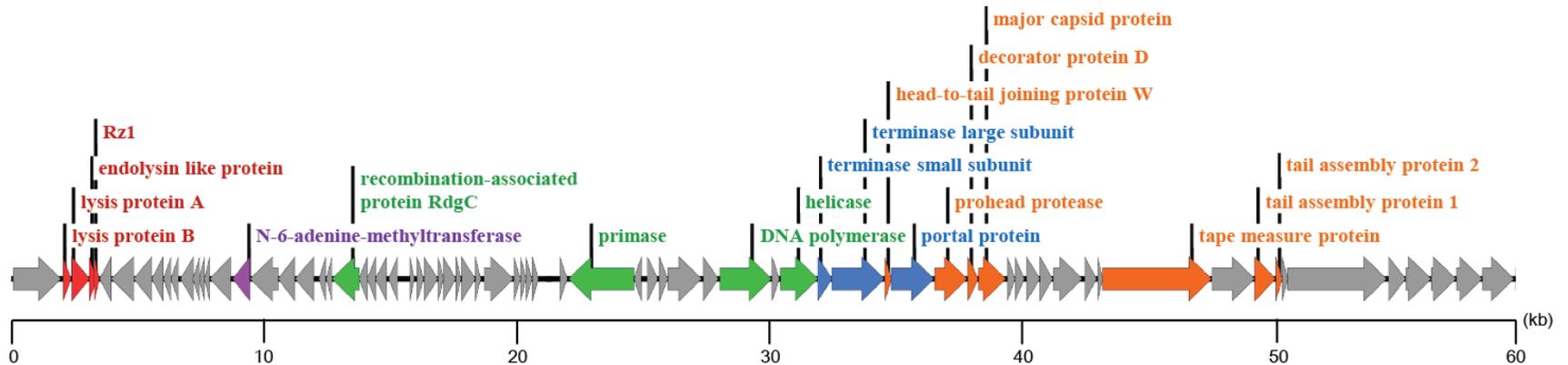


Figure. 2.6. Genome map of bacteriophage Chi. Functional ORFs were classified into five groups. Red, purple, green, blue, and orange arrows indicate host lysis, additional function, DNA manipulation, packaging, and structure-related ORFs, respectively. The scale unit is kilo base pairs (kb).

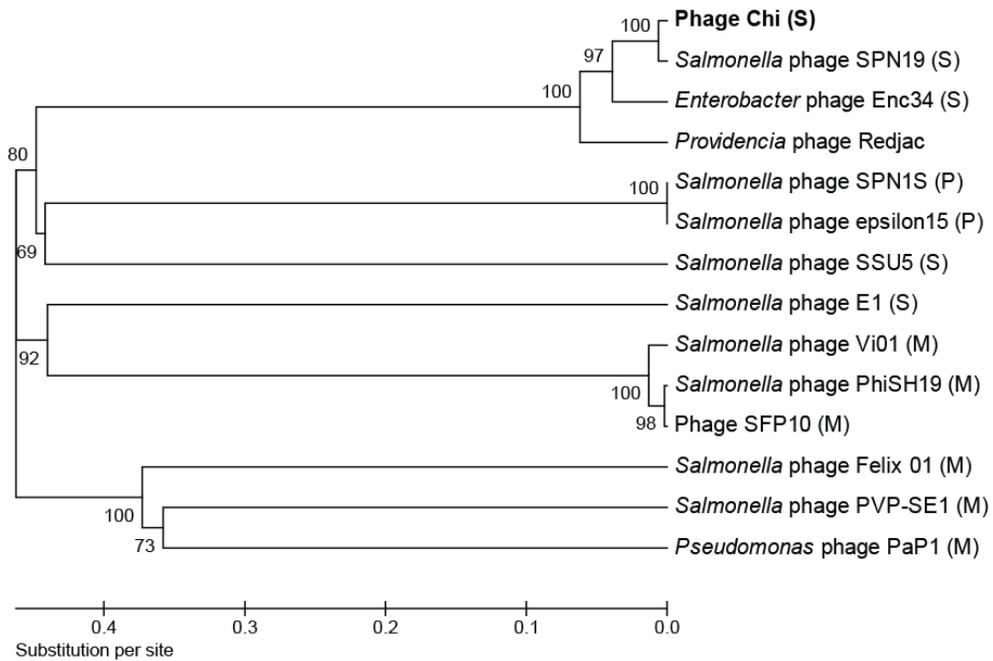


Figure. 2.7. Phylogenetic analysis of MCPs in phage Chi and other various bacteriophages. MCPs were compared by ClustalW alignments (16) and the phylogenetic tree was generated by the neighbor-joining method with *P* distance values using MEGA5 (15). *S*, *Siphoviridae*; *P*, *Podoviridae*; *M*, *Myoviridae*.

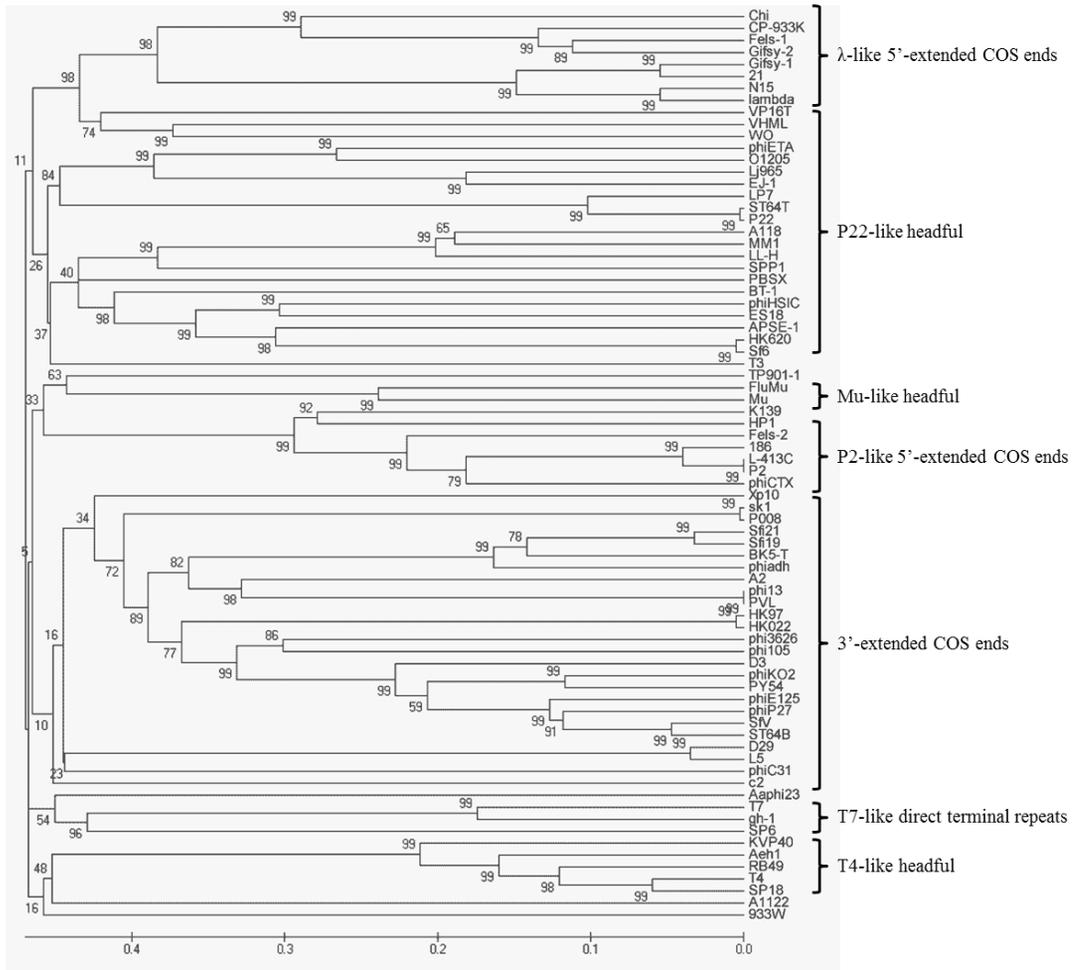


Figure. 2.8. Comparative phylogenetic analysis of terminase large subunits in phage Chi and other various bacteriophages. Amino acid sequences of terminase large subunits were compared by ClustalW alignments and the phylogenetic tree was generated by the neighbor-joining method with *P* distance values using MEGA5.

II-6-1-3. References

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II-6-2. Complete Genome Sequence of *Salmonella* Bacteriophage

SPN3US

(Published in Journal of Virology, 2011, 85: 13470-13471)

II-6-2-1. Abstract

Salmonella bacteriophage SPN3US was isolated from a chicken fecal sample. It is a virulent phage belonging to the *Myoviridae* family, showing effective inhibition against *Salmonella enterica* and a few *E. coli* O157:H7. Here, we announce the completely sequenced first genome of a *Salmonella* phage using flagella as a receptor. It is the largest genome among *Salmonella* phages to date and major findings from its annotation are described.

II-6-2-2. Main text

Salmonellosis is one of the most serious diseases caused by foodborne pathogens (8). While antibiotics have been broadly used to treat this disease, emergence of antibiotics resistance in *Salmonella* is getting more problematic (4). Bacteriophage is now considered as a good alternative biocontrol agent to inhibit this pathogen (9). Because the bacteriophage treatment gained the status of “Generally Recognized as Safe” by US FDA in 2006 (3), this approach as a phage therapy could be useful to inhibit pathogenic *Salmonella*.

The *Salmonella* bacteriophage SPN3US was isolated from chicken feces using a host strain *S. enteria* serovar Typhimurium LT2. The receptor study revealed that this phage infects *Salmonella* using flagella as a receptor (data not shown). Although flagella were previously reported as the phage receptor (6), the complete genome sequence of the *Salmonella* flagella-targeting phage has never been reported yet.

The genomic DNA was extracted from the stock using alkaline lysis method (11). A pyrosequencing approach was used with the Genome Sequencer FLX (GS-FLX) Titanium by Macrogen in Korea (55X coverage) and the quality filtered reads were assembled into a complete genome sequence using 454 Newbler 2.3 assembler. Prediction of open reading

frames (ORFs) was performed using GAMOLA automatic annotation program (1) and predicted ORFs were confirmed using Glimmer 3.02 (5), GeneMark.hmm (7) and FgeneV software (<http://www.softberry.com>). Annotation of predicted ORFs was conducted using the results of BLASTP (2) and InterProScan analyses (12).

The complete genome of phage SPN3US revealed 240,413 bp lengths with GC content of 48.54%, 264 ORFs and two tRNAs, suggesting the largest genome among *Salmonella* phages to date (Fig. 2.9) (10). The gene average length is 855 bp and gene density is 1.098 genes per kb. 87.5% of ORFs are positioned in one of two DNA strands.

While the gene coding percentage is 93.9% in the genome, 79.2% of ORFs were annotated to be hypothetical, probably due to insufficient database information on the functional genes of *Salmonella* phage genomes. This genome contains functional genes related to phage structure and packaging (major capsid protein, unknown phage structure proteins and terminase), tail structure for host interaction (tail fiber protein, tail sheath protein and tail-associated protein), replication/transcription (helicase, DNA-directed RNA polymerases, SbcCD nuclease, endodeoxyribonuclease, ribonuclease H and transcription regulator), host lysis (endolysin without holin) and additional functions (phage DNA adenine methylase for

protection from host restriction-modification system and dihydrofolate reductase/thymidylate kinase/thymidylate synthase for probably folate metabolism). However, these functional genes do not position in the same gene clusters but they are scattered over the genome. Interestingly, the repeats of six RNA polymerase beta subunits suggest that transcription of phage genes may be dominant rather than gene transcription of the host. This phage genome has only one phage fiber protein, probably interacting with the host flagella for infection. The complete genome analysis of this phage provides a new insight into its characteristics and interactions with *Salmonella*.

Nucleotide sequence accession number. The complete genome sequence of *Salmonella* phage SPN3US is available in GenBank under the accession number JN641803.

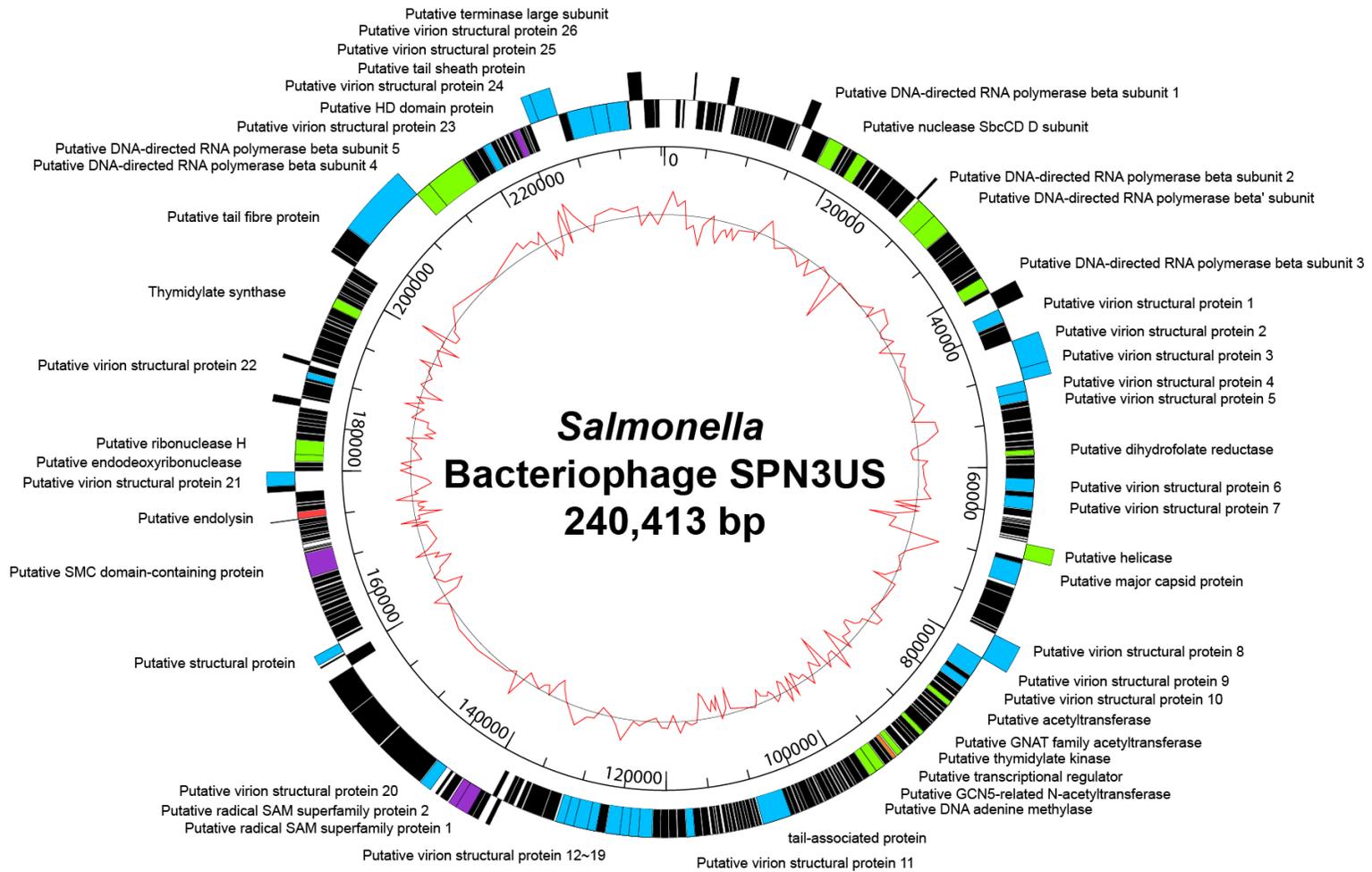


Figure 2.9. Genome map of bacteriophage SPN3US. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories such as phage structure and packaging (blue), regulation (orange), replication/transcription (green), host lysis (red) and additional functions (purple). The inner circle with red line indicates the G+C content. Scale unit is base pair.

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II-6-3. Complete Genome Sequence of *Salmonella enterica* serovar

Typhimurium Bacteriophage SPN1S

(Published in Journal of Virology, 2012, 86: 1284-1285)

II-6-3-1. Abstract

To understand the interaction between pathogenic *Salmonella enterica* serovar Typhimurium host and its bacteriophage, the bacteriophage SPN1S was isolated from environmental water. It is a lysogenic phage in the family of *Podoviridae*, using O-antigen of lipopolysaccharides (LPS) as a host receptor. Comparative genomic analysis between phage SPN1S and *S. enterica* serovar Anatum-specific phage ϵ 15 revealed the different host specificities probably due to low homology of host specificity-related genes. Here, we report the complete circular genome sequence of *S. Typhimurium*-specific bacteriophage SPN1S and its analysis results are shown.

II-6-3-2. Main text

Salmonella outbreak is one of the most common foodborne illnesses (more than 30% of all bacterial foodborne poisoning) (4, 5). More than 1.4 million cases of foodborne *Salmonella* outbreak have been reported every year in the United States and they have increased by 10% in recent years (4, 9, 13). Moreover, emergence of multi-drug resistant *Salmonella* such as *S. Typhimurium* phage type DT104 has been getting more problematic (7, 14). To control these drug-resistant *Salmonella* strains, applications of *Salmonella*-specific bacteriophage have been proposed (8, 15). Therefore, it is important to understand the infection mechanism between *Salmonella* host and *Salmonella*-specific phages. To increase our knowledge on this interaction, a *S. Typhimurium*-specific phage SPN1S was isolated from environmental water and its genome was completely sequenced.

The genomic DNA of phage SPN1S was isolated using an alkaline lysis method (16) and sequenced using the Genome Sequencer FLX (GS-FLX) Titanium technology in Macrogen, Korea with 130X coverage. Sequence assembly of quality filtered reads was performed using 454 Newbler 2.3 assembler. From the complete genome sequence of phage SPN1S, open reading frames (ORFs) were predicted using GAMOLA automatic annotation program (1) and confirmed using GeneMarkS (3),

Glimmer 3.02 (6) and FgenesV (<http://www.softberry.com>). Conserved protein domain analysis was conducted using BLASTP (2), InterProScan (17) and NCBI Conserved Domain Database (CDD) (12). Prediction of tRNAs was carried out using tRNAscan-SE program (11).

Bacteriophage SPN1S has a circular genome consisting of 38,684 bp with GC content of 50.16%, 52 ORFs but no tRNA (Fig. 2.10). The annotation of this genome reveals that phage packaging (terminase small and large subunits), morphogenesis (a phage head-tail connector protein, an endoprotease, a major capsid protein and a minor structural protein), host specificity (a tailspike protein), conversion of host LPS (a GtrA and two copy of lipopolysaccharide modification acyltransferase), host lysis (a holin, an endolysin, a Rz-like protein and a Rz1), DNA replication/modification (a DNA replication protein, an integrase, an exonuclease VIII/RecE-like protein and an adenine methylase) and transcription regulation (a transcriptional activator and transcriptional regulators).

Comparative genome analysis of phage SPN1S and *S. enterica* serovar Anatum-specific phage ϵ 15 (GenBank accession number AY150271) revealed that while these two phages are closely related in the DNA level, host specificity-related genes encoding tailspike/tail fiber proteins are quite different. In addition, the receptor study of phage SPN1S showed that the

tailspike protein (SPN1S_0022) interacts with O-antigen of LPS in *S. Typhimurium*, suggesting that this phage infects the host strain via LPS as a host receptor (Fig. 2.11). Interestingly, Rz1 and Rz-like protein collaborate with the endolysin for host lysis. A single gene expression of the endolysin gene using *E. coli* gene expression system does not lyse the host strain, but co-expression of the genes encoding endolysin, Rz1 and Rz-like protein really do (10). The genome study of this phage SPN1S would increase our knowledge on interaction between *S. Typhimurium* host and its bacteriophages.

Nucleotide sequence accession number. The complete genome sequence of *S. enterica* serovar Typhimurium phage SPN1S is available in GenBank under the accession number JN391180.

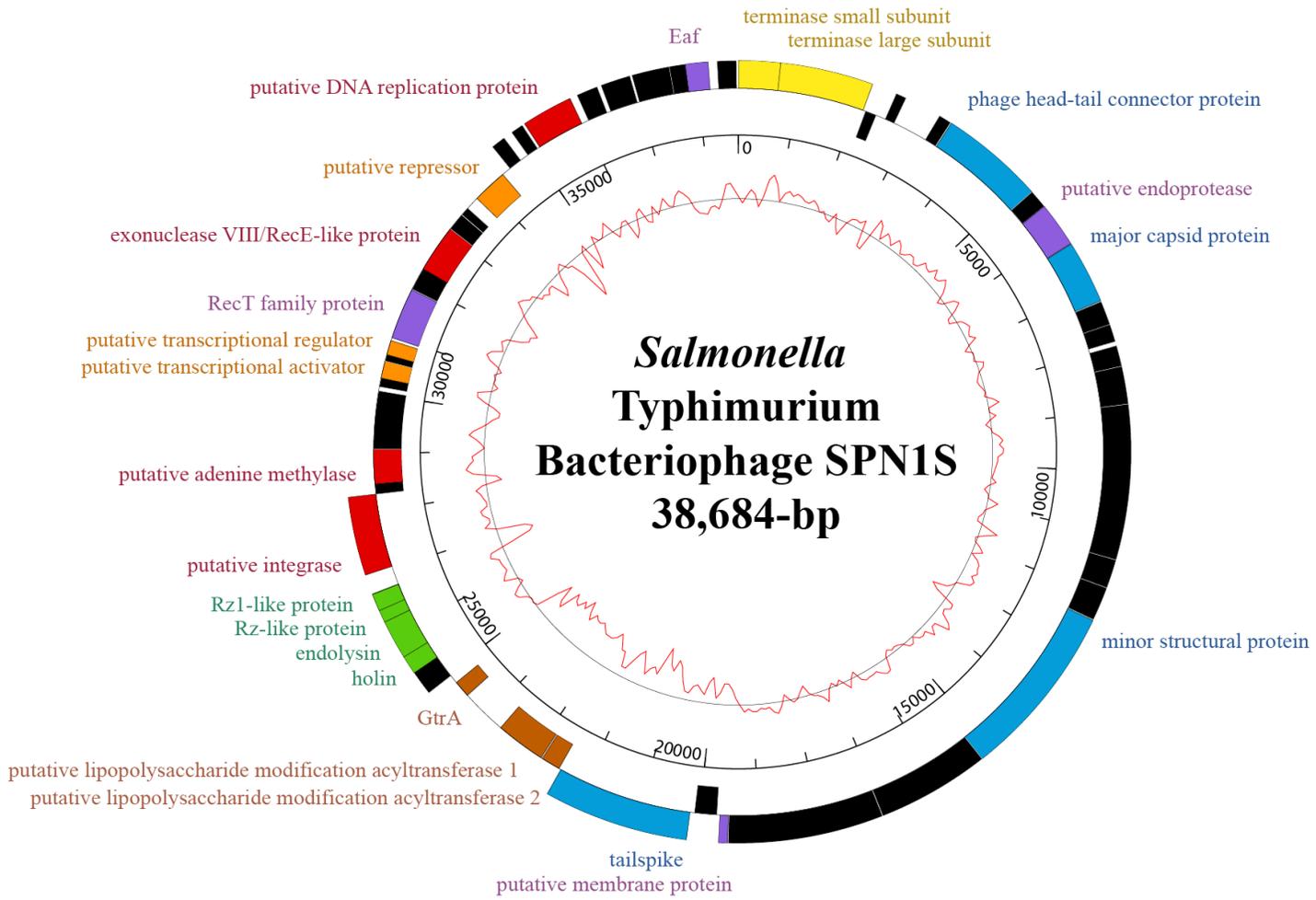
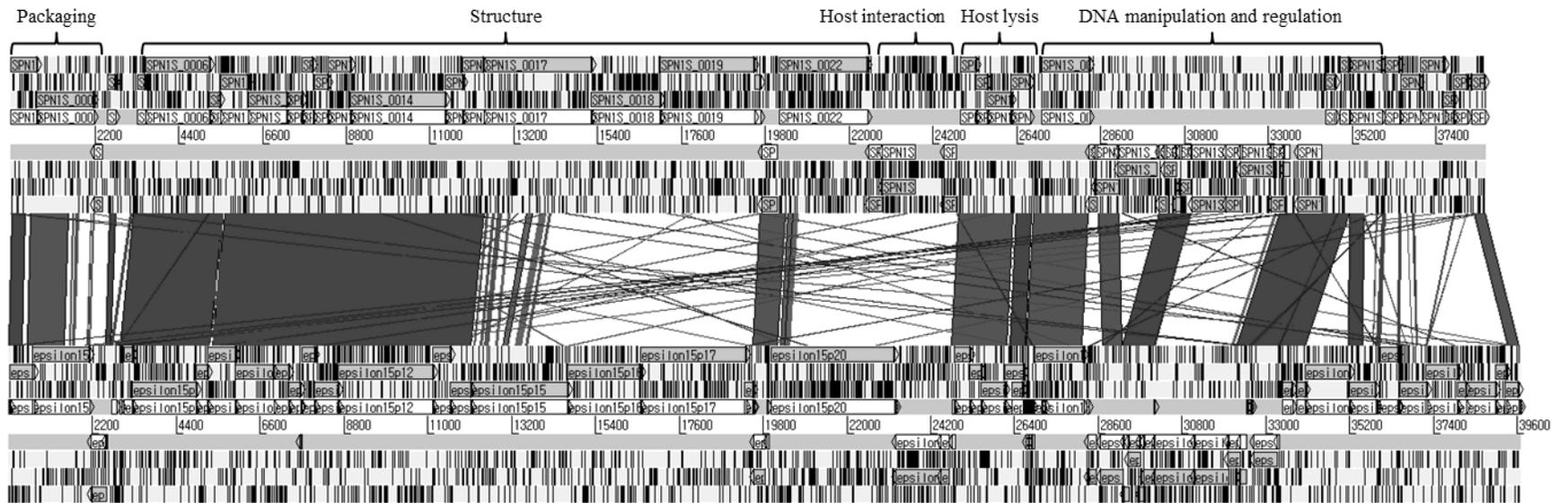


Figure 2.10. Genome map of bacteriophage SPN1S. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories. Yellow, packaging; Blue, structure; Brown, host interaction; Green, host lysis; Red, DNA manipulation; Orange, regulation; Purple, additional functions. The inner circle with red line indicates the G+C content. Scale unit is base pair.

SPN1S



Epsilon15

Figure 2.11. Comparative genomic analysis of SPN1S phage and Epsilon15 phage. Comparative analysis of complete genome sequences of SPN1S (upper) and Epsilon15 (bottom) using BLASTN and ACT10.

II-6-3-3. References

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II-6-4. Complete Genome Sequence of *Salmonella enterica* serovar

Typhimurium Bacteriophage SPN3UB

(Published in Journal of Virology, 2012, 86: 3404-3405)

II-6-4-1. Abstract

Salmonella is one of the major pathogenic bacteria causing food poisoning. To elucidate the host infection mechanism of *S. enterica* serovar Typhimurium-targeting phages, the bacteriophage SPN3UB was isolated from a chicken fecal sample. This phage morphologically belongs to *Siphoviridae* family and infects the host via O-antigen of LPS. To further understand its infection mechanism, the genome was completely sequenced and analyzed. Here, we announce its complete genome sequence and show major findings from the genomic analysis results.

II-6-4-2. Main text

Salmonella is a pathogenic bacterium causing salmonellosis via contaminated foods (6, 7). To develop phages as a biocontrol agent for controlling this pathogen in foods, understanding of the host infection mechanism of *Salmonella* phages is important (2, 3, 10). While O-antigen of lipopolysaccharides (LPS) is a common host receptor for infection in *Myoviridae* (FelixO1) (11) and *Podoviridae* (P22, ε34) families (4, 13, 14), the phages in *Siphoviridae* family using this host receptor such as SETP3 are rare (8). *S. Typhimurium*-targeting SPN3UB phage belonging to *Siphoviridae* family could not infect the *rfaL* (O-antigen ligase)-deficient mutant strain of *S. Typhimurium* SL1344 (data not shown), suggesting that it infects the host via O-antigen of LPS (12). To further understand its host infection mechanism, the genome was completely sequenced and analyzed.

Phage genomic DNA was isolated using standard alkaline lysis method (15) and sequenced using the Genome Sequencer FLX Titanium by Macrogen, Korea. Assembly of quality filtered reads was performed using a 454 Newbler 2.3 assembler and open reading frames (ORFs) were predicted using GeneMarkS (5), Glimmer 3.02 (9), and FgenesV (Softberry, Inc., Mount Kisco, KY) and their ribosomal binding sites were confirmed using RBSfinder (J. Craig Venter Institute, Rockville, MD). Annotation of the

predicted ORFs was conducted using BLASTP (1) and InterProScan (16).

SPN3UB phage has a circular dsDNA-based genome consisting of 47,355-bp length with a GC content of 49.61% and 71 ORFs but no tRNA, indicating that it is the largest genome sequence in *Siphoviridae* phage using O-antigen of *Salmonella* LPS as a host receptor (Fig. 2.12). This phage genome encodes head/tail structure proteins (major capsid protein, tape measure protein, minor tail protein M and L, tail assembly protein K and I, and tail fiber protein J), phage packaging (terminase large and small subunits), integration and recombination (integrase, excisionase-like protein, RecT recombinase, and RecE exodeoxyribonuclease), lysogeny control (Cro, CI, and CII), phage replication (PrpO replication protein and DnaC DNA replication protein), antitermination (antitermination protein Q), host cell lysis (endolysin and Rz/Rz1 endopeptidases), and additional functions (Arc-like DNA binding protein, antirepressor family protein, Eaa protein, DinI DNA damage-inducible protein, NinG, and Kila-N domain protein). Because this phage has only one tail fiber protein J, it may play an important role in the host infection via O-antigen of LPS. The lysogeny control proteins and antitermination protein Q may contribute to formation of lysogen during infection and reconstruction of the phage from the lysogen was confirmed by mitomycin C induction (data not shown). Interestingly,

some of replication proteins such as helicase, primase, etc. are missing. Probably, this phage takes advantage of host replication proteins or they are annotated to hypothetical proteins due to too low identity with other phage replication proteins in GenBank database. The complete genome sequence of *S. Typhimurium* SPN3UB phage provides extended information about host infection and interaction mechanisms with this phage.

Nucleotide sequence accession number. The complete genome sequence of *S. Typhimurium* bacteriophage SPN3UB is available in GenBank under accession number JQ288021.

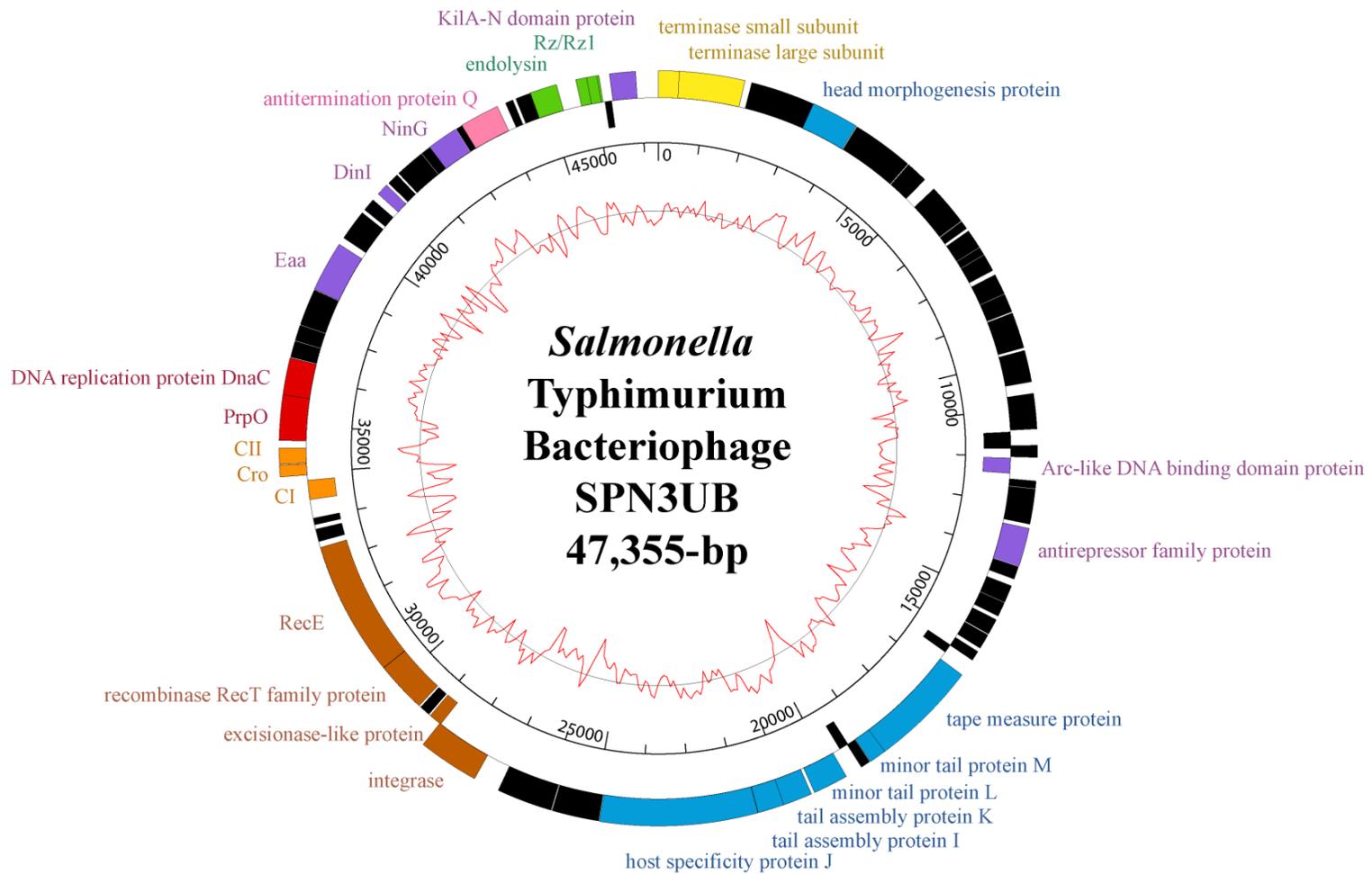


Figure 2.12. Genome map of bacteriophage SPN3UB. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories. Yellow, packaging; Blue, structure; Brown, integration and recombination; Green, host lysis; replication, DNA manipulation; Pink, antitermination; Orange, regulation; Purple, additional functions. The inner circle with red line indicates the G+C content. Scale unit is base pair.

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**Chapter III. Genomic Investigation of Lysogen
Formation and Host Lysis Systems of the *Salmonella*
Temperate Bacteriophage SPN9CC**

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III-1. Introduction

Food poisoning is generally caused by uptake of foods or drinks contaminated by foodborne pathogenic bacteria, such as *Salmonella*, *E. coli*, *Listeria*, and *Campylobacter* (44). Among these bacteria, *Salmonella* is one of the most common pathogens. The bacterium causes salmonellosis with various symptoms, such as diarrhea, vomiting, high fever, and even death (3, 11). In the United States, more than 1.4 million cases of *Salmonella* have been reported, and the number has increased by more than 10% annually in recent years (11, 22, 44). Although antibiotics have been widely used to control this pathogen and salmonellosis, multi-drug resistant *Salmonella* strains, such as *S. Typhimurium* DT104, have appeared (12, 53).

Due to the emergence of antibiotic-resistant *Salmonella*, a novel approach using bacteriophage has been proposed to control them (21, 48). To take advantage of the phage treatment against salmonellosis, it is necessary to characterize *Salmonella* phages phenotypically and genotypically. Moreover, understanding of infection mechanisms between *Salmonella* host and *Salmonella*-targeting phages is important for this purpose. The major host-phage infection processes include the phage attachment via host receptor, control of the host lytic-lysogenic cycle, and

the host lysis mechanism.

Several *Salmonella* host receptors for phage infection have been experimentally determined and characterized, such as flagella (30, 62), Vi capsular antigen (52), LPS (60), and host outer membrane proteins (OmpC (26), BtuB (29, 31), TolC (58), and FhuA (9)). These receptors play a role in the determination of host specificity of the phages, suggesting that host receptor study would be able to provide novel insights in the infection mechanisms between *Salmonella* host and the phages. Lambdoid lysogenic phages generally contain a lysogeny control region consisting of *cro*, *cI*, *cII*, *cIII*, *N*, and *Q* (6, 67). Constitutive bacteriophage promoters, P_L and P_R , express N and Cro proteins. N protein binds to all terminators for antitermination. During this early gene expression, CII, CIII and Q proteins are produced. Among these proteins, CII-CIII complex activates P_{RE} and P_I promoters, resulting in the lysogenic cycle by production of integrase and CI protein, which are related to phage genome integration and blocking of all phage gene expressions. At this point, if the host HflA proteolytic enzyme is activated in the presence of low concentration of cAMP due to a sufficient supply of glucose to the host, it digests CII protein such that CII-CIII complex cannot produce CI protein, resulting in the prevention of the lysogenic cycle. Furthermore, Q protein activates gene expression related to

phage structure and host cell lysis. Therefore, the study of the lysogeny control region is important to understand the phage lytic/lysogenic cycles in the host. Holin and endolysin are known to be important for host cell lysis (73). Holin creates holes in the cytoplasmic membrane. This hole is used as a transport channel for endolysin, which digests the peptidoglycan layer. In addition, Rz/Rz1-like proteins often enhance endolysin activity as endolysin accessory proteins (34).

Salmonella-targeting P22 phage belongs to the family of *Podoviridae* morphologically and has been well-characterized to develop genetic transfer tools via lysogenization (6, 67). Host receptor studies have revealed that the phage tailspike protein plays a role in the interaction with the host by interacting with the O-antigen of LPS in *S. Typhimurium* (4, 69). The complete genome sequence analysis of P22 phage also revealed the presence of functional genes related to lysogenization and host specificity determination (6, 51). In addition, comparative genomic analysis of P22 and closely related phages revealed the presence of the P22-like phage group (10). This group includes ϵ 34, ST104, ST64T, SE1, c341, and HK620. They share phage morphogenesis and assembly genes for similar morphology and generally infect *Salmonella*, *E. coli*, and *Shigella* in the *Enterobacteriaceae* family. However, while ant moron regions in phage P22 have been known to

be involved in the regulation of gene expression, these regions are completely or partially missing in other P22-like phages (72). Although the role of this region is not clearly understood yet, it may be related to lysogeny conversion (41). Further genome studies of these P22-like phages indicate that morphogenesis-related genes are highly conserved, but other genes are variable, suggesting that even though they have similar phage morphologies, the host specificity of these P22-like phages may differ among them. Therefore, further study of these P22-phages would provide new information on the host infection of the phages in this group.

To understand the infection mechanisms of the bacteriophage at the genomic level, the complete genome of SPN9CC was analyzed and compared with P22-like phage genomes. In addition, a ΔcI mutant of the lysogen-forming P22-like SPN9CC phage was constructed and characterized. This study will be useful for increasing our knowledge of the host infection and lysis mechanisms of P22-like phages, including SPN9CC.

III-2. Materials and Methods

III-2-1. Bacterial strains and growth conditions

The bacterial strains used in this study and gene knockout mutant strains for the host receptor study are listed in Table 3.1. Prophage-free *Salmonella enterica* serovar Typhimurium LT2C was used for the isolation and propagation of *S. Typhimurium*-targeting phages (19) (Cancer Research Center, Colombia, MO, USA). All of the bacteria listed in Table 3.1 were cultivated at 37°C for 12 h in Luria-Bertani (LB) broth medium (Difco, Detroit, MI), and the agar medium was prepared with 1.5% agar supplementation (Difco) of the broth medium.

Table 3.1. Host range of SPN9CC bacteriophage

Bacterial host	SPN9CC ^a	Source ^b or reference
<i>Salmonella enterica</i> serovar Typhimurium		
LT2	+++	(43)
LT2C	+++	(19)
SL1344	+++	NCTC
UK1	+++	(16)
ATCC 14028s	+++	ATCC
DT104	+	(53)
ATCC 43174	++	ATCC
<i>Salmonella enterica</i> serovar Enteritidis		
ATCC 13076	+	ATCC
<i>Salmonella enterica</i> Paratyphi		
A IB 211	++	IVI
B IB 231	-	IVI
C IB 216	-	IVI
<i>Salmonella enterica</i> Dublin		
IB 2973	+	IVI
<i>E. coli</i>		
K-12 MG1655	-	(25)
DH5 α	-	ATCC
O157:H7 ATCC 35150	-	ATCC
O157:H7 ATCC 43890	-	ATCC
Gram-negative bacteria		
<i>Shigella flexneri</i> 2a strain 2457T	-	IVI
<i>Shigella boydii</i> IB 2474	-	IVI
<i>Vibrio fischeri</i> ATCC 700601	-	ATCC
<i>Pseudomonas aeruginosa</i> ATCC 27853	-	ATCC
<i>Cronobacter Sakazakii</i> ATCC 29544	-	ATCC
Gram-positive bacteria		
<i>Enterococcus faecalis</i> ATCC 29212	-	ATCC
<i>Staphylococcus aureus</i> ATCC 29213	-	ATCC
<i>Bacillus cereus</i> ATCC 14579	-	ATCC
<i>Listeria monocytogenes</i> ATCC 19114	-	ATCC
Mutants of <i>S. Typhimurium</i> SL1344		
Δ flgK	+++	(63)
Δ btuB	+++	(31)
Δ rfaL	-	(50)
Δ rfaL (pUHE21-lacI ^U ::rfaL)	+++	(50)

^a, +++, EOP 1 to 00.1; ++, EOP 0.01 to 0.0001; +, EOP, less than 0.001; -, no susceptible to SPN9CC.

^b, NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; IVI, International Vaccine Institute.

III-2-2. Bacteriophage isolation and propagation

Commercially processed broiler skin samples were collected from the Moran traditional market, Seongnam, South Korea and used for isolation of *S. Typhimurium*-targeting bacteriophage SPN9CC with *S. Typhimurium* LT2C strain. The basic procedures for the isolation and propagation of bacteriophage SPN9CC were previously described by Shin *et al.*(63).

III-2-3. Lysogen induction

Selected SPN9CC lysogens of *S. Typhimurium* LT2C were cultivated at 37°C until the OD_{600 nm} reached 1.0, and 0.5 µg/ml of mitomycin C (Sigma, St. Louis, MO) was added to the cultures. Then, these cultures were additionally incubated at 37°C for 2 h. After incubation, the cells were removed by centrifugation and filtration, and the supernatant was collected. The spotting assay of this supernatant with *S. Typhimurium* LT2C was conducted to confirm the presence of induced SPN9CC phage.

III-2-4. Electron microscopy

A transmission electron microscope (TEM) was used for morphological analysis of purified SPN9CC phage. This TEM analysis was performed using the procedure described by Shin *et al.* (63). The

morphological classification of SPN9CC phage was conducted according to the guidelines of the International Committee on Taxonomy of Viruses (20).

III-2-5. Host range determination by spotting assay

The host range and comparative efficiency of plaquing (EOP) test of SPN9CC phage were determined using a spotting assay with *S. Typhimurium*, *S. Paratyphi*, *E. coli*, and other gram-negative and gram-positive bacterial strains using the procedure previously described by Park *et al.* (50).

III-2-6. Genome sequencing and bioinformatics analysis

Genomic DNA of SPN9CC phage was isolated and purified as described by Sambrook *et al.* (61). The construction of a genomic DNA library and pyrosequencing using Genome Sequencer FLX (GS-FLX) Titanium (Roche, Mannheim, Germany) were conducted by Macrogen, Korea. The prediction of open reading frames (ORFs) was conducted using Glimmer 3.02 (18), GeneMarkS (5), and FgenesV (Softberry, Inc., Mount Kisco, NY). The prediction of ribosomal binding sites (RBS) of ORFs was performed using RBSfinder (J. Craig Venter Institute, Rockville, MD). The annotation of predicted ORFs was conducted using BLASTP (2) and

InterProScan (75) with conserved protein domain databases. The GenBank data file was generated using GAMOLA (1) and Sequin programs (National Center for Biotechnology Information, Bethesda, MD). The phylogenetic analysis of major capsid proteins from bacteriophages, including SPN9CC, was performed using MEGA5 with the neighbor-joining method using *p*-distance values (35). The program Mobylye was used for comparative codon usage analysis of the *S. Typhimurium* SL1344 host and SPN9CC phage (46). Comparative genomic analysis of SPN9CC with other P22-like phages and visualization were conducted using BLASTN (2) and ACT12 (7).

III-2-7. Gene expression of the host lysis gene cluster

The SPN9CC_0042, SPN9CC_0043, SPN9CC_0044 and SPN9CC_0044_1 genes encoding holin, endolysin, and Rz/Rz1 endopeptidases, respectively, in the host lysis gene cluster were amplified using PCR with the primers listed in Table 3.2. These PCR products were double-digested using EcoRI and SalI and cloned into the multiple cloning site (MCS) of pBAD18 (24) individually or in combination with more than two genes. These cloned plasmids are listed in Table 3.3. *S. Typhimurium* SL1344 and *E. coli* MG1655 were used as gene expression hosts of the cloned pBAD18 plasmids after transformation. The expression of the cloned

genes was induced by addition of 0.2% arabinose (final concentration) after a 2 h incubation of the sub-inoculated cultures. To test the lysis activity of host lysis proteins during incubation and the induction of the cultures, optical density was measured at 600 nm wavelength every hour.

Table 3.2. Primers used in this chapter

Primer	Sequence (5' to 3')^a	Reference
SPN9CC_0042_F	TAAAAGA <u>AATTC</u> AAATCCCCTCAATAAAGGGGGTAGAG	This study
SPN9CC_0042_R	TTTTT <u>GTCGACTT</u> ATCGCCGCTATTACGCTATTTTC	This study
SPN9CC_0043_F	AAAAAGA <u>AATTC</u> AAACGCAAAGAGCGTGAGGACAG	This study
SPN9CC_0043_R	TTTTT <u>GTCGACATA</u> ATCGCGGTTACTCTGCTCATTG	This study
SPN9CC_0044_F	AAATT <u>GAATTC</u> TTGAGCGTGAAGTCTGTTTGTGGG	This study
SPN9CC_0044_R	AAAAA <u>GTCGACT</u> TATGTGATGGAAATTATTTTCAGGCATTG	This study
9CC-BRED_C	TCTTAAAAGTGA <u>ACTCAT</u> CACCACATAACCTTGCAATGCA AAAAGCTTCGCTATGTCATAACCAGTTCATTTTCATCCTTAA ATTATACA	This study
9CC-BRED_CF	TTGTAGGAATACTTGTCCGCTGTCTTTGATGAGCTTCTTAA AAGTGA <u>ACTCAT</u> CATGTAGGCTGGAGCTGCTTCG	This study
9CC-BRED_CR	TTTACGATTTGTGACTGTTCTTGTTTGATACAAATTGTATAA TTAAGGATGAAAATTCGGGGATCCGTCGACC	This study
9CC-BRED_conf_F	TATCTCATCAGGCCATTGGCTGGCTACAAC	This study
9CC-BRED_conf_R	TAATGACAAACTGCACCACGCGTACAACCG	This study

^a, Underbars indicate specific restriction enzymes for cloning. Forward and reverse primers contain EcoRI and SalI sites, respectively.

Table 3.3. Plasmids used in this chapter

Plasmid	Description	Reference
pBAD18	<i>P_{araC}</i> , <i>ColE1 ori</i> , and Amp ^R	(24)
pBAD18-42	pBAD18 expressing SPN9CC_0042	This study
pBAD18-43	pBAD18 expressing SPN9CC_0043	This study
pBAD18-44	pBAD18 expressing SPN9CC_0044	This study
pBAD18-42/43	pBAD18 expressing SPN9CC_0042 and SPN9CC_0043	This study
pBAD18-43/44	pBAD18 expressing SPN9CC_0043 and SPN9CC_0044	This study
pBAD18-42/43/44	pBAD18 expressing SPN9CC_0042, SPN9CC_0043, and SPN9CC_0044	This study
pUHE21-2 <i>lacI^q</i>	pMB1 <i>ori</i> , Amp ^R , and <i>lacI^q</i>	(64)
pUHE21-2 <i>lacI^q::rfaL</i>	pUHE21-2 <i>lacI^q</i> expressing <i>rfaL</i>	(50)
pUHE21-2 <i>lacI^q::flgK</i>	pUHE21-2 <i>lacI^q</i> expressing <i>flgK</i>	(63)
pACYC184	p15A <i>ori</i> , Cm ^R , and Tet ^R	(13)
pMS100	pACYC184 expressing <i>btuB</i>	(31)

III-2-8. Deletion of *cI* gene in SPN9CC genome using BRED

The *cI* gene of SPN9CC was specifically deleted using Bacteriophage Recombineering of Electroporated DNA (BRED) method previously described by Marinelli *et al.* (42). To delete *cI* gene using the BRED method, a 200-bp dsDNA substrate containing a 100-bp region upstream and the other 100-bp region downstream the *cI* target gene was PCR amplified using primers (9CC-BRED_C, 9CC-BRED_CF and 9CC-BRED_CR, listed in Table 3.2). A *S. Typhimurium* SL1344 electroporation host with pKD46 encoding recombinase was induced with arabinose and used for electrocompetent cell preparation (17). The phage genomic DNA and 200-bp DNA substrate were co-electroporated into the arabinose-induced electrocompetent cells for homologous recombination. After a 1 h shaking incubation of the transformants at 37°C, 6 ml of 0.4% molten LB top agar containing 200 µl of the transformant culture was overlaid on the 1.5% LB base agar and incubated overnight. Plaques were randomly picked and plaque-PCR was performed with specific primers (9CC-BRED_conf_F and 9CC-BRED_conf_R, listed in Table 3.2). The plaque-PCR products were partially sequenced with the same primers to confirm the deletion of *cI* gene. The *cI* gene-deleted phage (SPN9CCM) was purified using the single-picking method and was propagated previously described above.

III-2-9. One-step growth curve and bacterial challenge test

S. Typhimurium SL1344 was used as a host strain in these assays.

The overall procedures for the performed one-step growth curve assay and challenge test followed the protocol described by Park *et al.* (50).

III-2-10. Nucleotide sequence accession number

The GenBank accession number for the complete genome sequence and annotation information of bacteriophage SPN9CC is JF900176.

III-3. Results

III-3-1. Isolation and morphology of SPN9CC phage

For the development of new biocontrol agents, *Salmonella*-targeting bacteriophages were isolated from a commercially processed broiler skin sample using a host strain of *S. Typhimurium* LT2C. Among these phages, SPN9CC phage produced distinct clear plaques with cloudy centers (Fig. 3.1A), suggesting the possibility of lysogen formation in the cloudy center. Mitomycin C treatment of the colonies isolated from the cloudy centers of the clear plaques revealed the induction of SPN9CC phage, confirming lysogen formation (data not shown). TEM morphological observation revealed that this phage has the typical short tails belonging to the *Podoviridae* family (Fig. 3.1B).

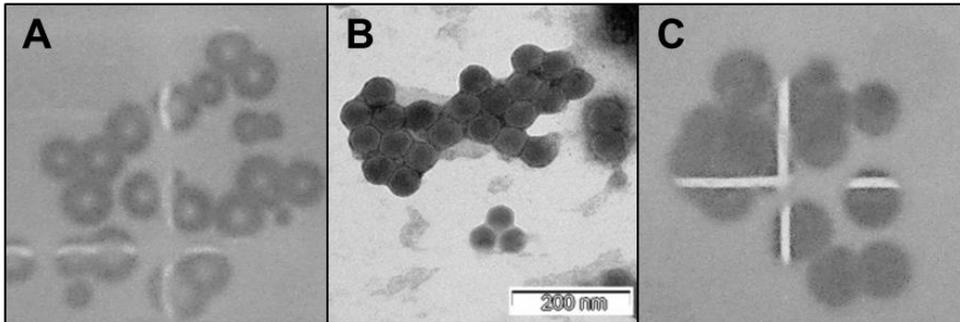


Figure 3.1. Morphological characteristics of SPN9CC phage. Bulls-eye shape plaque morphology in dotting assay (A) and TEM morphology (B). (C) Plaque morphology of SPN9CCM phage in dotting assay.

III-3-2. Host range and host receptor study

The host range test of SPN9CC phage demonstrated specific inhibition against *S. Typhimurium*, *S. Paratyphi*, and *S. Dublin*. However, various gram-positive and gram-negative bacteria, including other *Salmonella* strains, were not inhibited by this phage, suggesting that this phage specifically infects certain strains of *Salmonella* (Table 3.1). To determine the host receptor for SPN9CC phage, previously constructed mutants of *S. Typhimurium* SL1344 were used, including *ΔflgK* (encoding flagellar hook-associated protein), *ΔbtuB* (encoding a vitamin B₁₂-uptake protein), and *ΔrfaL* (encoding O-antigen ligase) (31, 50, 63). Only the *ΔrfaL* mutant displayed resistance to SPN9CC phage, suggesting that the O-antigen of LPS is a host receptor for phage infection. Subsequent complementation of this mutant with the pUHE21-*lacI^q*::*rfaL* expression vector (50) confirmed O-antigen as a receptor of SPN9CC (Table 3.1).

III-3-3. Bacteriophage genome analysis

The complete genome sequencing of SPN9CC was performed with approximately 90 times coverage using the next-generation sequencing (NGS) technology with a 454 pyrosequencer, revealing 40,128-bp with a GC content of 47.33%, 63 putative ORFs, and 2 tRNAs (tRNA_{Thr} and

tRNA_{Asn}) (Fig. 3.2). The comparative codon usage preference analysis of tRNA_{Thr} between *S. Typhimurium* SL1344 host and SPN9CC phage indicated a different preference in threonine, suggesting that this tRNA may play a role in the translation of phage mRNA, and not of the host mRNA. In addition, gene density was observed to be 1.545 genes per kb, and the coding percentage was 90.9%. The average length of each ORF was determined to be 588-bp. Comparative phylogenetic analysis using major capsid proteins from various phages revealed that SPN9CC phage is closely related to *Salmonella*-targeting P22-like phages, such as P22, ST64T, ST104, and ε34 (Fig. 3.3).

Annotation and functional analysis of ORFs in this genome revealed that 44 of 63 ORFs have putative functions. Functional categorization of these genes revealed 14 groups, such as LPS modification and superinfection exclusion (O-antigen conversion proteins, GtrABC and superinfection exclusion protein B), integration (phage integrase), P22 *ea* genes (Eaa and Eai), recombination (Erf recombination protein, Abc1 and Abc2 anti-RecBCD proteins), antitermination (antitermination protein N and Q), lysogeny control (Cro, CI, and CII), replication (DNA replication protein and helicase), *nin* genes (NinABEFHXZ), host cell lysis (holin, endolysin, and Rz/Rz1 endopeptidases), DNA packaging (terminase large

and small subunits), head (portal protein, scaffolding protein, and major capsid protein), tail (DNA stabilization proteins/tail accessory proteins (Gp4, Gp10, and Gp26), head assembly protein, and DNA transfer proteins/ejection proteins)), Ant moron (Mnt regulatory protein), and host specificity (tailspike protein).

The roles of LPS modification proteins (GtrABC) and superinfection exclusion protein B are the prevention of other phage infections after lysogen formation via modification of its host O-antigen of LPS (6, 67). Among the recombination proteins, Abc1 and Abc2 anti-RecBCD proteins are involved in phage recombination and protect both ends of linear phage genome from host RecBCD exonuclease, and Erf recombination protein circularizes this linear genome by ligation of both ends of the phage genome (54). Lysogeny control and antitermination determine the phage lytic/lysogenic cycles depending on the host status. Replication proteins are produced during early gene expression, and they are responsible for phage genome replication. However, the functions of *ea* genes and *nin* genes are unknown (15). Host cell lysis proteins such as holin, endolysin, and Rz/Rz1-like proteins are suggested to cooperate in bursting the host cell after replication and reconstruction of the phage (34). Holin creates pinholes in the host inner membrane and subsequent secretion of

endolysin via these pinholes results in the host cell lysis. Although the regions of ant moron have been found in P22-like phages, these regions are highly variable among them (72), and the function of ant moron is not clearly understood. SPN9CC phage also has only one gene in this region, a *mnt* gene encoding repressor protein, which is very similar to ST104 and ST64T phages. This Mnt repressor was suggested to control the expression of *ant* gene encoding an anti-repressor (71). As with other P22-like phage of *Podoviridae* members, SPN9CC phage has only a tailspike protein without a tail fiber protein. This tailspike protein is homologous to other tailspike proteins observed in certain P22-like phages targeting *S. Typhimurium*.

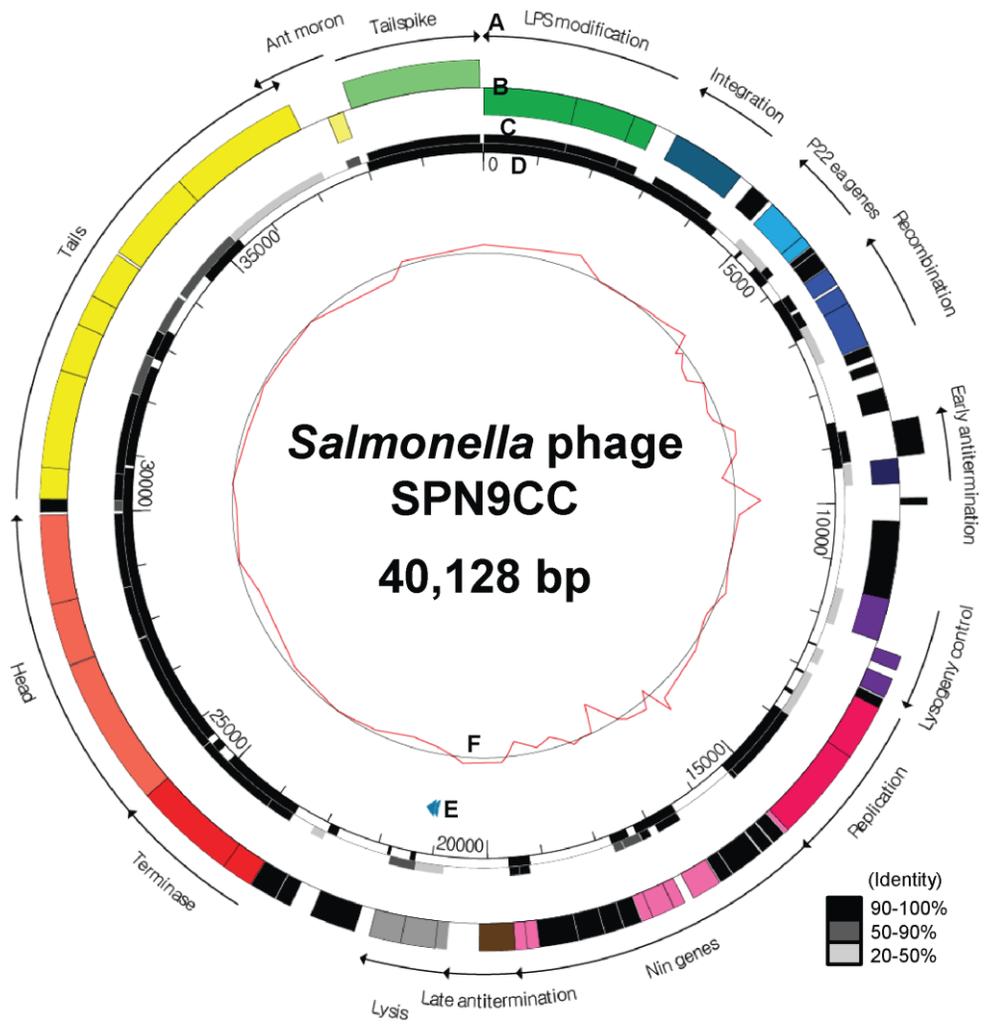


Figure 3.2. Genome map of SPN9CC phage. (A) Functions of gene clusters (B) Predicted ORFs by strand. The colors indicate the function of each gene cluster. Black-colored ORFs encode hypothetical proteins. (C) Comparative ORF analysis of SPN9CC phage with P22 phage at the amino acid sequence level. ORF homology between SPN9CC and P22 phages indicates different darkness of each block (See the figure legend). (D) Comparative genomic analysis of SPN9CC phage with P22 phage at the DNA sequence level. (E) tRNA prediction is indicated by blue arrows. (F) GC content of SPN9CC phage. The scale unit is in base pairs.

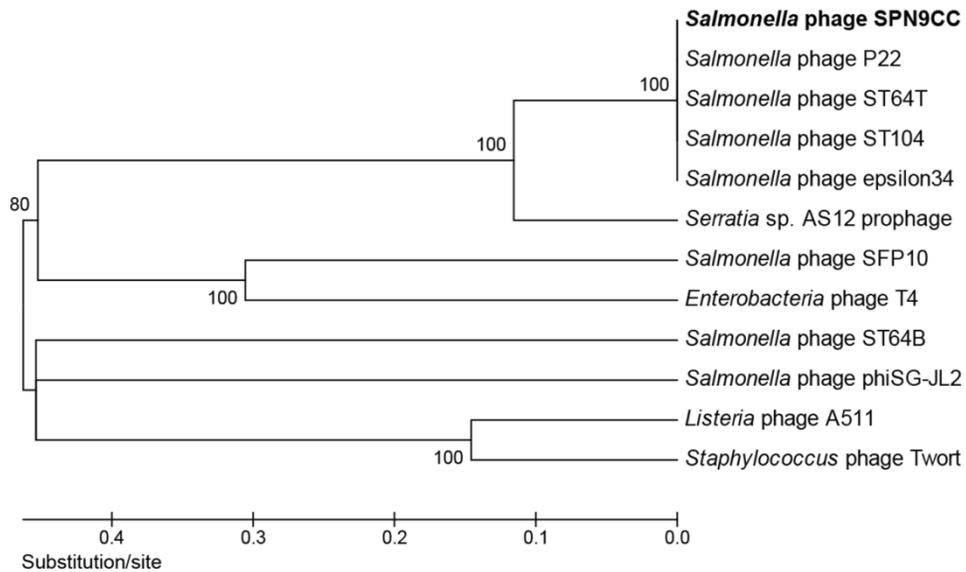


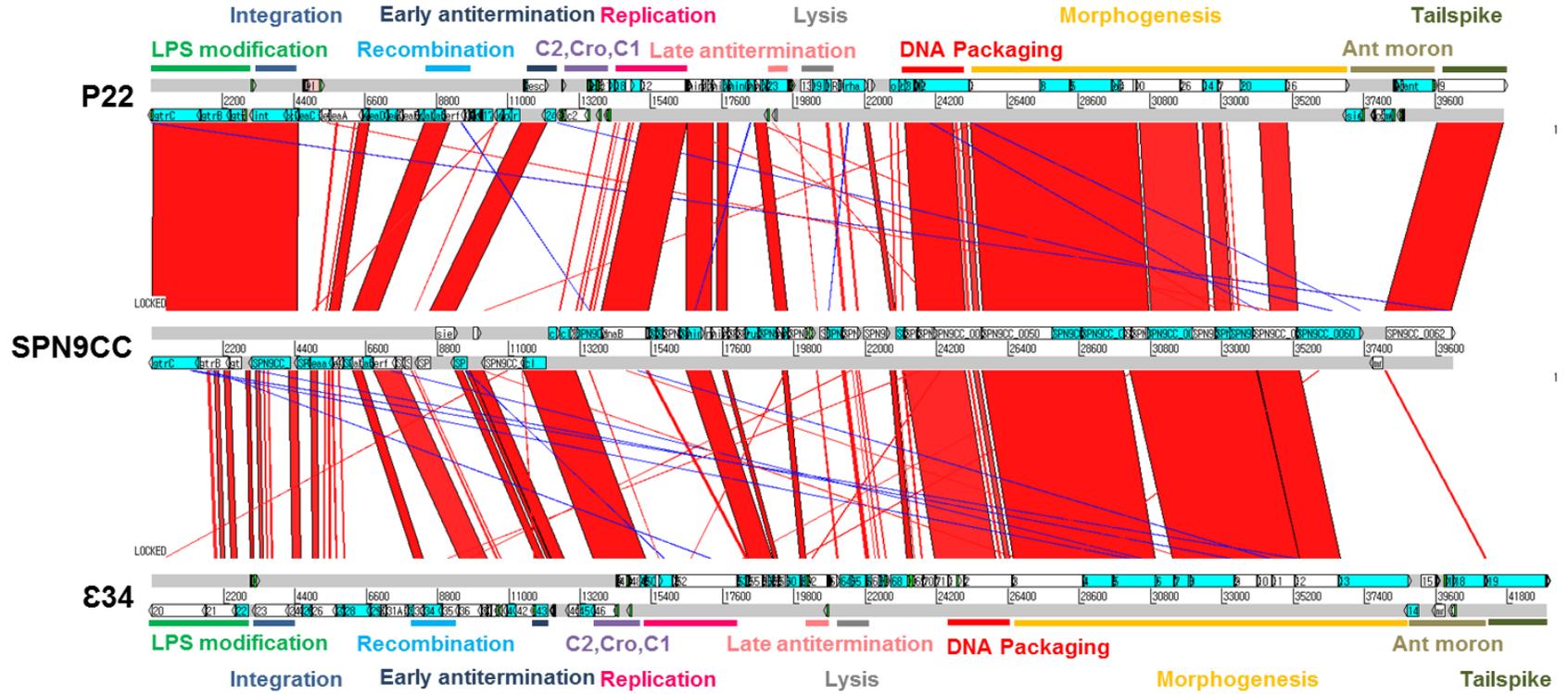
Figure 3.3. Comparative phylogenetic analysis of major capsid proteins (MCPs) from various bacteriophages. The MCPs were compared with ClustalW multiple alignment algorithm, and the phylogenetic tree was generated by MEGA5 using the neighbor-joining method with *P* distance values.

III-3-4. Comparative genomic analysis of SPN9CC with P22-like phages

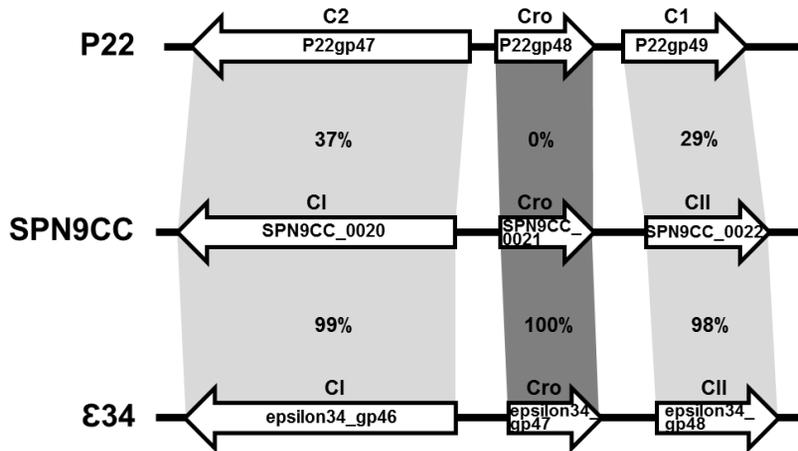
Comparative genomic analysis of SPN9CC phage with P22-like phages such as P22 and ϵ 34 revealed that DNA packaging and morphogenesis (heads and tails) gene clusters are highly conserved, indicating that P22-like phages commonly share phage structure genes and belong to *Podoviridae* family (Fig. 3.4A). Recent comparative genomic study of P22-like phages supports our analysis result (10). However, the tailspike protein of ϵ 34 differs enough from those of P22 and SPN9CC phages that it most likely has different host specificity (Fig. 3.4A). While host range analysis of P22 and SPN9CC phages displayed the same inhibition spectrum, the specific infection of ϵ 34 phage to *S. Anatum* substantiates this (68). The lysogeny control region (Cro, CI, and CII) of SPN9CC phage differs from P22 phage but similar to ϵ 34 phage, suggesting that SPN9CC and ϵ 34 may share lytic/lysogenic decision and lysogen formation mechanisms (Fig. 3.4B). Comparative analysis of the host lysis gene clusters in SPN9CC, P22, and ϵ 34 phages revealed that they are not conserved among them, suggesting that they most likely lyse the host strains in different manners (Fig. 3.4C). Although the functions of genes in this gene cluster of P22 phage were experimentally confirmed (8, 32, 47), the function of each gene in the host lysis gene cluster of SPN9CC phage

cannot be deduced from those in the gene cluster of P22 phage due to low amino acid sequence identities of these genes between P22 and SPN9CC phages. To understand the host cell lysis mechanism of SPN9CC phage, the function of each gene in the host cell lysis gene cluster of SPN9CC phage should be confirmed experimentally. Interestingly, the genes in this gene cluster of SPN9CC phage are similar to those of ST104 and even *E. coli* K-12 prophage DLP12, suggesting that they may share the same mechanism for host cell lysis (Fig. 3.4D). Successful cell lysis results from *S. Typhi* using endolysin from *E. coli* phage DLP12 supports this (65). However, whereas the amino acid sequence identities of host lysis proteins, such as holin, endolysin, and Rz/Rz1-like proteins, between two host lysis gene clusters in SPN9CC and ST104 phages are extremely high, the gene functions in the gene cluster of ST104 are not experimentally confirmed. Therefore, the expression of these genes in *S. Typhimurium* and *E. coli* host strains needs to be examined to elucidate the function of each gene in the host cell lysis gene cluster of SPN9CC phage and their cooperation effect for host cell lysis.

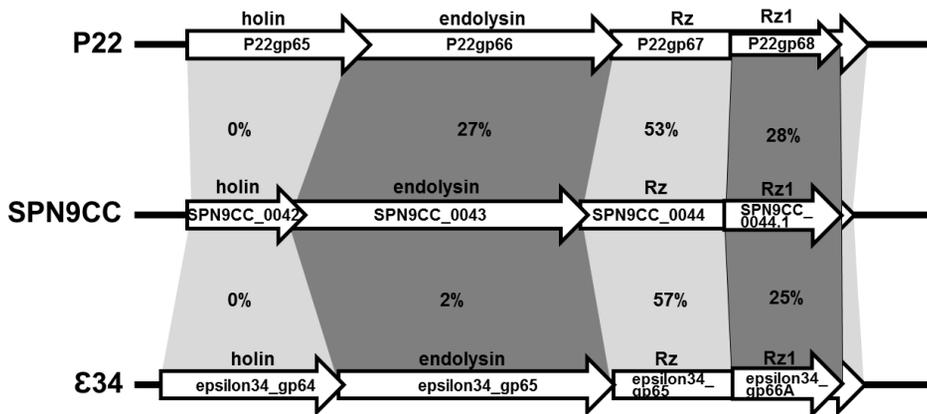
A



B



C



D

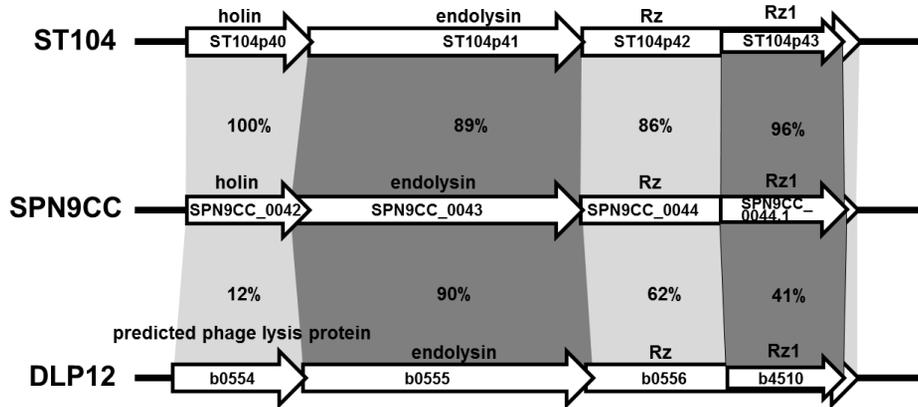


Figure 3.4. Comparative genomic analysis of P22-like phages (SPN9CC, P22, ST104, and ϵ 34) and *E. coli* K-12 prophage DLP12. (A)

Comparative analysis of complete genome sequences of SPN9CC, P22, and ϵ 34 using BLASTN and ACT12. Various colors were used to indicate each function of gene clusters in genomes. Comparative analyses of lysogeny control regions (B) and host cell lysis gene clusters (C) in SPN9CC, P22, and ϵ 34. (D) Comparative analysis of host cell lysis gene clusters in SPN9CC, ST104, and *E. coli* K-12 prophage DLP12. The identities of amino acids between homologous genes are indicated as percentages.

III-3-5. Function of host cell lysis gene cluster

Interestingly, high amino acid sequence identity of host lysis proteins (except for holin) encoded in the host cell lysis gene clusters of *S. Typhimurium*-targeting SPN9CC phage and *E. coli* K-12 DLP12 prophage suggests that host cell lysis proteins encoded by the genes in this cluster of SPN9CC phage should function in both *Salmonella* and *E. coli*. To elucidate the host cell lysis mechanism of this phage, each gene in this cluster was cloned in pBAD18 and transformed into *S. Typhimurium* and *E. coli* hosts, respectively.

The expression of a single gene encoding holin (SPN9CC_0042) in *S. Typhimurium* resulted in host cell lysis (Fig. 3.5A). However, the expression of a single gene encoding endolysin (SPN9CC_0043) or encoding Rz/Rz1-like proteins (SPN9CC_0044) in *S. Typhimurium* did not, suggesting that the endolysin needs holin for crossing the cytoplasmic membrane. To elucidate their cooperation effects for host cell lysis of *S. Typhimurium*, various combinations for the expression of more than two genes were prepared, and those genes are co-expressed in *S. Typhimurium*. The gene expression combinations of holin and endolysin or all four cell lysis proteins (holin + endolysin or holin + endolysin + Rz/Rz1-like proteins) in *S. Typhimurium* resulted in much higher host cell lysis efficiency than

expression of holin gene alone (Fig. 3.5A). However, gene expression combinations without holin (endolysin + Rz/Rz1-like proteins) did not lyse the host cells, suggesting that holin is a key protein for lysis of *S.*

Typhimurium (Fig. 3.5A).

However, the expression of these genes in *E. coli* host displayed different host cell lysis patterns (Fig. 3.5B). As for the *S. Typhimurium* host, endolysin alone did not contribute to cell lysis of *E. coli* host, but the co-expression of endolysin and other proteins (endolysin + holin or endolysin + Rz/Rz1-like proteins) in *E. coli* host did result in host cell lysis, suggesting that endolysin may need support for crossing the *E. coli* cytoplasmic membrane and that either holin or Rz/Rz1-like proteins could help endolysin to cross the membrane (Fig. 3.5B). It is intriguing to note that the main difference of host cell lysis patterns between *E. coli* and *Salmonella* by the SPN9CC lysis gene cluster is the role of Rz/Rz1-like proteins, which function only in *E. coli*.

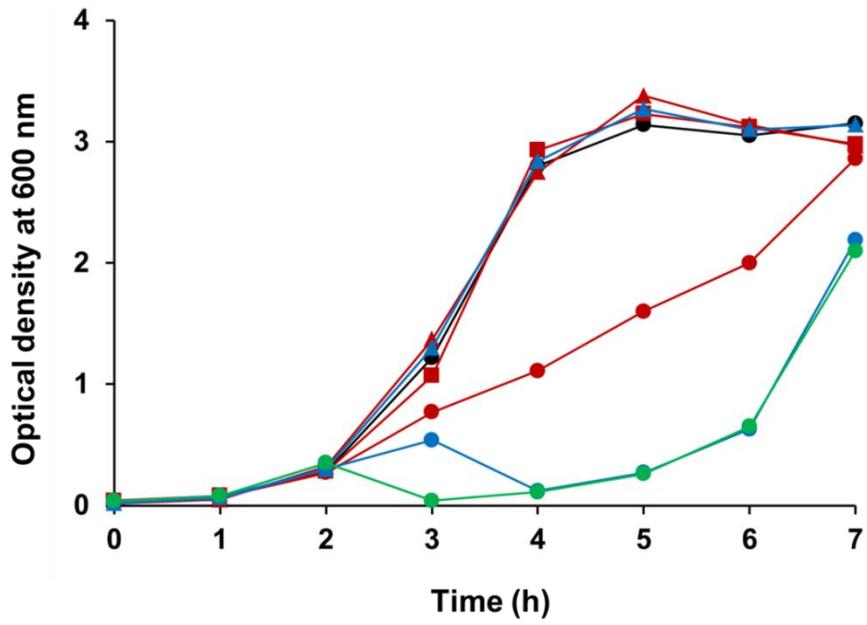
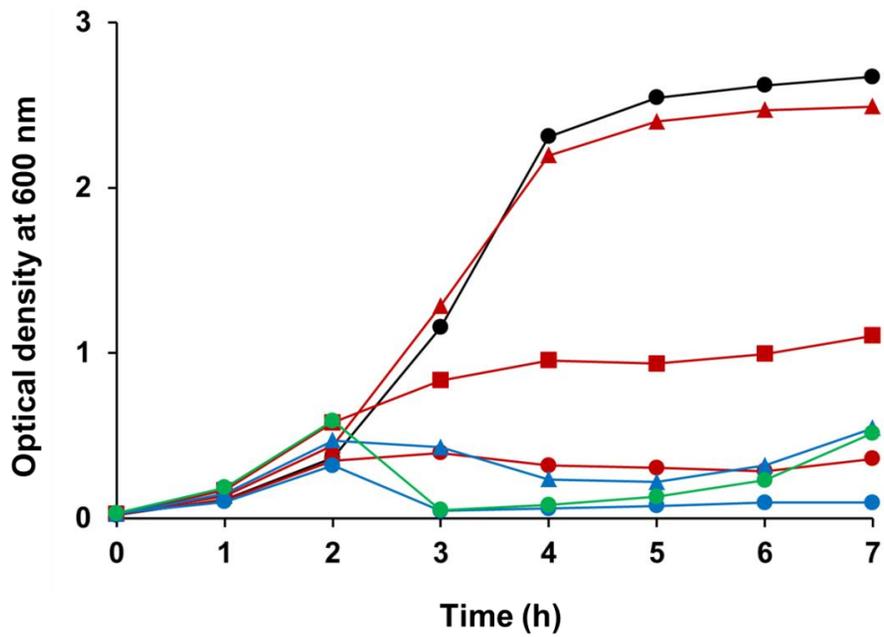
A**B**

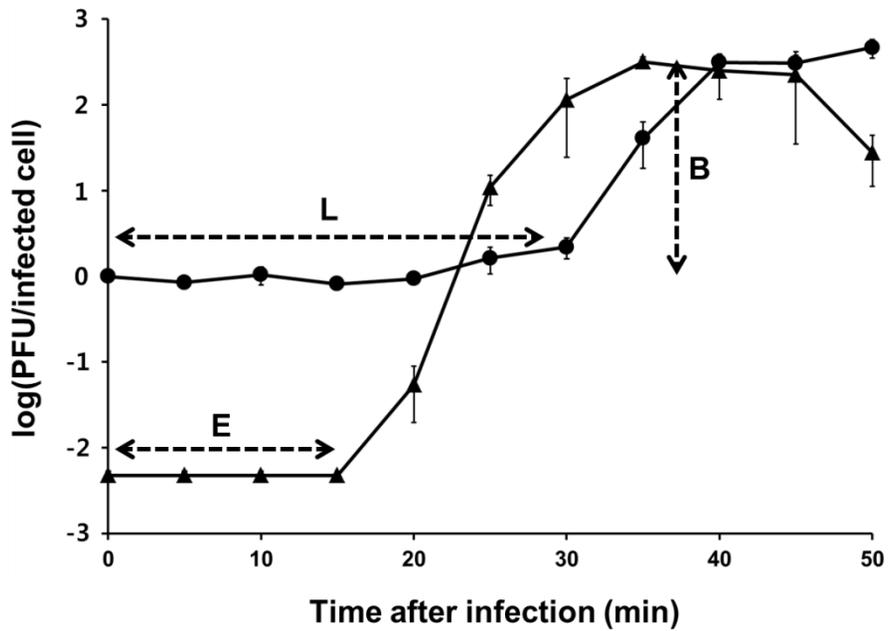
Figure 3.5. Confirmation of host lysis system of SPN9CC phage via expression of host lysis genes encoding holin, endolysin, and Rz/Rz1 in *S. Typhimurium* SL1344 (A) and *E. coli* MG1655 (B). The black, red, blue, and green colors indicate negative control without gene expression, single gene expression, co-expression of two genes, and the co-expression of all of the genes in this cluster, respectively. The red circle, red triangle, and red square indicate the gene expressions of SPN9CC_0042 (holin), SPN9CC_0043 (endolysin), and SPN9CC_0044/0044.1 (Rz/Rz1), respectively. The blue circle and blue triangle indicate co-expression of SPN9CC_0042/0043 and SPN9CC_0043/0044/0044.1, respectively. Green circle indicates co-expression of all genes, SPN9CC_0042/0043/0044/0044.1.

III-3-6. Conversion of phenotypes in SPN9CC phage by deletion of *cI* gene

CI, CII, and Cro, are key proteins in the lysogeny control region (27, 55, 74). Among them, CI is a repressor for termination of gene expression in the phage genome. Therefore, mutation of the *cI* gene can inhibit lysogen formation. The effects of *cI* gene deletion on the life cycle of SPN9CC phage was studied by constructing the ΔcI mutant phage with the BRED method (42). Interestingly, whereas SPN9CC phage generates distinct clear plaques with cloudy centers as lysogens, the ΔcI mutant phage SPN9CCM does not produce cloudy centers in the plaques, suggesting that the phenotype of ΔcI mutant phage may be converted from temperate to virulent (Fig. 3.1C). To further understand the plaque morphology change by *cI* deletion, one-step growth analysis and bacterial challenge assay of SPN9CC and SPN9CCM phages were compared. The one-step growth analysis revealed that while SPN9CC phage has relatively long eclipse/latent periods and a small burst size, SPN9CCM phage has much shorter eclipse/latent periods and larger burst sizes (Fig. 3.6). The eclipse/latent periods of SPN9CC and SPN9CCM were 15/30 min and 10/20 min, respectively. The burst sizes of SPN9CC and SPN9CCM phages were 220 and 280 PFU per cell, respectively, suggesting that the efficiency of phage multiplication was

increased for SPN9CCM most likely due to an inability to form lysogens. Furthermore, bacterial challenge assays of SPN9CC and SPN9CCM phages with *S. Typhimurium* SL1344 demonstrated that the inhibition activity of SPN9CCM phage is much higher than that of SPN9CC phage (Fig. 3.7).

A



B

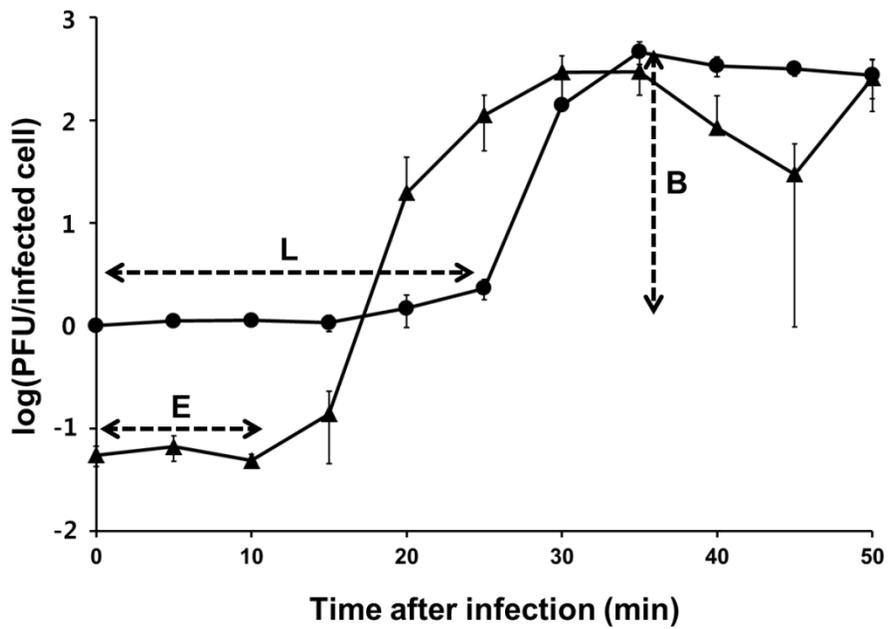


Figure 3.6. One-step growth curve analysis of SPN9CC (A) and SPN9CCM (B) phages. The circle indicates chloroform-untreated samples, and the triangle indicates chloroform-treated samples. The error bars indicate the standard deviation in triplicate experiments. E, eclipse period; L, latent period; B, burst size.

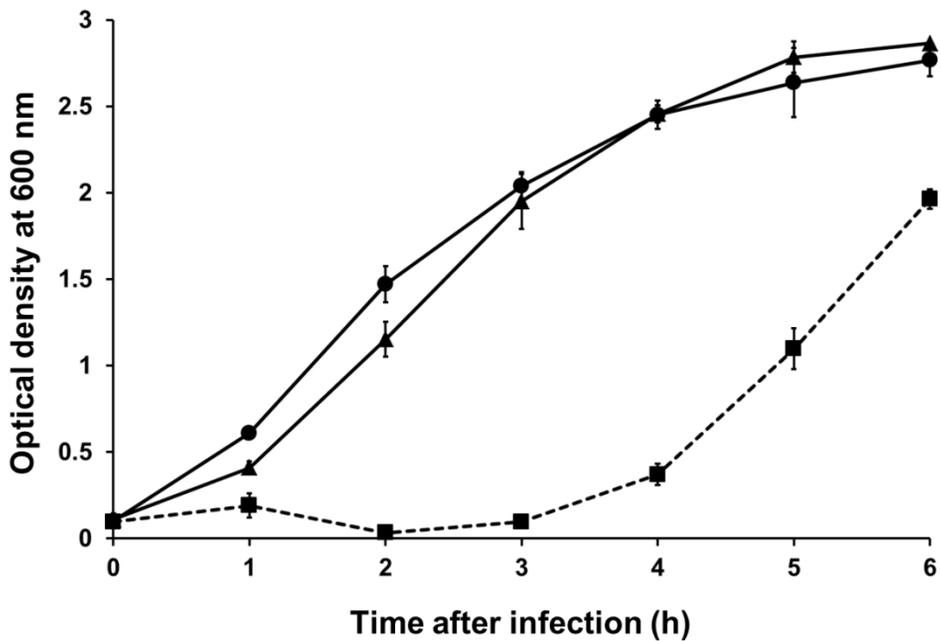


Figure 3.7. Bacterial challenge assay of SPN9CC and SPN9CCM phages against *S. Typhimurium* LT2C. The circle indicates phage-untreated samples, and the triangle indicates SPN9CC-treated samples, and a rectangle with dotted line indicates SPN9CCM-treated sample.

III-3-7. Bacterial challenge test of SPN9CC phage

Growth curve analysis and subsequent viable cell counting of SPN9CC-sensitive *S. Typhimurium* SL1344 after infection of SPN9CC were performed to determine the bacteriophage-insensitive mutants (BIMs). Thirty minutes after phage infection with different MOIs (namely, 1, 10, and 100), viable cell numbers were reduced by 0.81 log(CFU/ml) average, and they were recovered in an additional 3 h incubation, suggesting the generation of BIMs (Fig. 3.8). In addition, the BIM frequencies of *S. Typhimurium* SL1344 with different MOIs (1, 10, and 100) were 7.78×10^{-1} , 4.19×10^{-1} , and 5.62×10^{-1} , respectively. These very low viable cell reduction and high BIM frequencies may be due to the formation of lysogen during phage infection. Subsequent induction of infected *S. Typhimurium* cells 30 min after infection supports this conclusion (data not shown). In addition, the viable cell reduction at MOI = 10 was maximum at 30 min after phage infection and not at MOI = 100, suggesting that high MOI promotes lysogen formation, resulting in higher cell viability against phage infection (33, 45) (Fig. 3.8). Based on this result, the presence of highly concentrated phages in the center of SPN9CC phage plaques may promote lysogen formation, resulting in the formation of unusual clear plaques with cloudy centers.

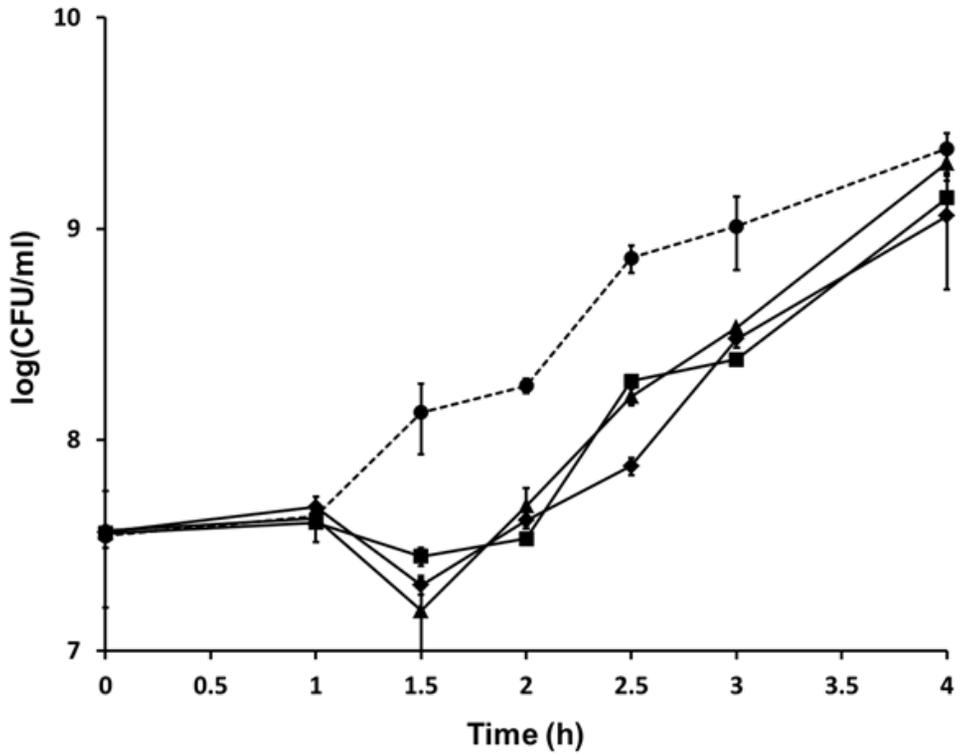


Figure 3.8. Bacterial challenge test of SPN9CC phage with *S.*

***Typhimurium* LT2C.** The circle, square, triangle, and diamond indicate an SPN9CC-uninfected sample (dotted line) and SPN9CC-infected samples with MOI = 1, 10, and 100, respectively. The error bars indicate the standard deviation in triplicate experiments.

III-4. Discussion

Salmonellosis is one of the most common types of food poisoning caused by foodborne pathogens all over the world. To reduce this food poisoning, the bacteriophage approach has recently been appearing more attractive than antibiotics treatment due to the emergence of multidrug-resistant *Salmonella* strains (12, 53). To maximize the efficiency of this phage approach, further understanding of phage infection and host lysis mechanisms is required (31, 37). P22 phage has been studied in the context of the development of a molecular transduction tool (6, 67), the identification of host specificity and receptor (4, 69), the tail structure for host interaction (14, 38), the lysogeny control region (39, 56), superinfection exclusion (28, 57), and other areas. The P22-like phage group was previously proposed based on the homology of virion assembly genes, which includes ST104, ε34, ST64T, L, Sf6, c341, and HK620, among others. (10). Recent improvement of genome sequencing and analysis technologies, such as next-generation sequencing and bioinformatic tools, enabled us to analyze the full genome sequences of these P22-like phages and to study their characteristics in genomic level. Recent comparative genomic analysis revealed that while their genomic characteristics are diverse, most likely due

to horizontal gene transfer/exchange in the group, morphogenesis and DNA packaging are highly conserved (10, 72). However, the diversity of other genomic features may determine the specific characteristics of each phage in the group, such as host specificity, lysogeny control region, and host cell lysis system, involved in the host infection and lysis mechanisms.

The *Salmonella*-targeting temperate phage SPN9CC was isolated from a commercially processed broiler skin sample, and its complete genome analysis suggests that SPN9CC phage is in the P22-like phage group. One-step growth analysis of SPN9CC phage revealed a longer latent period and smaller burst size than other lytic phages, such as T7-like *Podoviridae* phages (Fig. 3.6A) (36, 49, 70), suggesting that lysogen formation during phage infection may affect the host cell lysis activity of SPN9CC phage. A high frequency of observed bacteriophage-insensitive mutants (BIMs) of this phage during bacterial challenge test also supports this (Fig. 3.8). It is well known that the host receptor is modified once the host is lysogenized phage (6, 59, 72). SPN9CC has LPS modification proteins homologous to the GtrABC (SPN9CC_003, 002, and 001, respectively), which modify LPS to prevent super-infection of SPN9CC lysogen. Furthermore, the resistance activity of the host lysogen caused by LPS modification during lysogenization may contribute to forming cloudy

centers in SPN9CC plaques (Fig. 3.1A). In the center of the plaques, high phage concentration may promote lysogenization of SPN9CC phage, similar to the P22 phage (33, 45) (Fig. 3.1A).

Complete genome sequence and comparative genomic analyses of SPN9CC phage with other P22-like phages revealed a diversity of phage infection and host lysis mechanisms in the group (Fig. 3.2 and 3.4A). P22-like phages are in the family *Podoviridae* and have short tails, indicating that the tailspike protein is a major determinant for host specificity in P22-like phages (23, 66, 69). However, the tailspike protein is variable in the group, suggesting that host specificity and host range for P22-like phage infection could be variable. Whereas P22, ST104, ST64T phages with homologous tailspike proteins infect *S. Typhimurium*, ϵ 34 and Sf6 phages with different types of tailspike proteins infect *S. Anatum* and even *Shigella*, respectively, supporting the notion of a variable host range and specificity (10, 40, 68). Comparative analysis of the lysogeny control regions in P22-like phages indicated that the region in SPN9CC is nearly identical to that of ϵ 34 phage but quite different from that of P22 phage, suggesting that members of P22-like phages may have diverse lytic/lysogenic decision mechanisms (Fig. 3.4B).

The characterization of the host cell lysis gene cluster of SPN9CC

is important to understanding the host cell lysis mechanism of SPN9CC. The host cell lysis gene cluster encodes putative holin, endolysin, and Rz/Rz1-like proteins. This gene cluster of SPN9CC phage is quite different from those of P22 and ϵ 34 phages but very similar to those of ST104 and even *E. coli* K-12 DLP12 prophage, suggesting the possibility of *E. coli* cell lysis via the activity of lysis proteins that are encoded in the gene cluster of SPN9CC phage. The expression of these genes individually or expression of their combinations in *S. Typhimurium* or *E. coli* hosts revealed that holin is a key protein for both host cell lysis, but endolysin could not achieve lysis by itself (Fig. 3.5A and 3.5B). These results indicate that endolysin of SPN9CC requires holin to cross the cytoplasmic membrane to act on the peptidoglycan in the periplasm. Rz/Rz1-like proteins are known accessory proteins of endolysin (34), and Rz/Rz1-like proteins alone or in combination with endolysin in *S. Typhimurium* did not exhibit cell lysis activity. Interestingly, Rz/Rz1-like proteins alone and even their combination with endolysin resulted in growth inhibition of *E. coli* even though the degree of inhibition was relatively low. However, the lysis activity of Rz/Rz1-like proteins in *E. coli* host is not fully understood. Comparative functional analysis of this host cell lysis gene cluster between *S. Typhimurium* and *E. coli* revealed that this lysis protein combination works better in *E. coli* than

in *S. Typhimurium* (Fig. 3.5A and 3.5B).

In this study, comparative analysis of SPN9CC phage with P22-like phages provided novel insights into phage infection and host lysis mechanisms with *S. Typhimurium* host strains. This study probably contributes to a better understanding of the new approach for bacteriophage treatment to inhibit foodborne pathogens as well as to the development of newly optimized phages for therapy.

III-5. References

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**Chapter IV. Characterization and Comparative
Genomic Analysis of Bacteriophages Infecting the
Bacillus cereus Group**

Submitted to Archives of Virology

(H. Shin, J.-H. Lee, and S. Ryu, 2013, Archives of Virology)

IV-1. Introduction

Since the discovery of bacteriophages in 1915 (65), bacteriophages have been known to be viruses of prokaryotes, which invade specific bacterial hosts, replicate using the host DNA replication and protein biosynthesis systems, and lyse the hosts for propagation (34, 60, 63). The lifestyles of bacteriophages include a lysogenic cycle (for phage genome integration into the host chromosome) and a lytic cycle (for lysis the bacterial host because of bactericidal activity) (25, 34, 63). Bacteriophages occur everywhere in the biosphere and are frequently found in the ocean and soil. The total biomass of phages and number of phage species in nature have been estimated at over 10^{30} particles and more than 10^6 species, respectively (10, 48). Bacteriophages are classified by the International Committee on Taxonomy of Viruses (ICTV) according to phage morphology and nucleic acid type (26). Approximately 96% of all bacteriophages belong to the order *Caudovirales* and the remaining belong to the order *Ligamenvirales* and an unassigned order including *Tectiviridae* family (2). The bacteriophages in the order *Caudovirales* belong to three different families including *Myoviridae* with a contractile tail, *Siphoviridae* with a non-contractile tail, and *Podoviridae* with a short and non-contractile

tail (26, 34).

The *Bacillus cereus* group consists of *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* (28, 69). Among them, *B. cereus*, *B. anthracis*, and *B. thuringiensis* have been suggested to be a single species of *B. cereus sensu lato* (14, 24). This classified species is a pathogen that infects humans, animals, and even insects. *B. cereus* is a well-known, food-borne pathogen, which produces enterotoxins that causes diarrhea, vomiting, and nausea (9, 21). In addition, *B. anthracis* is a category A bio-threat agent, causing the fatal anthrax in humans and animals (22). In 2001, its endospore was used as a biological weapon, resulting in a mortality rate higher than 45% in those exposed (7). To generally control bacterial pathogens, various antibiotics have been widely used. However, *B. cereus* is generally insusceptible to penicillin-related antibiotics (because of its production of β -lactamase) and to additional antibiotics, such as erythromycin and tetracycline (31, 50). In addition, long-term antibiotic treatment with various antibiotics against *B. anthracis* resulted in its rapid development of antibiotic resistance (5). Therefore, because of the emergence of antibiotic resistant strains, alternative biocontrol approaches against these pathogens are needed. Employing bacteriophages to infect *B. cereus* or *B. anthracis* could be a

good candidate to control these pathogens. An additional member of the *Bacillus cereus* group, *B. thuringiensis* (Bt), is highly characterized and used as a biological pesticide for the biocontrol of insect pests. It produces insecticidal crystal proteins (ICPs), which are highly toxic to the pest larvae but not to humans and animals (4, 51). Whereas Bt has been widely used for insect pest control, bacteriophage contamination causes damage to Bt production via fermentation. To overcome this problem, bacteriophages infecting *B. thuringiensis* should be studied to understand their infection mechanism and their lytic/lysogenic determination mechanism.

Resulting from the recent developments in genome sequencing and bioinformatic technologies, bacteriophage studies at the genomic level have been booming, allowing for further applications in novel biocontrol agents and phage therapy. In this review, we describe the general features of the *B. cereus* group phages and genomic insights resulting from comparative and functional genomic analyses. This genomic information is useful for extending our understanding of their general genomic characteristics and their various applications in the control of bacterial pathogens and for phage therapy.

IV-2. Results and Discussion

IV-2-1. The general genomic features and classification of *B. cereus* group bacteriophages using comparative genomics

To date, 30 complete genome sequences of *B. cereus* group bacteriophages (18 for *B. cereus* phages, four for *B. anthracis*, and eight for *B. thuringiensis*) are available in the GenBank database. The general features of all 30 complete genome sequences are listed in Table 4.1. Three different morphological families are present in all 30 *B. cereus* group bacteriophages such as *Myoviridae*, *Siphoviridae* (belonging to the order *Caudovirales*), and *Tectiviridae* (belonging to the unassigned order) families. Interestingly, the phage genome size may be related to the family morphology of the *B. cereus* group bacteriophages. The ranges of phage genome sizes in the family *Myoviridae*, *Siphoviridae*, and *Tectiviridae* are 94 to 219-kb, 36 to 53-kb, and 14.3 to 14.9-kb, respectively (Table 4.1). Furthermore, the phage life cycle may also be related to the family morphology of the *B. cereus* group bacteriophages. Whereas all *Myoviridae* family bacteriophages are virulent, *Siphoviridae* and *Tectiviridae* family bacteriophages are temperate, supporting this hypothesis. Therefore, a close relationship may exist

between the family morphology, genome size, and life cycle of the *B. cereus* group phages. Based on these results, there may be three potential phage groups in the 30 *B. cereus* group bacteriophages. To further classify these 30 different *B. cereus* group bacteriophages, the comparative phylogenetic analyses using the phage major capsid proteins (MCPs) and phage terminase large subunits were conducted and revealed that there are three evolutionary groups, phage group I, II and III (Fig. 4.1A and 1B). A subsequent comparative dot plot analysis of all 30 bacteriophage genomes at the DNA level also showed that the phage genomes in each phage group are similar, consistent with the phage classification (Fig. 4.2). Recently, Lavigne *et al.* suggested that the *Myoviridae* family is divided into the three subfamilies *Peduovirinae*, *Teequatrovirinae*, and *Spounavirinae* (36). Based on the phylogenetic tree in the report, phage group I in the *Myoviridae* family belongs to the subfamily *Spounavirinae*. This *Spounavirinae* subfamily was also suggested to have two genera such as Spo1-like virus and Twort-like virus. However, the phage group I does not belong to these two genera but most likely belongs to another new genus, suggesting that this *Spounavirinae* subfamily may be more diverse (17).

Although 30 complete genome sequences of the *B. cereus* group bacteriophages are available, 34.9 to 93.5% of the annotated open reading

frames (ORFs) are putatively annotated as hypothetical proteins, most likely because of insufficient phage gene annotation data in the sequence databases (Table 4.1). Therefore, a genomic study of *B. cereus* group bacteriophages is required to extend our understanding to allow further applications in the development of biocontrol agents and phage therapy.

Table 4.1. General genomic features of the *B. cereus* group bacteriophages

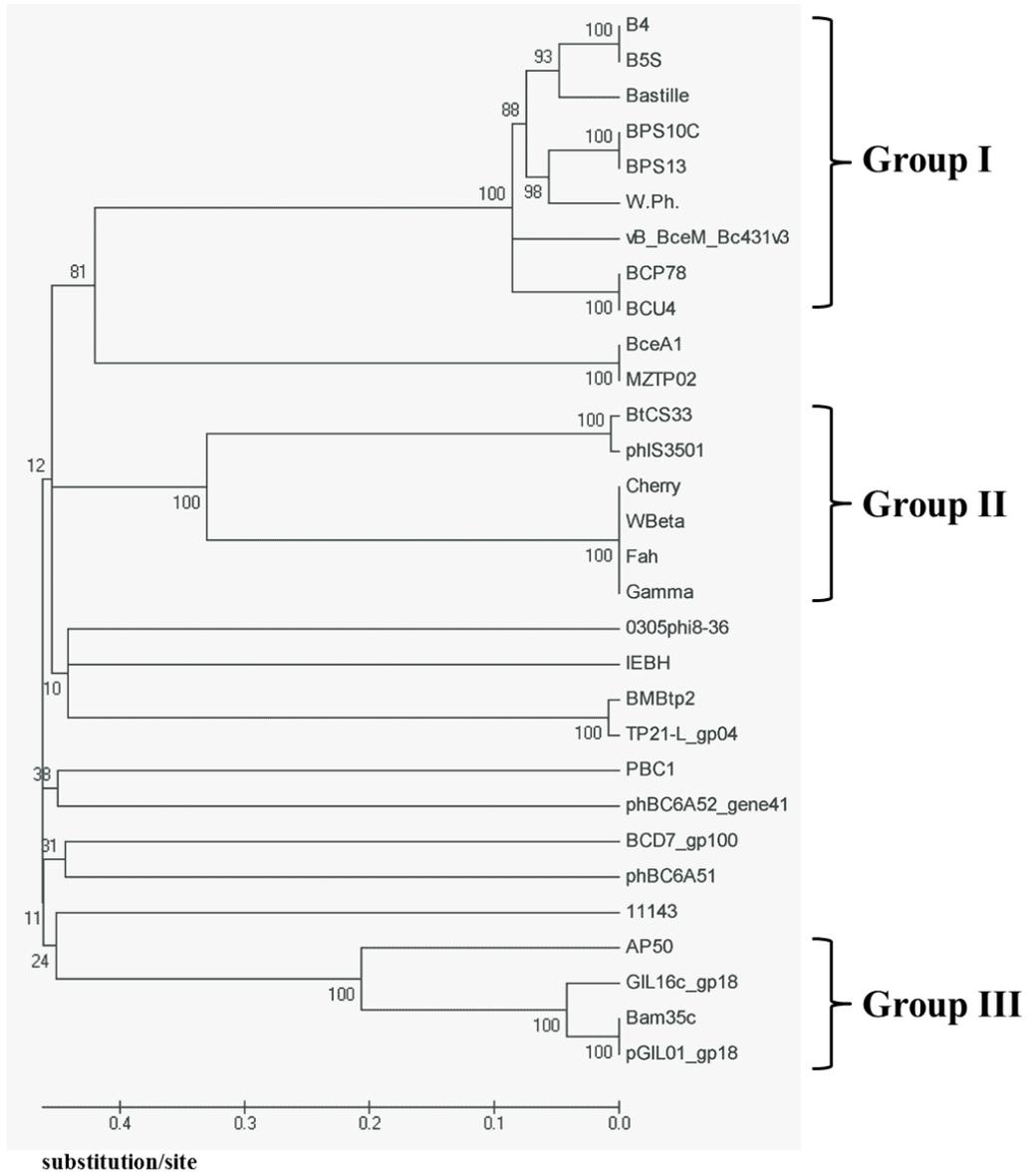
Phage	Host	Genome size (bp)	GC%	Predicted ORFs	No. Hypo. (%) ^a	Coding (%)	No. tRNAs	Morphology	Life style	Accession number	Group	Reference
B4	<i>B. cereus</i>	162,596	37.71	277	228 (82.3)	90.6	0	<i>Myoviridae</i>	Virulent	NC_018863	I	(37)
B5S	<i>B. cereus</i>	162,598	37.71	272	230 (84.6)	90.5	0	<i>Myoviridae</i>	Virulent	JN797796	I	This study
Bastille	<i>B. cereus</i>	153,962	38.14	273	231 (84.6)	92.6	7	<i>Myoviridae</i>	Virulent	NC_018856	I	(42)
W.Ph.	<i>B. cereus</i>	156,897	36.45	274	250 (91.2)	92.4	0	<i>Myoviridae</i>	N/D	NC_016563	I	-
BPS10C	<i>B. cereus</i>	159,590	38.74	271	232 (85.6)	91.6	0	<i>Myoviridae</i>	Virulent	JN654439	I	(in review)
BPS13	<i>B. cereus</i>	158,305	38.75	268	231 (86.2)	89.4	0	<i>Myoviridae</i>	Virulent	NC_018857	I	(in review)
vB_BceM_Bc431v3	<i>B. cereus</i>	158,621	39.98	238	165 (69.3)	90.6	21	<i>Myoviridae</i>	Virulent	JX094431	I	(17)
BCP78	<i>B. cereus</i>	156,176	39.86	227	181 (79.7)	90.0	18	<i>Myoviridae</i>	Virulent	NC_018860	I	(38)
BCU4	<i>B. cereus</i>	154,371	39.86	223	171 (76.7)	89.9	19	<i>Myoviridae</i>	Virulent	JN797798	I	This study
BMBtp2	<i>B. thuringiensis</i>	36,932	37.79	53	39 (73.6)	86.9	0	<i>Siphoviridae</i>	Temperate	NC_019912		(16)
TP21-L	<i>B. cereus</i>	37,456	37.8	56	N/D	89.3	0	<i>Siphoviridae</i>	N/D	NC_011645		(42)
IEBH	<i>B. cereus</i>	53,104	36.42	86	60 (69.8)	85.8	0	<i>Siphoviridae</i>	Temperate	NC_011167		(56)
phBC6A51	<i>B. cereus</i>	61,395	37.69	75	56 (74.7)	83.0	0	N/D	N/D	NC_004820		(27)
BCD7	<i>B. cereus</i>	93,839	38.04	140	107 (76.4)	90.2	0	<i>Myoviridae</i>	Virulent	NC_019515		This study
PBC1	<i>B. cereus</i>	41,164	41.68	50	29 (58.0)	92.0	0	<i>Siphoviridae</i>	Virulent	NC_017976		(32)
BceA1	<i>B. cereus</i>	42,932	35.66	63	22 (34.9)	88.1	0	<i>Siphoviridae</i>	Temperate	HE614282		(62)
MZTP02	<i>B. thuringiensis</i>	15,717	37.55	20	11 (55.0)	78.8	0	<i>Siphoviridae?</i>	Temperate	AY894696		(41)

Table 4.1. General genomic features of the *B. cereus* group bacteriophages (continued)

Phage	Host	Genome size (bp)	GC%	Predicted ORFs	No. Hypo. (%) ^a	Coding (%)	No. tRNAs	Morphology	Life style	Accession number	Group	Reference
AP50	<i>B. anthracis</i>	14,398	38.65	31	14 (45.2)	96.2	0	<i>Tectiviridae</i>	Temperate	NC_011523	III	(58)
GIL16c	<i>B. thuringiensis</i>	14,844	40.07	31	26 (83.9)	98.8	0	<i>Tectiviridae</i>	Virulent ^b	NC_006945	III	(67)
Bam35c	<i>B. thuringiensis</i>	14,935	39.72	32	30 (93.8)	99.7	0	<i>Tectiviridae</i>	Virulent ^c	NC_005258	III	(59)
GIL01	<i>B. thuringiensis</i>	14,931	39.73	30	24(80.0)	96.7	0	<i>Tectiviridae</i>	Temperate	AJ536073	III	(68)
0305phi8-36	<i>B. thuringiensis</i>	218,948	41.8	246	142 (57.7)	95	0	<i>Myoviridae</i>	Virulent	NC_009760		(64)
phBC6A52	<i>B. cereus</i>	38,472	34.72	49	31 (63.3)	80.4	0	N/D	N/D	NC_004821		(27)
11143	<i>B. cereus</i>	39,077	34.96	49	23 (46.9)	85.3	0	<i>Siphoviridae</i>	Temperate	GU233956		(40)
BtCS33	<i>B. thuringiensis</i>	41,992	35.22	57	29 (50.9)	85.1	0	<i>Siphoviridae</i>	Temperate	NC_018085	II	(70)
phIS3501	<i>B. thuringiensis</i>	44,401	34.86	53	25 (47.2)	75.7	1	<i>Siphoviridae</i>	Temperate	NC_019502	II	(44)
Cherry	<i>B. anthracis</i>	36,615	35.26	51	29 (56.9)	91.5	0	<i>Siphoviridae</i>	Virulent ^d	NC_007457	II	(19)
Fah	<i>B. anthracis</i>	37,974	34.94	50	18 (36.0)	89.6	0	<i>Siphoviridae</i>	Virulent ^d	NC_007814	II	(43)
Gamma	<i>B. anthracis</i>	37,253	35.22	53	30 (56.6)	90.7	0	<i>Siphoviridae</i>	Virulent ^d	NC_007458	II	(19, 53)
Wbeta	<i>B. cereus</i>	40,867	35.26	53	27 (50.9)	90.2	0	<i>Siphoviridae</i>	Temperate	NC_007734	II	(53)

^a, the number of hypothetical proteins; ^b, clear plaque mutant of Bam35 phage; ^c, clear plaque mutant of GIL16; ^d, lytic variants of Wbeta phage.

(A)



(B)

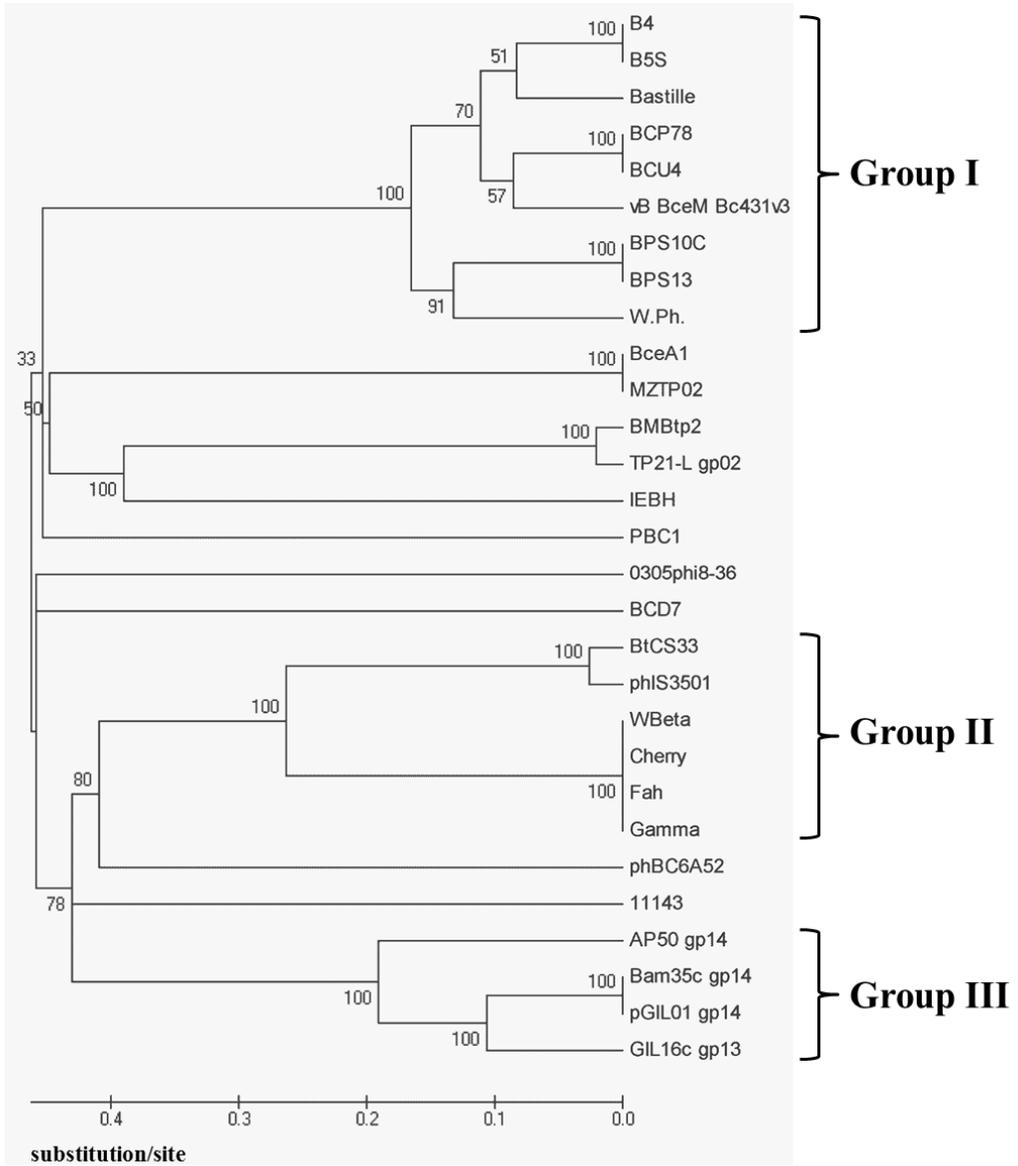


Figure 4.1. A comparative phylogenetic analysis of major capsid proteins (A) and terminase large subunits (B) using MEGA5 (33) and ClustalW (35) programs with a neighbor-joining method.

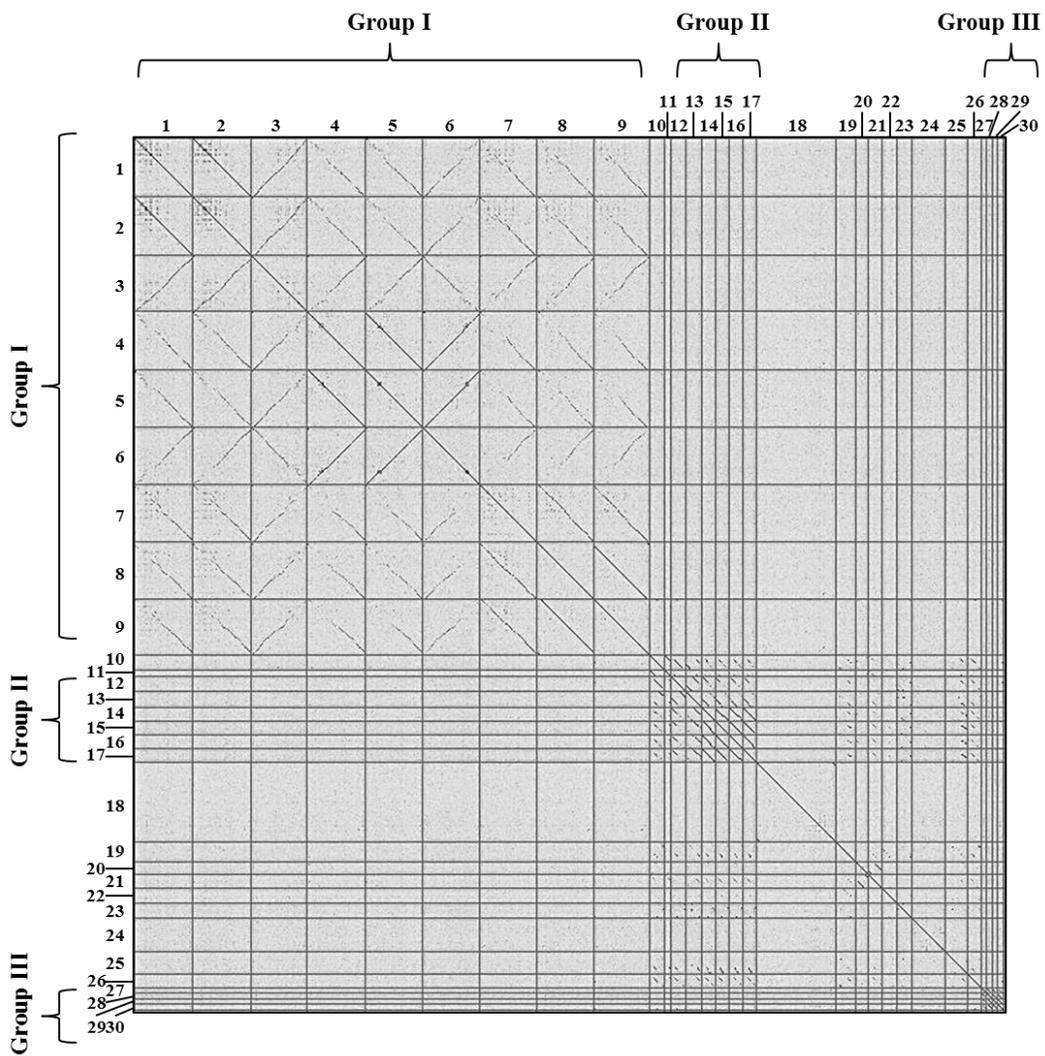


Figure 4.2. A comparative dot plot analysis of all 30 bacteriophage genomes using the JDotter program (11) with a maximum plot size for 700. 1, B4; 2, B5S; 3, Bastille; 4, BPS10C; 5, BPS13; 6, W.Ph.; 7, vB_BceM_Bc431v3; 8, BCP78; 9, BCU4; 10, BceA1; 11, MZTP02; 12, BtCS33; 13, phIS3501; 14, Cherry; 15, WBeta; 16, Fah; 17, Gamma; 18, 0305phi8-36; 19, IEBH; 20, BMBtp2; 21, TP21-L; 22, PBC1; 23, phBC6A52; 24, BCD7; 25, phBC6A51; 26, 11143; 27, AP50; 28, GIL16c; 29, Bam35c; 30, pGIL01.

IV-2-2. *B. cereus sensu lato* phage group I

According to the phage classification, the *B. cereus sensu lato* phage group I includes all *Myoviridae* family bacteriophages except for BCD7 and 0305phi8-36. Whereas these two distinct bacteriophages belong to the *Myoviridae* family, their genome sizes are different from the phages in group I. However, all phages in the *Myoviridae* family are virulent and predominantly infect *B. cereus*. A comparative genomic analysis of the phages in group I showed that functional gene clusters are located in identical positions, such as packaging, host lysis, DNA manipulation, phage structure, and additional functions, suggesting that genome arrangements in phage group I are identical (Fig. 4.3). In addition, a dot plot analysis also supports this similarity in the genome arrangements (Fig. 4.2). To further understand their functional characteristics, predicted functional genes were categorized into seven functional groups such as packaging, host lysis, regulation, host interaction, DNA manipulation, phage structure, and additional functions (Table 4.2). All predicted functional genes are shared by all bacteriophages in phage group I, except for the genes encoding a thioredoxin, flavodoxin, and a transcriptional regulator in the phage Bastille (Table 4.2). In addition, the putative ribose-phosphate pyrophosphokinase

was also missing in the phage W.Ph. (Table 4.2).

Table 4.2. Core gene analysis in the bacteriophage group I

Functional group	Predicted function	W.Ph.	BPS10C/BPS13	Bastille	B4/B5S	Bc431v3	BCP78/BCU4
Packaging	terminase large subunit	P	P	P	P	P	P
	portal protein	P	P	P	P	P	P
Lysis	endolysin*	I	I	II	II	III	VI
	putative holin	P	P	P	P	P	P
Regulation	DNA-binding protein	P	P	P	P	P	P
	transcriptional regulator 1	P	P	P	P	P	P
	transcriptional regulator 2	P	P	-	P	P	P
Host interaction	sporulation sigma factor SigF-like protein	P	P	P	P	P	P
	putative RNA polymerase sigma factor	P	P	P	P	P	P
DNA manipulation	DNA polymerase 1	P	P	P	P	P	P
	DNA recombination/repair protein	P	P	P	P	P	P
	DNA polymerase 2	P	P	P	P	P	P
	DNA primase	P	P	P	P	P	P
	exonuclease	P	P	P	P	P	P
	helicase 1	P	P	P	P	P	P
	helicase 2	P	P	P	P	P	P

*, *B. cereus* bacteriophage Group I have different type of endolysins.

Table 4.2. Core gene analysis in the bacteriophage group I (continued)

Functional group	Predicted function	W.Ph.	BPS10C/BPS13	Bastille	B4/B5S	Bc431v3	BCP78/BCU4	
Structure	adsorption associated tail protein/ tail fiber	P	P	P	P	P	P	
	baseplate J protein	P	P	P	P	P	P	
	baseplate protein	P	P	P	P	P	P	
	minor structural protein	P	P	P	P	P	P	
	minor structural protein/ putative tail fiber	P	P	P	P	P	P	
	tail lysin 1	P	P	P	P	P	P	
	tail lysin 2	P	P	P	P	P	P	
	tail sheath protein	P	P	P	P	P	P	
	major capsid protein	P	P	P	P	P	P	
	prohead protease	P	P	P	P	P	P	
	Additional function	3D domain-containing protein	P	P	P	P	P	P
		thymidylate synthase	P	P	P	P	P	P
		dephospho-CoA kinase	P	P	P	P	P	P
dihydrofolate reductase		P	P	P	P	P	P	
metal-dependent hydrolase		P	P	P	P	P	P	
thioredoxin		P	P	-	P	P	P	
flavodoxin		P	P	-	P	P	P	
putative ribose-phosphate pyrophosphokinase		-	P	P	P	P	P	
ribonucleotide-diphosphate reductase subunit beta		P	P	P	P	P	P	
ribonucleotide-diphosphate reductase subunit alpha		P	P	P	P	P	P	
deoxyuridine 5'-triphosphate nucleotidohydrolase		P	P	P	P	P	P	

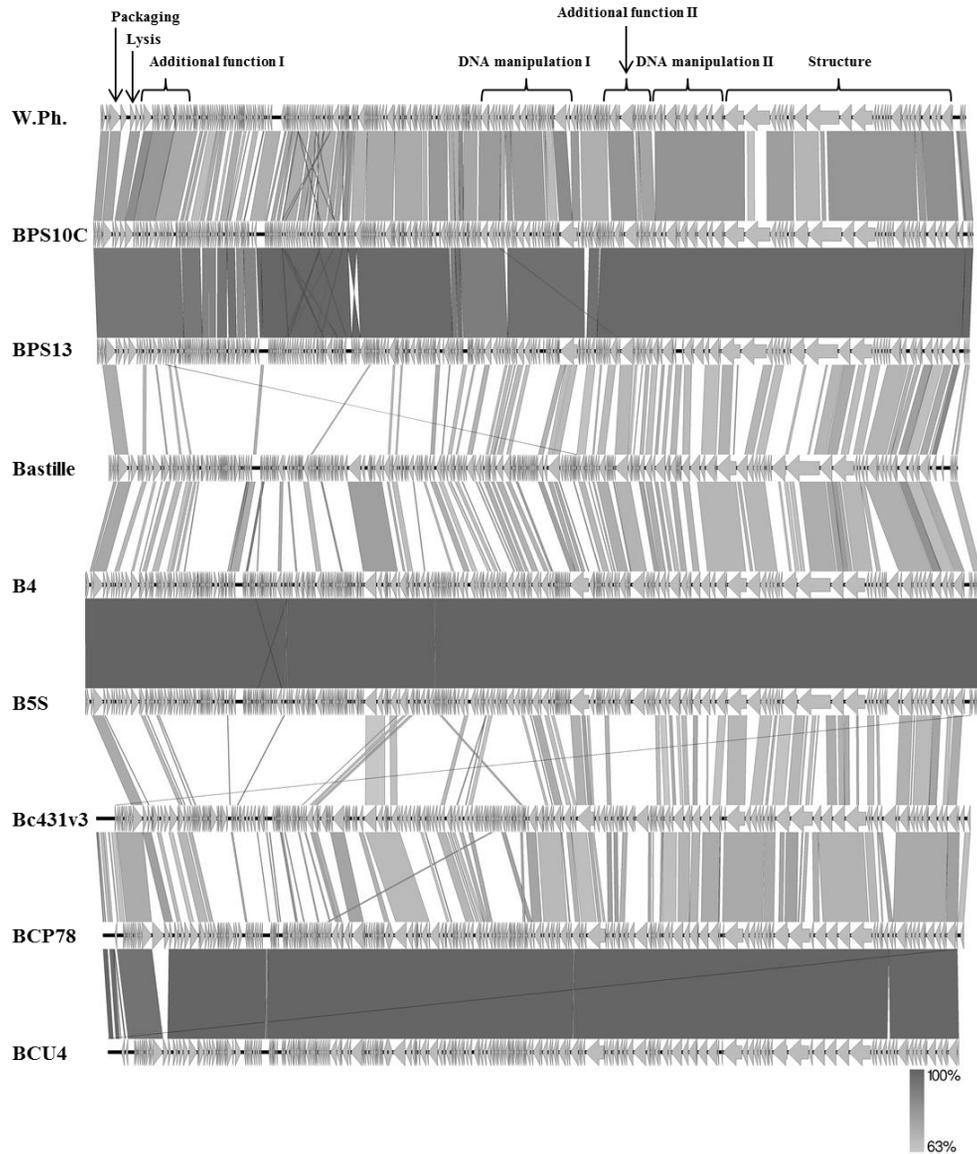


Figure 4.3. A comparative genomic analysis of phages in the *B. cereus sensu lato* phage group I using Easyfig program (61) at the DNA level.

A comparative protein domain analysis of endolysins in the phage group I showed that four different homologous endolysin groups are present: endolysin group I containing N-acetylmuramoyl-L-alanine amidase and SH3-like domain (phages W.Ph., BPS10C, and BPS13), endolysin group II containing cell wall hydrolysis/autolysin and SH3-like domain (phages Bc431v3, BCP78, and BCU4), endolysin group III containing peptidase M15B/M15C and SH3-like domain (phages B4 and B5S), and endolysin group IV containing glycoside hydrolase family 25 and N-acetylmuramoyl-L-alanine amidase (phage Bastille) (Fig. 4.4). All endolysin groups, except for group IV, share the SH3-like domain (PF08460), which has been predicted to be a cell wall binding domain. However, the phage Bastille maintains a different type of N-acetylmuramoyl-L-alanine amidase (PF12123) from other phages in endolysin group I (PF01510). Furthermore, the endolysin of the phage Bastille does not have a SH3-like cell wall binding domain, indicating that the phage Bastille may have a different type of cell wall binding domain in the endolysin. Loessner *et al.* suggested that the C-terminal of endolysin in the phage Bastille has a 77-amino acid repeat sequences (located in 211-287 and 288-364 a.a.) and may be involved in cell wall binding, such as the SH3-like domain (42). Endolysins in endolysin group I have an N-acetylmuramoyl-L-alanine amidase domain, probably

involved in cell wall lysis. To characterize this amidase domain (PF01510), Park *et al.* purified the endolysin of the phage BPS13 and tested the cell wall lysis mechanism (46). This endolysin cleaves the bond between N-acetylmuramic acid and L-alanine in the cell wall by its amidase activity. In addition, Son *et al.* purified endolysin of the phage B4 and tested its cell wall lysis to characterize peptidase M15B/M15C domain (PF02557), revealing that this peptidase domain acts like a L-alanoyl-D-glutamate endopeptidase by cutting the peptide bond between L-alanine and D-glutamate (57). Therefore, these two endolysin domains, N-acetylmuramoyl-L-alanine amidase and peptidase M15B/M15C, performs host cell wall lysis. However, the other two endolysin domains, the cell wall hydrolase/autolysin (PF01520) and glycoside hydrolase family 25 (PF01183), are predicted to perform host cell lysis, but their cell wall lysis mechanisms should be experimentally confirmed (Fig. 4.4).

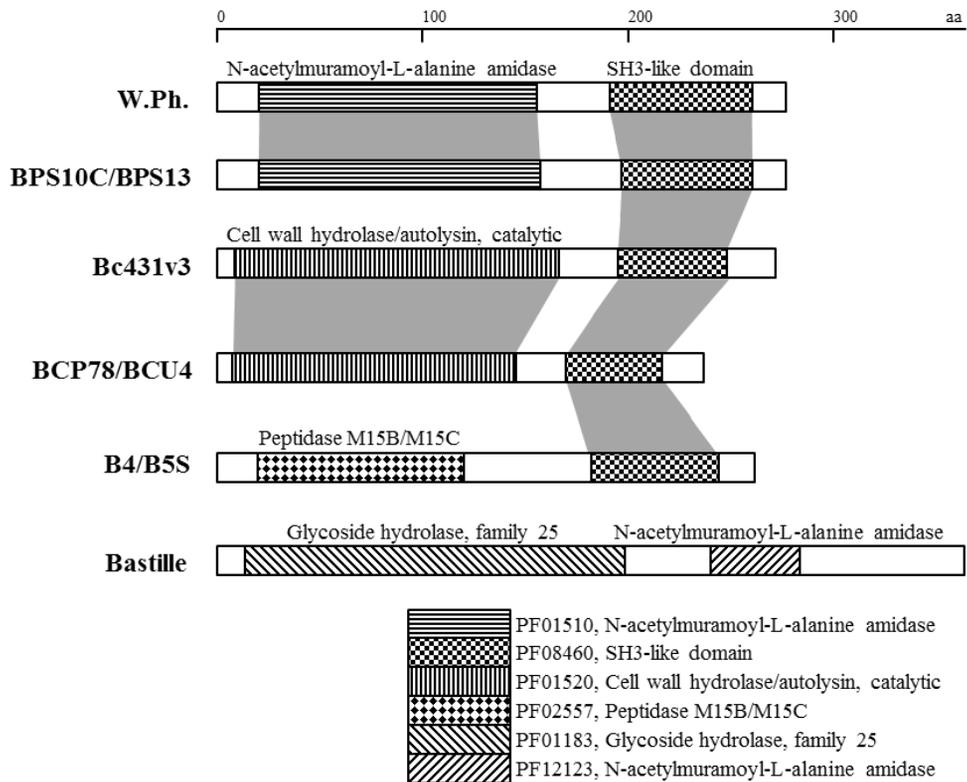


Figure 4.4. A comparative functional domain analysis of endolysins in the *B. cereus sensu lato* phage group I phages using the InterProScan program (72).

Two *B. cereus* phages, BPS10C and BPS13, were characterized and compared. The host range analysis for these two phages showed that they completely inhibit the growth of *B. cereus* group strains over 6 h (Unpublished, H. Shin, J.-H. Lee, J. Park, and S. Ryu). These two phages have a wide range of pH (5 to 8) and temperature (<50°C) stabilities. Whereas these two phages showed very similar characteristics, the phage BPS13 has higher pH and temperature stabilities than the phage BPS10C. As discussed above, the endolysin of the phage BPS13, LysBPS13, belongs to the endolysin phage group I. In the presence of glycerol, the lytic activity of LysBPS13 is displayed in temperatures up to 100°C, suggesting high temperature stability (46). A comparative genome sequence analysis of these two phages revealed that they have an endolysin and two copies of tail lysins. This gene set encoding an endolysin and two tail lysins is present in all nine phage genomes of the *B. cereus sensu lato* phage group I (Table 4.2). Whereas a few endolysins were previously characterized, the characteristics of these two tail lysins are still unknown. Therefore, further experiments characterizing these tail lysins would extend our understanding about phage infection and host lysis mechanisms by the *B. cereus sensu lato* phage group I. The host range of the phage Bc431v3 showed a relatively broad growth inhibition spectrum including *B. cereus* group strains and even *B.*

licheniformis, *B. psychrosaccharolyticus*, and *B. megaterium*, but not *B. subtilis* (17). Bc431v3 has a long latent period (85 min) with a high burst size (>300 PFU). A comparative genome analysis of the phages BCP78 and BCU4 revealed that their genomes are nearly identical (>96% DNA sequence identity) (Fig. 4.3). The endolysin group II phages (Bc431v3, BCP78, and BCU4) and endolysin group IV (Bastille) have multiple numbers of tRNAs in their genomes (Table 4.2). Comparative codon usage analysis of *B. cereus* strains (AH187 and ATCC 14597) and the phage BCP78 showed different codon preference in asparagine, phenylalanine, and serine, suggesting that extra tRNAs in the phage genome may play a role in translation of phage genes, not of host genes (38). Experimental characterization and a complete genome sequence analysis of the *B. cereus* phage B4 showed that it has a short eclipse/latent period (10/15 min) and a high burst size (>200 PFU) (37). Moreover, a bacterial challenge assay showed complete growth inhibition of *B. cereus* up to 20 h and its endolysin, LysB4, also showed efficient host cell lysis in 15 min (57), suggesting that the phage B4 could be a good candidate as a novel biocontrol agent against *B. cereus*. Furthermore, a host range analysis of the phage B4 revealed that it can inhibit the growth of *B. cereus* group strains and *B. subtilis* (Shin *et al.* 2013. Arch. Virol.). The *B. cereus* phage B5S has almost identical genome

sequence (>99% DNA sequence identity) with the phage B4. Further experimental characterization of the phage B5S also showed it has almost identical host cell lysis activity. In addition, characterization of the endolysin in the phage Bastille, PlyBa showed the efficient lysis of *B. cereus* and *B. thuringiensis* (42).

The *B. cereus sensu lato* phage group I contains all virulent phages with high host lysis activity against *B. cereus*. Therefore, the phages in this group may be good candidates for various applications as biocontrol agents against *B. cereus*. However, most of the phages (except for the phage Bastille) in this group were only recently isolated and their genomic annotation information is not sufficient. Further research on the phages in this group is needed to increase our understanding and to develop applications in the inhibition of the growth of *B. cereus*.

IV-2-3. *B. cereus sensu lato* phage group II

According to the phage classification, *B. cereus sensu lato* phage group II includes the phages BtCS33, phIS3501, and Wbeta (W β) with virulent variants of Cherry, Fah, and Gamma (W γ) in the *Siphoviridae* family. Their genome sizes (36 to 53-kb) are smaller than those of the *B. cereus sensu lato* phage group I, but larger than those of the *B. cereus sensu lato* phage group III. The phages BtCS33 and phIS3501 were isolated from induced strains of *B. thuringiensis*, and phage Wbeta was isolated from induced strains of *B. cereus* (44, 70).

The phage Wbeta and its variants inhibit the growth of *B. anthracis* (Table 4.1). The phages in this group have been widely used for phage typing. Furthermore, the phage Wbeta was used to construct a reporter phage containing *luxAB* for rapid detection of *B. anthracis* within 1 h after infection (52). Phage Gamma is a variant of the phage Wbeta and it can infect *B. cereus* W strain with phage Wbeta in the genome as a prophage, whereas phage Wbeta cannot re-infect *B. cereus* W (12, 53). Therefore, phage Gamma was obtained from re-infection of phage Wbeta lysate to the host strain, *B. cereus* W. Whereas phage Wbeta cannot infect capsulated *B. anthracis*, phage Gamma infects both encapsulated and non-encapsulated *B.*

anthracis, indicating that phage Gamma can infect a broad range of *B. anthracis* (1). Because of this broad host range, phage Gamma has been widely used for identification and indirect detection of *B. anthracis* (1, 47). In addition, phage Gamma has been used to inhibit the growth of *B. anthracis*. Endolysin of the phage Gamma, PlyG was characterized, showing that it has two conserved protein domains such as N-terminal T7 lysozyme-like amidase domain for host cell lysis and C-terminal cell wall binding domain (29, 30, 49, 54). Notably, deletion of the binding domain abolished the host cell lysis activity of PlyG, suggesting that this binding domain may be important for specific host cell lysis by N-terminal catalytic domain (30). To further understand this binding domain, several motifs of the binding domain were chemically synthesized and their binding activities were investigated (49). This study elucidated that the short 10-amino acid sequence (LKMTADFILQ) is a key motif for host cell wall binding. This short sequence was coupled with Qdot-nanocrystals and used for rapid detection of *B. anthracis* (49). The host receptor study of phage Gamma showed that the host receptor of *B. anthracis* by phage Gamma is a surface-anchored protein containing a LPXTG motif, designated GamR (15). However, the genome sequence of *B. cereus* ATCC 14579 does not have this host receptor protein, GamR, explaining its narrow host specificity (15).

Phage Cherry has also been used for typing of *B. anthracis* but this phage is almost identical to phage Gamma in many aspects, such as phenotype, morphology and genome sequence (19). In addition, genome sequencing and gene expression studies of phage Fah showed the presence of unique viral promoters and a unique sigma factor, most likely involved in the host transcription event (43).

Phage BtCS33 was obtained by the induction of *B. thuringiensis* subsp. *kurstaki* CS33 (70). This phage efficiently lyses *B. thuringiensis* because of its endolysin, PlyBt33, containing conserved protein domains of an N-terminal glycoside hydrolase family 23 and a C-terminal amidase02_C. Notably, the C-terminal amidase02_C domain binds to the cell wall of *B. thuringiensis* and *B. subtilis* (71). Phage phIS3501 was isolated from *B. thuringiensis* var. *israelensis* ATCC 35646 after induction (44). The integration site of this phage is in *hlyII*, encoding hemolysin II. Therefore, this toxic hemolysin II may be activated by the induction of the prophage phIS3501.

The phages in the *B. cereus sensu lato* phage group II generally infect *B. anthracis* and *B. thuringiensis* and they have generally been used for typing of these species. The phage Gamma or its endolysin PlyG is a good candidate to control *B. anthracis*, thereby protecting against the fatal

anthrax disease. Therefore, these *B. anthracis*-infecting phages in this group should be further studied in the control of the bio-threat posed by *B. anthracis*. In addition, the study of *B. thuringiensis*-infecting phages would help to protect the Bt production via fermentation.

IV-2-4. *B. cereus sensu lato* phage group III

According to the phage classification, *B. cereus sensu lato* phage group III includes phages AP50, Bam35, GIL16C, and GIL10 in the *Tectiviridae* family. The order including the *Tectiviridae* family is not yet assigned. Phages in this *Tectiviridae* family have no head-tail structure, but instead they possess tail-like tubes and viral membranes consisting of a lipid bilayer with inner and outer capsids. Their genome sizes (14.3 to 14.9-kb) are the smallest among all *B. cereus* phages in this report. In 1972, the phage AP50 was originally isolated from a soil sample with *B. anthracis* Sterne as a host strain (3, 45). The host range analysis of this phage showed high host specificity to *B. anthracis*, indicating a narrow host range of the phage AP50. In addition, whereas phage AP50 makes turbid plaques, the variant AP50c showed clear plaques and the CsaB cell surface anchoring protein of *B. anthracis* may be involved in the phage adsorption (8). The phage Bam35 was initially isolated from *B. thuringiensis* (3). Notably, this phage has a discrete phage entry mechanism. N-acetyl-muramic acid of the host membrane is essential for binding of the phage Bam35 to the host, and the Bam35 virion has peptidoglycan hydrolysis activity. In addition, the phage Bam35 requires divalent cations, such as Ca^{2+} and Mg^{2+} , for phage

penetration (20). The temperate phage GIL01 was isolated from *B. thuringiensis*, and the phage genome was observed to be present in the host strain as a linear plasmid form (68). Notably, the induction of the lytic cycle in the phage GIL01 was reported to be associated with the host cellular SOS response to DNA damage (18). Notably, unlike endolysins of other phages, two ORFs encoding Mur1 and Mur2 were expressed, and their functions were experimentally confirmed to show peptidoglycan hydrolase activity during host lysis (66). In addition, the genome sequence and characteristics of the phage GIL16 showed high similarity with the phage GIL01 (67).

The phages in the *B. cereus sensu lato* phage group III infect *B. anthracis* and *B. thuringiensis*. In this group, the *B. anthracis*-infecting phage AP50 may be useful for *B. anthracis* typing and biocontrol. For example, the application of the endolysin from AP50 may serve as a host specific biocontrol. However, *B. thuringiensis*-infecting phages negatively affect the Bt production via fermentation. Therefore, studies of host-phage interactions, infection mechanisms, and lytic/lysogenic decision mechanisms may be important to provide extended information about the characteristics of *B. thuringiensis*-infecting phages (or prophages), ultimately describing a possible technique to prevent phage contamination of Bt.

IV-2-5. Other *B. cereus* bacteriophages

As previously discussed, we categorized the *B. cereus* group-infecting phages into three phage groups. We discussed these three groups in terms of their general and genomic features. However, several phages are not categorized into these groups when using comparative phylogenetic analysis (Fig. 4.1). A few phages have their own distinct characteristics, such as the *B. cereus*-infecting phages BCD7 and PBC1 and the *B. thuringiensis*-infecting phage 0305phi8-36. Phage BCD7 has the smallest genome whereas phage 0305phi8-36 has the largest genome in the *Myoviridae* family infecting *B. cereus* group (Table 4.1). Whereas phage PBC1 belongs to *Siphoviridae* family, the other two phages, BCD7 and 0305phi8-36, belong to the *Myoviridae* family. The virulent phage PBC1 in *B. cereus* group is the first *Siphoviridae* phage of which complete genome sequence was reported. A genome analysis of phage PBC1 showed the absence of lysogeny-related genes, supporting that it is a virulent phage (32). The phage BCD7 was isolated from a soybean sample, and displayed a high host lysis and growth inhibition activity in broth culture over 20 h (Unpublished, H. Shin, J.-H. Lee, and S. Ryu). Its genome has two copies of host cell wall hydrolases with a holin. However, a comparative dot plot

analysis of the phage BCD7 showed that its genome sequence is not homologous to other phage genomes in the *B. cereus* group-infecting phages (Fig. 4.2). In addition, the genome sequence of the phage 0305phi8-36 also showed no homology with other phage genomes (23, 64), suggesting that phages BCD7 and 0305phi8-36 may have evolved from different ancestors from other *Myoviridae* phages in the *B. cereus sensu lato* phage group I. The different genome sizes of these phages (94-kb for BCD7 and 219-kb for 0305phi8-36) support this hypothesis (Table 4.1).

IV-3. Conclusion

The pathogens *B. cereus* and *B. anthracis* and the insect pathogen *B. thuringiensis* are designated as a single species of *B. cereus sensu lato* (14, 24). *B. cereus* is a well-known food-borne pathogen and *B. anthracis* causes anthrax (9, 21, 22) (7). Therefore, control of these pathogens is important in the prevention of food poisoning and bio-threats. Antibiotics have been widely used to control them. However, penicillin-related antibiotics are ineffectual because of the production of β -lactamase (31, 50). Therefore, an alternative phage based approach has been suggested for their control. *B. thuringiensis* (Bt) has been widely used as a natural pesticide to kill harmful insect pests (4, 51). However, phage contamination negatively affects the Bt production via fermentation. Therefore, a Bt phage study is required to investigate a process to protect Bt from phage contamination.

Bacteriophages are bacterial viruses that specifically invade and kill the host bacteria (34, 60, 63). Therefore, phages have been applied to control the pathogenic *B. cereus* and *B. anthracis*. To control these pathogens, generally two approaches have been studied, such as direct growth inhibition of these pathogens and host cell lysis using phage endolysins. As an example, two *B. cereus* phages, FWLBc1 and FWLBc2,

were isolated from a soil sample and treated with mashed potatoes, resulting in a more than 5 log reduction in the cell numbers of *B. cereus*. This suggests that applying phages to foods may be useful in the control of food-borne pathogens, including *B. cereus* (39). In addition, the *B. cereus* phage BCP1-1 showed a high host specificity and inhibited only *B. cereus*, not other fermentative bacteria such as *B. subtilis* in Korean fermented soybean food, suggesting selective growth inhibition of only the target bacterium (6). In investigating the lysis of the host by phages, several endolysins, such as LysB4, LysBPS13, Ply12, Ply21, PlyBa (for *B. cereus*), and PlyG (for *B. anthracis*), have been characterized (42, 46, 54, 57). Generally, endolysins have the two conserved protein domains of a host cell wall binding domain (CBD) and an enzymatic activity domain for peptidoglycan lysis (EAD). The CBD may provide host specificity, transfer of endolysin, and cell wall binding to the specific membrane. The EAD lyses the peptidoglycan in the host membrane, but the cleavage site of each endolysin depends on the type of EAD domain. To control *B. anthracis*, PlyG was characterized. This endolysin recognizes and cleaves a neutral polysaccharide (NPS) comprised of galactose (Gal), N-acetylglucosamine (GlcNAc), and N-acetylmannosamine (Man-NAc) (13, 55). The application of PlyG (20 U) showed the almost sterilization of *B. anthracis* in 15 min, suggesting that

purified endolysin may be useful in the control of pathogens (54). In addition to the phage applications, the *B. anthracis* phages have been used for the typing of this pathogen. As an example, the phage Gamma is a virulent phage that specifically infects and lyses *B. anthracis* with >95% accuracy (1). In addition, *B. anthracis* can be detected using real-time PCR with the phage Gamma in 5 h with a detection limit of 207 CFU/ml, suggesting that the Gamma phage is a good detection tool for *B. anthracis* (47). Furthermore, the phage Wbeta was used to construct the reporter phage that contains the *luxAB* genes. This reporter phage was able to produce light in the *B. anthracis* host in 1 h (52). The detection limit of *B. anthracis* using this reporter phage system was 10^3 CFU/ml.

Because of recent developments in genomic technologies and bioinformatics, phage genomics is becoming more popular. Genomics provides further information about the physiology, genetics, and host-phage infection/interaction mechanisms of the phage. Therefore, 30 complete phage genome sequences of the *B. cereus* group in the families *Myoviridae*, *Siphoviridae*, and *Tectiviridae* are currently available (Table 4.1). The categorization of the phages into three groups revealed that morphology, genome size, and lifestyle may be associated (Table 4.1). Three *B. cereus* phage groups showed different genome size (group I > group II > group III),

phage family association (*Myoviridae* for group I, *Siphoviridae* for group II, and *Tectiviridae* for group III), and lifestyle (virulent phenotype for group I and temperate phenotype for group II and III). Although 30 complete genome sequences for phages of the *B. cereus* group are available in the GenBank database, the functions of the proteins encoded by 34.9 to 93.8% of the genes in the phage genomes are still unknown (Table 4.1). More than 69% of the genes in the *Myoviridae* phage genomes are hypothetical, most likely because of insufficient annotation information for their genomes. They were recently isolated and their genome sequences were reported. The insufficient annotation of the genomes highlighted the shortage of available information in the GenBank database (Table 4.1). However, annotation information about core genes of the *Myoviridae* phages, generally involved in host infection/interaction and phage replication/reconstruction, is available, and the core genes are shared in all phages in group I, suggesting that they may have been evolved from a common ancestor (Table 4.2). Notably, two phages, BCD7 and 0305phi8-36, belong to the *Myoviridae* family but they are not in phage group I. A comparative phylogenetic and comparative dot plot analysis of phage genomes showed that the genome sequences of phages BCD7 and 0305phi8-36 are not homologous to those of other phages in the phage group I (Fig. 4.1 and 4.2). This finding suggested

that their ancestors are different from one another and even different from the common ancestor of the phage group I, even though they belong to the *Myoviridae* family and have virulent phenotypes (Table 4.1). The different genome sizes of phages BCD7 (94-kb) and 0305phi8-36 (219-kb) support this hypothesis (Table 4.1).

In this review, we compared *B. cereus* group phages at the genomic level. A comparative genomic analysis of these phages showed that the *B. cereus* group phages can be categorized into three different groups with each group maintaining its own set of specific features. Whereas *B. anthracis* phages have been applied in the biocontrol, typing, and rapid detection of *B. anthracis*, the recently isolated and analyzed *B. cereus* phages were not well-suited for the biocontrol of the food-borne pathogen *B. cereus*. Nevertheless, phage group I generally inhibited the growth of *B. cereus* and are all virulent phages. This group of phages may be useful for the efficient biocontrol of *B. cereus* via the infection by the phages or the application of the purified endolysins. However, more than 36% of the *B. cereus* group phages are not assigned in this grouping, based on the comparative phylogenetic and dot plot analyses. When more phage genome sequences are available in the GenBank database, new phage groups could be generated from a further comparative phylogenetic analysis, and the phages

that are not assigned to a group may belong to these new phage groups. As discussed previously, insufficient genome analyses have been conducted for the *B. cereus* group phages. Therefore, further genome sequencing and bioinformatic analyses should be performed to overcome the lack of genome annotation information, to extend our understanding of these phages, and to successfully apply them in the biocontrol of the pathogens *B. cereus* and *B. anthracis*.

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IV-5. Appendix :
Characterization and Genomic Analysis of Bacteriophages
Targeting *Bacillus cereus*

**IV-5-1. Characterization and Complete Genome Sequence of a Virulent
Bacteriophage B4 Infecting Food-borne Pathogenic *Bacillus cereus***
(Published in Archives of Virology, 2013, in press)

IV-5-1-1. Abstract

Bacillus cereus causes food poisoning such as vomiting and diarrhea by production of enterotoxins. To control this food-borne pathogen, the virulent bacteriophage B4 was isolated and characterized. Bacterial challenge assays showed that phage B4 effectively inhibited growth of *B. cereus* group as well as even *B. subtilis*, and retained its growth inhibition for over 20 h. One-step growth analysis also revealed host lysis activity of phage B4 with relatively short eclipse/latent times (10/15 min) and high burst size (>200 PFU). The complete genome of phage B4 containing a 162-kb DNA with 277 ORFs was analyzed. The endolysin encoded by phage B4 genome have accounted for this cell lysis activity. These results suggest that phage B4 has potential as a biological agent to control *B. cereus* propagation.

IV-5-1-2. Introduction

Bacillus cereus is a food-borne pathogen producing enterotoxins such as hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K (20). Due to these toxins, the uptake of contaminated foods can cause vomiting, diarrhea, and nausea (5, 15). Generally, a high number of *B. cereus* cells (10^4 to 10^9 CFU per gram of contaminated food) is required for disease outbreak (24). However, responses to infection in humans such as diarrheal syndrome (8 to 16 h) and emetic syndrome (0.5-6 h) are relatively quick, due to toxins produced and released by *B. cereus* (8, 24). The outbreaks of *Bacillus* species in the European Union were reported up to 1.4% of all food-borne pathogenic outbreaks in 2005 (2), and the number of *B. cereus* outbreaks has been recently increasing in the developed countries, highlighting the importance to control *B. cereus* levels in foods (9, 17, 20).

The antibiotic resistance of *B. cereus* has been reported. Previous studies showed that *B. cereus* is generally insusceptible to penicillin-related antibiotics due to production of β -lactamase and sometimes even to erythromycin and tetracycline (18, 31). Therefore, an alternative bacteriophage approach has been suggested to be effective to reduce this pathogen in various foods (10). Due to this positive potential of bacteriophage application in foods, some bacteriophages infecting *B. cereus*

have been studied and reported (3) 12, (19) (23) (32). Two phages with highly specific host ranges, FWLBc1 and FWLBc2, were isolated and characterized to develop biocontrol agents in foods (23). Bandara *et al.* (3) reported that divalent cations such as Ca^{2+} , Mg^{2+} or Mn^{2+} are required to enhance the host lysis activity of the bacteriophage in fermented foods. Interestingly, about 40% of the fermented foods contain *B. cereus*-infecting phages, suggesting that *B. cereus* is prevalent in fermented foods where bacteriophages probably inhibit its growth and consequently, limit contamination (32).

To inhibit the growth of *B. cereus*, a novel bacteriophage B4 was isolated from an environmental sample and characterized using host range test, bacterial challenge assay, and one-step growth curve analysis. In addition, whole genome of phage B4 was completely sequenced and analyzed. In this report, we describe a novel potential biocontrol agent bacteriophage B4 and provide genomic information about this phage for further applications in foods.

IV-5-1-3. Materials and Methods

IV-5-1-3-1. Bacterial strains and growth condition

B. cereus ATCC 10876 was used as an isolation and propagation host for bacteriophage B4. Bacterial strains used for host range test are described in Table 4.3. All the bacterial strains were grown at 37°C with shaking in Luria-Bertani (LB) broth medium (Difco, Detroit, MI, USA).

IV-5-1-3-2. Isolation and propagation of bacteriophage B4

Mud samples from Seoul Grand Park (Gwacheon, South Korea) were used to screen for bacteriophages that infect *B. cereus* using strain ATCC 10876 as a host. In the case of solid samples, 25 g of the samples were homogenized in 225 ml sterile Butterfield's phosphate-buffered dilution water (0.25 M KH₂PO₄, pH 7.2) with a blender (BacMixer 400; Interscience Laboratory Inc., St. Nom, France). After homogenization, 25 ml of each homogenized sample was added to 25 ml of 2X LB broth and the mixture was incubated for 12 h at 37 °C with shaking at 220 rpm. The incubated culture was centrifuged at 9,000 × g, 4°C for 10 min and the supernatant was filtered to remove bacterial cells using 0.22 μm pore size filters (Millipore, Billerica, MA). Ten milliliter of the filtrate was mixed with 50 ml LB broth containing 1% overnight culture of *B. cereus* ATCC 10876 (final concentration) and the mixture was incubated at 37°C for 12 h

with shaking. After incubation, the mixed culture was centrifuged at $9,000 \times g$, 4°C for 10 min and the supernatant was filtered again to remove bacterial cells using $0.22 \mu\text{m}$ pore size filters (Millipore). In order to confirm the presence of bacteriophages in the filtered supernatant, tenfold serial dilutions of the filtrate were spotted on molten 0.4% LB soft agar containing 1% *B. cereus* ATCC 10876 (final concentration). The plates were incubated overnight at 37°C and monitored for formation of plaques. Each single plaque was picked with a sterile tip and eluted in 1 ml of sterilized sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). This purification step was repeated at least three times.

The culture of *B. cereus* ATCC 10876 ($\text{OD}_{600} = 1.0$) was infected with bacteriophages at a multiplicity of infection (MOI) of 1 and incubated at 37°C with shaking until the culture became clear. Cell debris was removed by subsequent centrifugation at $9,000 \times g$ for 10 min and filtration using $0.22 \mu\text{m}$ pore size filters and phage particles were precipitated by treatment of polyethylene glycol (PEG) 6,000 (Sigma, St. Louis, MO, USA). Finally, cesium chloride (CsCl) density gradient ultracentrifugation (Himac CP 100 β , Hitachi, Japan) with different CsCl steps (step density = 1.3, 1.45, 1.5 and 1.7 g/ml) was carried out at $78,500 \times g$, 4°C for 2 h. Viral particles were recovered and dialyzed with stirring using Spectra/Por 4 dialysis membrane tube (Spectrum, Rancho Dominguez, CA, USA) and SM buffer

for 1 h at 4°C.

IV-5-1-3-3. Bacteriophage host range

A hundred microliter of each test bacterial culture in stationary phase was added to 5 ml of the molten 0.4% LB agar and the mixture was overlaid on the 1.5% LB agar plate. And then 10 µl of each serially diluted B4 phage suspension from 10^2 to 10^{11} PFU/ml was spotted on the overlaid plates and these plates were incubated at 37°C. After incubation, appropriate titers forming single plaques were selected and the sensitivity of test bacteria to B4 phage was determined. The efficiency of plating (EOP) was calculated and determined from comparison of titers between the selected test bacterium and the host strain *B. cereus* ATCC 10876.

IV-5-1-3-4. Transmission electron microscopy (TEM)

Diluted CsCl-purified bacteriophage B4 in SM buffer was put on carbon-coated copper grids and negatively stained with 2% aqueous uranyl acetate (pH 4.0) for 2 min. Electroscope microscopy of prepared samples was carried out using a transmission electron microscope (LIBRA 120, Carl Zeiss, Switzerland) at 80 kV. Bacteriophage B4 was identified and classified into its relative family according to the guidelines of the International Committee on Taxonomy of Viruses based on the morphology of phages (13).

IV-5-1-3-5. Bacterial challenge assay

An exponentially growing *B. cereus* ATCC 10876 culture was infected with phage B4 (MOI = 1.0) to confirm its lytic activity. After adding phages to *B. cereus* cultures ($OD_{600\text{ nm}} = 1.0$), the optical density was monitored at 600 nm every hour, and the culture without phage infection was used as a control.

IV-5-1-3-6. One-step growth curve

When the $OD_{600\text{ nm}}$ of the culture of the same reference strain reached 1.0, 50 ml of the culture was harvested. B4 phage was added at a MOI of 0.01 and allowed to be adsorbed for 5 min at room temperature. The mixture was centrifuged and the supernatant was discarded to remove the residual phage. The cell pellet was then resuspended with the same volume of fresh LB broth medium and the resuspended culture was further incubated at 37°C with shaking. Two sets of samples were collected every 5 minutes. These two sets of samples were immediately diluted and plated for phage titration. However, in order to determine the eclipse period, the second set of samples was treated with 1% chloroform to release intracellular phages before the titration. Latent period and burst size were determined based on PFU number per cell.

IV-5-1-3-7. Isolation and purification of bacteriophage genomic DNA

Bacteriophage genomic DNA was isolated from the phage lysate as previously described by Wilcox *et al.* (36). Before purification of phage genomic DNA, phage lysate was treated with DNase and RNaseA at 37 °C for 1 h to remove bacterial DNA and RNA, respectively. Phage lysate was treated with lysis buffer (0.5% of Sodium dodecyl sulfate, 20mM of EDTA and 50 µg/ml of proteinase K, final concentration) for 2 h at 56°C. A standard phenol-chloroform DNA purification and ethanol precipitation were carried out (30).

IV-5-1-3-8. Genome sequencing of bacteriophage B4 and bioinformatics analysis

Extracted B4 phage DNA was sequenced with a Genome Sequencer FLX (GS-FLX) titanium sequencer (Roche, Mannheim, Germany) and assembled with GS de novo assembler software (Roche) at Macrogen Inc., South Korea. Prediction of open reading frames (ORFs) was carried out using GeneMarkS (4) and Glimmer v3.02 (11) and FgenesB softwares (Softberry, Inc. Mount Kisco, NY), and confirmed by RBSfinder (J. Craig Venter Institute, Rockville, MD). Prediction of tRNA genes was carried out using tRNAscan-SE program (25). Annotation of ORFs was performed using BLASTP (1) and InterProScan programs (37). The complete genome sequence and its annotation data were handled and edited by Artemis14 (7).

Evolutionary phylogenetic analysis of phage B4 was conducted using MEGA5 with neighbor-joining method (21).

IV-5-1-3-9. Nucleotide sequence accession number

The complete genome sequence of *B. cereus* phage B4 is available in GenBank database under accession number JN790865.

IV-5-1-4. Results and Discussion

IV-5-1-4-1. Isolation and characterization of bacteriophage B4

Bacteriophage B4 was isolated from a mud-sample as a clear-plaque former against strain *B. cereus* ATCC 10876. TEM analysis revealed that phage B4 had an isometric head with a nonflexible and contractile tail, suggesting that it belongs to the *Myoviridae* family (Fig. 4.5). Diameters of the isometric head and tail were about 85 nm and 21 nm, and non-contracted and contracted tail lengths were about 213 nm and 101 nm, respectively (Fig. 4.5). Morphological comparison of phage B4 and other *B. cereus* phages in *Myoviridae* family (3, 12) showed that the head size of phage B4 is smaller than those of BCP1-1 and BCP8-2 (>95 nm) but similar to that of Bc431v3 phage (85.4 ± 3 nm). However, the tail size of phage B4 is longer than that of Bc431v3 phage (180 ± 3 nm) but similar to those of BCP1-1 and BCP8-2 phages (220 nm and 210 nm), suggesting that phage B4 has relatively small head and long tails. A host range test of phage B4 revealed that phage B4 showed relatively broad inhibition against *B. cereus* group (*B. cereus*, *B. thuringiensis*, *B. mycooides*) and even *B. subtilis* (Table 4.3).

Table 4.3. Host range of bacteriophage B4

Bacterial isolate	Plaque formation^a	Source^b or reference
Gram positive bacteria		
<i>Bacillus cereus</i> ATCC 10876	CC	ATCC
<i>Bacillus cereus</i> ATCC 13061	C	ATCC
<i>Bacillus cereus</i> ATCC 14579	C	ATCC
<i>Bacillus cereus</i> ATCC 21768	C	ATCC
<i>Bacillus cereus</i> ATCC 27348	CC	ATCC
<i>Bacillus subtilis</i> ATCC 23857	CC	ATCC
<i>Bacillus mycoides</i> ATCC 6462	CC	ATCC
<i>Bacillus thuringiensis</i> subsp. <i>kurstaki</i> ATCC 35866	CC	ATCC
<i>Bacillus thuringiensis</i> ATCC 29730	CC	ATCC
<i>Enterococcus faecalis</i> ATCC 29212	-	ATCC
<i>Staphylococcus aureus</i> ATCC 29213	-	ATCC
<i>Staphylococcus epidermis</i> ATCC 35983	-	ATCC
<i>Listeria monocytogenes</i> ATCC 19114	-	ATCC
Gram negative bacteria		
<i>Salmonella enterica</i> serovar Typhimurium LT2	-	(26)
<i>Salmonella enterica</i> serovar Enteritidis ATCC13076	-	ATCC
<i>E. coli</i> K-12	-	(16)
<i>Shigella flexneri</i> 2a strain 2457T	-	(35)

^a, CC, EOP 1 to 0.1; C, EOP 0.1 to 0.001; -, no susceptible to phage B4.

^b, ATCC, American Type Culture Collection.

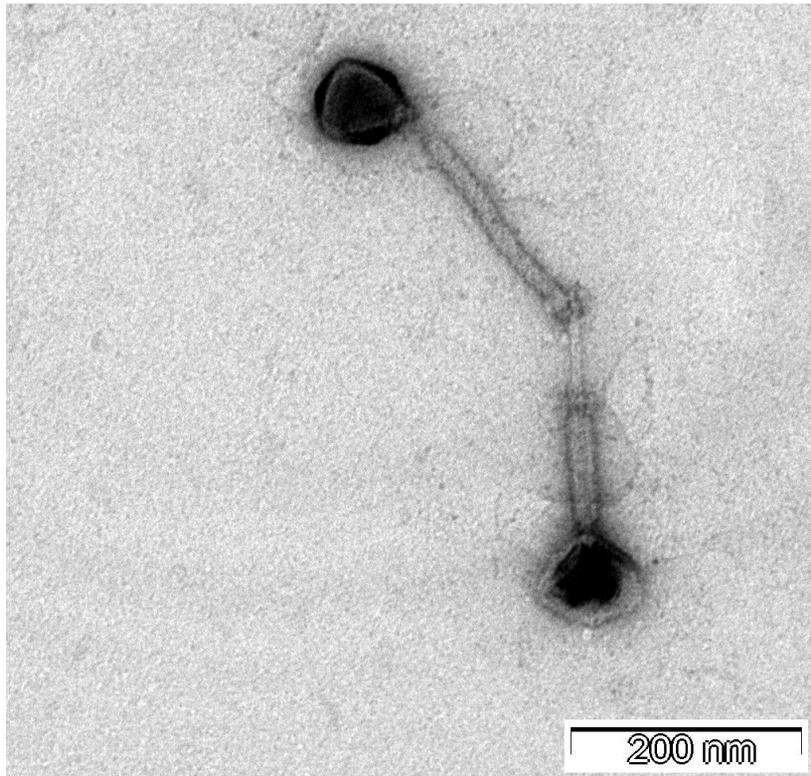


Figure 4.5. Transmission electron microscopy of bacteriophage B4 revealing that it belongs to *Myoviridae* family.

IV-5-1-4-2. Bacterial challenge assay

A bacterial challenge assay performed in liquid culture showed bacterial growth inhibition by phage B4. When phage B4 was added to the exponentially growing *B. cereus* ATCC 10876, the reduction of OD_{600 nm} was already observed within the first 30 min (Fig. 4.6). The growth inhibition activity was maintained for more than 20 h, indicating that the emergence of phage-resistant bacteria is low.

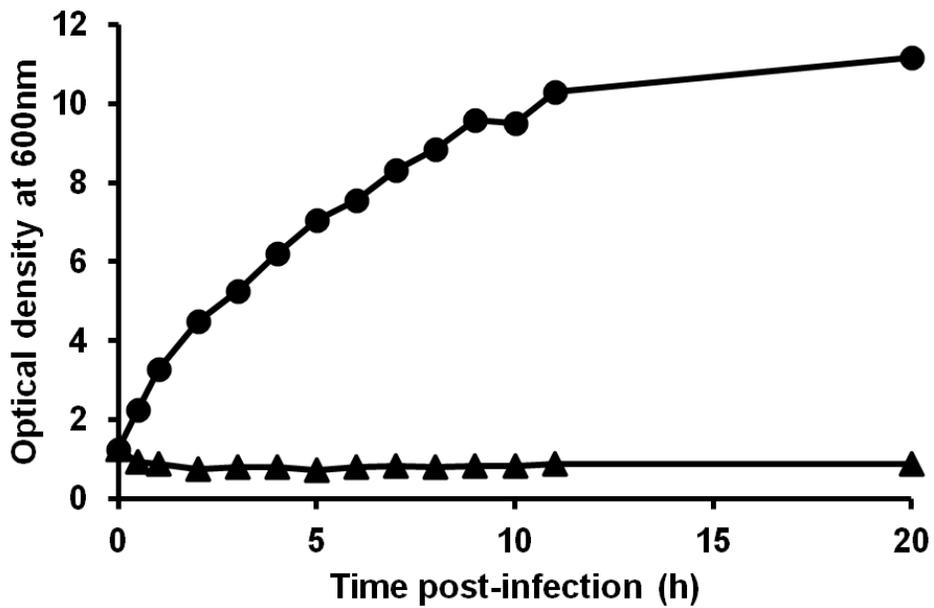


Figure 4.6. Bacterial challenge assay of phage B4 against *B. cereus* ATCC 10876 at a multiplicity of infection (MOI) of 1.0. Closed circle indicates non-phage treated *B. cereus* ATCC 10876 and closed triangle indicates phage treated *B. cereus* ATCC 10876.

IV-5-1-4-3. One-step growth curve of bacteriophage B4

The eclipse and the latent periods as well as the burst size of the B4 phage were determined by one-step growth curve analysis with *B. cereus* ATCC 10876 (Fig. 4.7). The eclipse and the latent periods of B4 phage were 10 min and 15 min, respectively. The burst size was more than 200 plaque forming unit (PFU) per infected host cell. The short latent period with large burst size indicates host lysis activity and propagation of this phage.

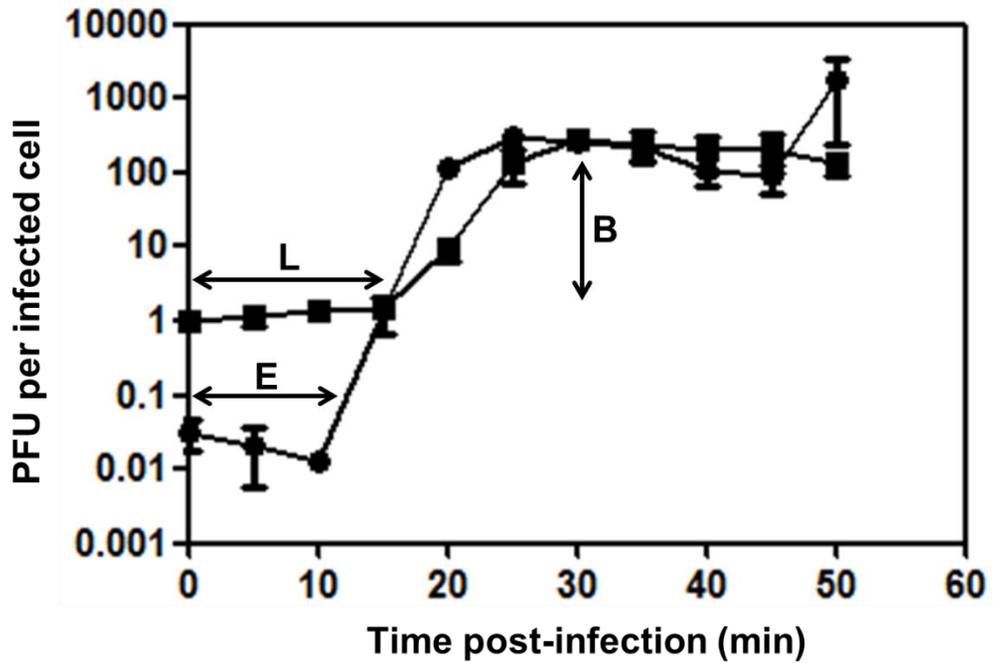


Figure 4.7. One-step growth curve analysis of *B. cereus* ATCC 10876 infected by B4 phage. E, eclipse period; L, latent period; B, burst size.

Closed circle indicates chloroform-treated sample and closed square indicates chloroform-untreated sample.

IV-5-1-4-4. Genomic analysis of bacteriophage B4

The complete genome of *B. cereus* bacteriophage B4 is 162,596-bp long with a GC content of 37.71%. A total of 277 ORFs was identified but no tRNA gene was detected (Fig. 4.8). The functional ORFs of phage B4 were categorized into eight groups such as packaging, lysis, regulation, host interaction, structure, DNA replication, RNA metabolism, and additional function and listed in Table 4.4. The phage genome encodes all necessary phage structural proteins including major capsid protein, structural protein, minor structural protein, portal protein, and several tail proteins (tail fiber protein, tail-lysins, tail sheath protein, putative tail protein, and baseplate proteins). In addition, this genome encodes many DNA replication proteins such as DNA polymerases, primase, helicases, exonucleases, and recombinase, implying that these proteins may collaborate with the host DNA replication proteins in replicating the phage genome. Interestingly, this genome also has its own putative sigma factors such as SigF-like protein (BCB4_0143) and phage RNA polymerase sigma factor (BCB4_0181). Although SigF is known to be involved in transcription of specific genes for sporulation, protein sequence identity of BCB4_0143 is less than 25% to those of other known *B. cereus* host SigF proteins, suggesting that its role for host sporulation cannot be deduced (12). However, phage RNA

polymerase sigma factor is relatively similar to other phage RNA polymerase sigma factors (40% protein sequence identity to that of *Enterococcus* phage phiEF24C), but no homology to *B. cereus* host RNA polymerase sigma factors, suggesting that this sigma factor may play a role in transcription of phage genes. Functions of sigma factors in these phages are not clearly understood yet, so further experiments may be needed to characterize them. Furthermore, the phage genome also encodes many additional functional genes such as metallophosphoesterase, ribonucleotide-diphosphate reductases, flavodoxin, thioredoxin, thymidylate synthase, dephospho-CoA kinase, and dihydrofolate. While their roles in the phage or in the host are not clearly understood yet, a few enzymes such as ribonucleotide-diphosphate reductases and thymidylate synthase were previously suggested to be involved in preparation of nucleotides for DNA synthesis (27). Additional evolutionary phylogenetic analysis of major capsid proteins (MCPs) of *Bacillus* bacteriophages revealed that phage B4 is closely related to other *Bacillus* phages such as Bastille (NC_018856), BPS13 (NC_018857) and BCP78 (NC_018860, (22)) phages in the *Myoviridae* family but quite different from other families such as *Siphoviridae* and *Podoviridae*, consistent with the previous morphological observation (Fig. 4.9). Furthermore, this genome does not have genes

associated with toxin production and bacterial virulence, suggesting the possibility of phage application to control pathogenic *B. cereus*.

Table 4.4. Functional grouping of predicted ORFs in bacteriophage B4

Functional Groups	Predicted function	Locus_tag
Packaging	terminase large subunit	BCB4_0004
	putative portal protein	BCB4_0270
Lysis	endolysin	BCB4_0006
	putative holin	BCB4_0179
Regulation	putative DNA-binding protein 1	BCB4_0089
	putative DNA-binding protein 2	BCB4_0168
	putative DNA-binding protein 3	BCB4_0208
	putative transcriptional regulator 1	BCB4_0234
	putative transcriptional regulator 2	BCB4_0235
	putative transcriptional regulator 3	BCB4_0272
Host interaction	cell division FtsK/SpoIIIE-like protein	BCB4_0127
	sporulation sigma factor SigF-like protein	BCB4_0143
	putative RNA polymerase sigma factor	BCB4_0181
Structure	Ig-like domain containing protein	BCB4_0133
	putative tail protein	BCB4_0238
	putative baseplate J protein	BCB4_0240
	putative baseplate protein	BCB4_0241
	putative minor structural protein	BCB4_0246
	putative tail fiber	BCB4_0247
	putative tail lysin 1	BCB4_0248
	putative tail lysin 2	BCB4_0249
	structural protein	BCB4_0258
	putative tail sheath protein	BCB4_0259
	putative capsid protein	BCB4_0266
putative prohead protease	BCB4_0268	

Table 4.4. Functional grouping of predicted ORFs in bacteriophage B4 (continued)

Functional Groups	Predicted function	Locus_tag
DNA replication	putative DNA polymerase 1	BCB4_0176
	DNA recombination/repair protein	BCB4_0184
	putative DNA polymerase 2	BCB4_0200
	putative primase	BCB4_0226
	putative exonuclease 1	BCB4_0228
	putative exonuclease 2	BCB4_0230
	putative helicase 1	BCB4_0233
	putative helicase 2	BCB4_0236
RNA metabolism	putative RNA ligase	BCB4_0100
Additional function	PhoH family protein	BCB4_0011
	thymidylate synthase	BCB4_0015
	dephospho-CoA kinase	BCB4_0017
	putative dihydrofolate reductase	BCB4_0021
	beta-lactamase superfamily hydrolase	BCB4_0138
	putative methyltransferase type 11	BCB4_0151
	band 7 protein	BCB4_0201
	thioredoxin	BCB4_0212
	putative flavodoxin	BCB4_0214
	ribonucleoside-diphosphate reductase	BCB4_0215
	ribonucleotide-diphosphate reductase subunit alpha	BCB4_0219
	putative dUTP pyrophosphatase	BCB4_0225
	putative metallophosphoesterase	BCB4_0231
	3D domain protein	BCB4_0253
	pectin lyase domain containing protein	BCB4_0257

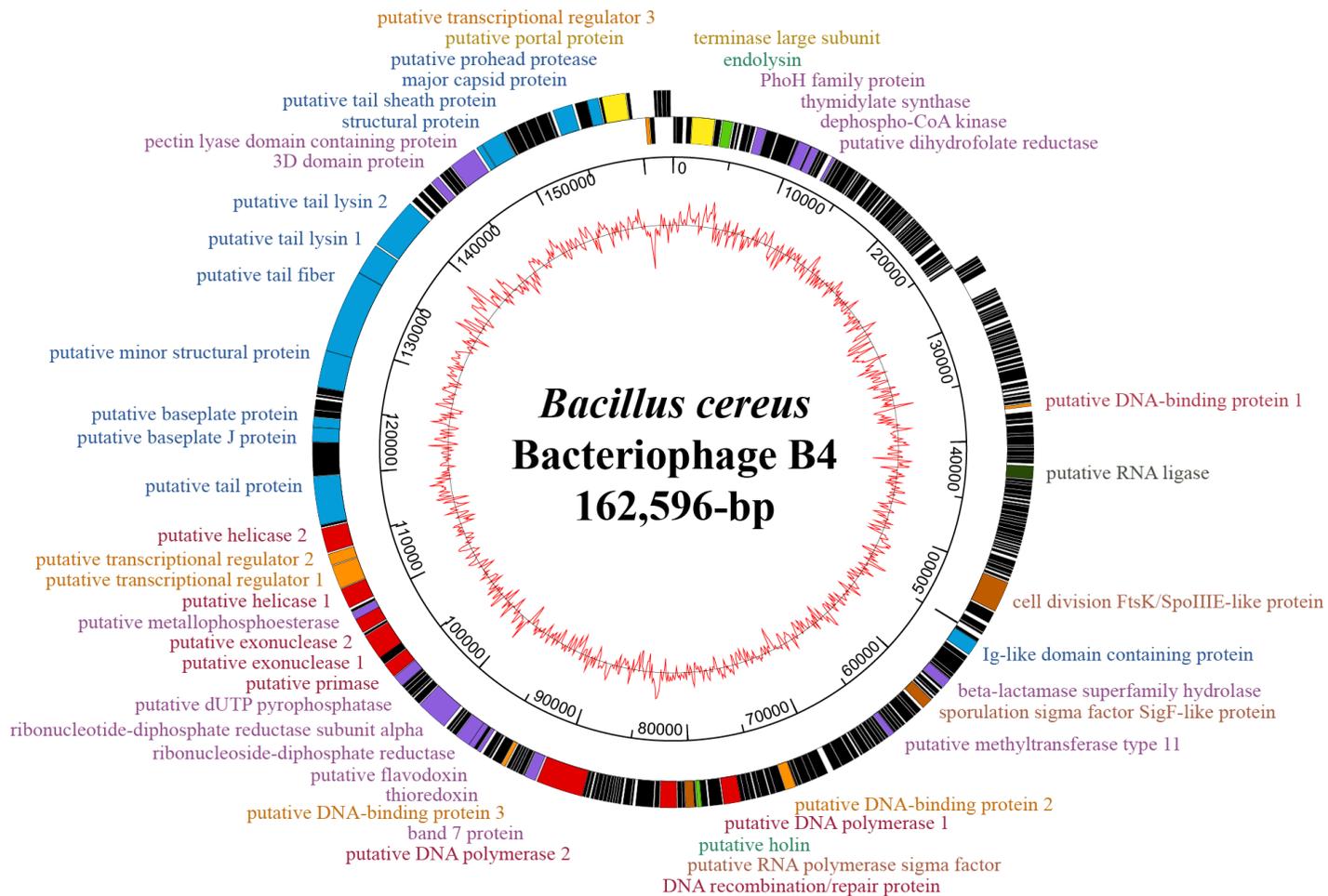


Figure 4.8. Genome map of phage B4. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories such as phage structure (blue), regulation (orange), host-phage interaction(brown), replication (red), cell lysis (green), packaging (yellow), and additional function (purple). The inner circle with red line indicates the G+C content. Scale unit is base pair.

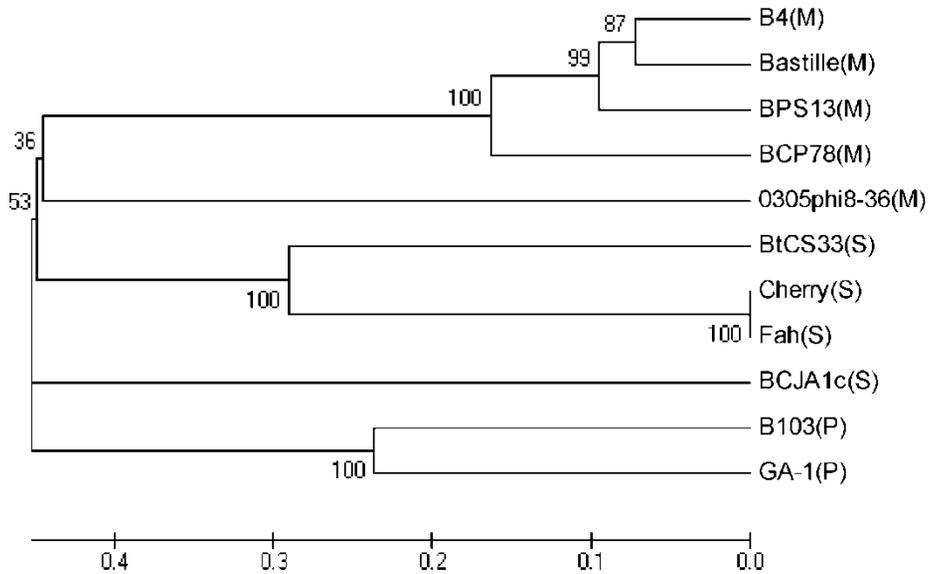


Figure 4.9. Phylogenetic tree of major capsid proteins (MCPs) in *Bacillus* bacteriophages. MCPs were compared by ClustalW multiple alignments and the phylogenetic tree was generated with the MEGA5 program using the neighbor-joining method with *P* distance values. (M), *Myoviridae*; (S), *Siphoviridae*; (P), *Podoviridae*.

IV-5-1-4-5. Host lysis of bacteriophage B4

The phage B4 genome encodes an endolysin (BCB4_0006) and a putative holin (BCB4_0179). While their respective genes lie unusually distant from each other in the genome (14, 28, 34), the highly lytic activity of phage B4 is most likely to be attributed to both enzymes. Interestingly, the endolysin of phage B4 (LysB4) has two conserved protein domains, PF08460 for cell wall binding and PF02557 for cell wall lysis (33). These conserved protein domains were frequently found in other bacteriophage endolysins against *Bacillus* and *Listeria*. Furthermore, LysB4 was experimentally characterized as an L-alanoyl-D-glutamate endopeptidase, showing optimum temperature and pH are 50°C and 8.5, respectively (33). The complete genome sequence of phage B4 also showed two genes, BCB4_0248 and BCB4_0249, encoding putative tail-lysins 1 and 2. Conserved protein analysis of these two tail-lysins showed that they may function in host lysis. Tail-lysins 1 has two conserved domains such as cd04129 (encoding Rho2 probably related to cell wall construction) and PF00877 (encoding cell wall-associated hydrolase probably related to cell wall lysis). Tail-lysins 2 also has a conserved domain, PF002901, coding for an endo- β -N-acetylglucosaminidase that is probably involved in peptidoglycan hydrolysis. However, it is necessary to experimentally

confirm that these tail-lysins are really involved in the host lysis. The broad host range and host lysis activity of phage B4 suggests that phage B4 can be a candidate as a novel biocontrol agent with its relatively high lysis activity against *B. cereus*.

IV-5-1-5. Conclusion

Due to its toxin production and antibiotic resistance, contamination with *B. cereus* is of increasing concern in the food industry and control methods other than classic antibiotic treatment are urgently needed. A bacteriophage-based approach has been suggested to control this kind of food-borne pathogens and one of bacteriophage applications (ListShield™ for control of *Listeria* in foods) has been approved by FDA (6, 29). To develop a novel biocontrol agent against *B. cereus*, bacteriophage B4 with relatively high host lysis activity was newly isolated and characterized. Subsequent complete genome sequence analysis of phage B4 revealed no genes associated with bacterial virulence or toxin and the presence of the host lysis system, suggesting that this phage may be suitable for host control. In this report, we suggest phage B4 as a possible biological candidate to control *B. cereus*.

IV-5-1-6. References

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**IV-5-2. Characterization and Genome Analyses of the *Bacillus cereus*-
infecting Bacteriophages BPS10C and BPS13**

(Submitted in Archives of Virology, 2013, In review)

IV-5-2-1. Abstract

Due to the emergence of antibiotic-resistant strains, bacteriophages are considered to be an alternative approach for the control of pathogens. In this study, the bacteriophages BPS10C and BPS13 were isolated and characterized to control food-borne pathogenic *B. cereus*. Phage BPS13 exhibits slightly higher host lysis activity compared with phage BPS10C. In addition, the analysis of their stability at various pH and temperature conditions revealed phage BPS13 exhibits higher stability. To extend our knowledge of the lysis of *B. cereus* by these phages, their genomes were completely sequenced and analyzed. The genome analysis results revealed that these phage genomes encode endolysin and two tail lysins, which are likely involved in the host invasion and lysis mechanisms. The combination of these host lysis-related proteins may increase the bactericidal activities of these phages, which suggests that these may be good candidates for the control of *B. cereus*.

IV-5-2-2. Introduction

Bacteriophages are bacteria-specific viruses that can lyse the host bacteria (5). Due to their host lysis activity, bacteriophages have been used for the control of specific bacteria in research and various other applications. Recently, bacteriophages are being studied to determine their potential as novel biocontrol agents against food-borne pathogens and antibiotic-resistant strains (4, 7, 18). These approaches are quite beneficial because phages target specific pathogens without affecting other beneficial bacteria in foods and even humans (20), as supported by the US Food and Drug Administration (FDA) approval of bacteriophage applications as food additives (4, 18). Therefore, the development of novel biocontrol agents using bacteriophages has been recently spotlighted. (4, 7, 18, 20).

Bacillus cereus is a Gram-positive food-borne pathogen that is frequently found in fresh vegetables and fruits grown in soil. This pathogen produces enterotoxins and cytotoxins that cause diarrhea and vomiting (3, 11, 14). Antibiotic treatment is not usually recommended because of the antibiotic resistance activity of this pathogen against penicillin-related antibiotics (12, 22). Therefore, the bacteriophage approach is an excellent candidate for the development of alternative biocontrol agents for the control of this pathogen. However, *B. cereus* phages have never been used

or applied for the biocontrol of this pathogen. Recently, a few *B. cereus*-targeting virulent phage genomes, such as BCP78, PBC1, Bc431v3, and B4, were completely sequenced and analyzed to extend our understanding of host-phage interaction and infection mechanisms (9, 13, 16).

IV-5-2-3. Materials and Methods

IV-5-2-3-1. Bacterial strains and growth condition

B. cereus ATCC 10876 was used to isolate and propagate the bacteriophages BPS10C and BPS13. The bacteria strains used for the determination of the antibacterial spectra of these bacteriophages are described in Table 4.5. All of the bacteria were cultivated in Luria-Bertani (LB) broth (Difco, Detroit, MI, USA) at 37°C with vigorous shaking, and the agar plate was prepared with a final agar (Difco) concentration of 1.5%.

IV-5-2-3-2. Bacteriophage isolation and purification

Food waste samples were collected from Mok-dong, Seoul in South Korea and used for the isolation of *B. cereus*-infecting bacteriophages. To isolate the bacteriophages, 25 g of each sample was mixed with 225 ml of Butterfield's phosphate-buffered dilution water (0.25 M KH₂PO₄, pH 7.2) in sterile bags. After homogenization, 25 ml of each diluted sample was mixed with 25 ml of 2X LB broth medium, and the mixture was incubated with shaking at 37°C for 12 h. Then, 0.5 ml of chloroform was added to the mixture, and the mixture incubated for 5 min at room temperature. The supernatant of the culture was collected by centrifugation at 6,000 × g for 10 min and filtered using 0.22-µm-pore-size filters (Millipore, Billerica, MA). Forty-five milliliters of each filtrate was mixed with an equal volume of LB

broth containing 10^7 CFU/ml *B. cereus* ATCC 10876, and the mixture was then incubated at 37°C for 12 h with shaking. After the incubation, the mixed culture was centrifuged at $6,000 \times g$ for 10 min, and the supernatant was filtered through 0.22- μm pore-size filters to remove the *B. cereus* cells. The filtered supernatant was used for plaque formation in molten 0.4% LB soft agar containing 10^7 CFU/ml *B. cereus* ATCC 10876. Each plaque was picked with a sterile tip and eluted with 1 ml of sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). This phage purification step was repeated at least five times.

For phage propagation, either BPS10C or BPS13 was added to a culture of *B. cereus* ATCC 10876 at a multiplicity of infection (MOI) of 1 when the optical density (OD) of the culture at 600 nm reached 1.0. The mixture was incubated at 37°C for 4 h with shaking. After the incubation of the mixture, the phage particles were recovered from the *B. cereus* cell debris by centrifugation at $6,000 \times g$ for 10 min and filtration using 0.22- μm -pore-size filters. The phage particles were then purified by precipitation with polyethylene glycol (PEG) 6,000 (Sigma, St. Louis, MO, USA) and CsCl density gradient ultracentrifugation (Himac CP 100 β , Hitachi, Japan) with different CsCl steps (step density = 1.3, 1.45, 1.5, and 1.7 g/ml) at $78,500 \times g$ and 4°C for 2 h. The purified phage particles were recovered, dialyzed using standard dialysis buffer (5 M NaCl, 1 M MgCl_2 , and 1 M Tris-HCl at pH 8.0), and stored at 4°C until further analysis.

IV-5-2-3-3. Transmission electron microscopy

The morphology of phages BPS10C and BPS13 were observed using Energy-Filtered Transmission Electron Microscope (EF-TEM). The phage samples were diluted with SM buffer, and 5 µl of each phage sample was applied to the surface of carbon-coated copper grids. The negatively stained samples with 2% uranyl acetate were allowed to absorb for 2 min. The prepared samples were observed using an EF-TEM (JEM-1010, JEOL, Tokyo, Japan) at 80 kV. The BPS10C and BPS13 phages were identified based on their morphology and classified into their relative family according to the guidelines of the International Committee on Taxonomy of Viruses (10).

IV-5-2-3-4. Bacteriophage host range test

Five milliliters of molten 0.4% LB top agar containing 100 µl of each test bacterial culture was overlaid on 1.5% LB base agar plates. Then, 10 µl of serially diluted phage solutions (10^2 to 10^{11}) were spotted on the overlaid agar plate and incubated at 37°C. The sensitivity of the test bacteria to each of the phages was determined based on whether a phage plaque formed. The efficiency of plating (EOP) was determined by a comparison of the titers between each selected test bacterium and the propagation strain *B. cereus* ATCC 10876.

IV-5-2-3-5. Bacterial challenge test

To confirm the host lysis activity of the phages, a *B. cereus* ATCC 10876 culture at OD_{600 nm} of 1.0 was infected with the corresponding phage (BPS10C or BPS13) at an MOI of 1.0. The optical density of the mixture was monitored at 600 nm at 1-h intervals. A *B. cereus* culture without phage infection was used as a control. This test was performed in triplicate.

IV-5-2-3-6. Bacteriophage DNA purification

The genomic DNAs of phages BPS10C and BPS13 were isolated as previously described by Wilcox *et al.* (25). Before the isolation of the phage genomic DNA, the phage particles were treated with DNase I and RNase A at 37°C for 1 h to remove the bacterial DNA and RNA, respectively. To isolate the phage genomic DNA, the phage particles were lysed with lysis buffer (1% sodium dodecyl sulfate (SDS), 0.5 mol/l EDTA, and 10 mg/ml proteinase K) for 2 h at 56°C. A standard phenol-chloroform DNA purification and ethanol precipitation was performed (21).

IV-5-2-3-7. Bacteriophage genome sequencing and bioinformatics analysis

The purified phage genomic DNAs were sequenced using a Genome Sequencer FLX (GS-FLX) instrument (Roche, Mannheim,

Germany), and the filtered sequence reads were assembled with Newbler 2.3 (Roche) at MacroGen Inc. (Seoul, South Korea). The prediction of all of the open reading frames (ORFs) was conducted using Glimmer v3.02 (8), GeneMarkS (2), and FgenesB (Softberry, Inc. Mount Kisco, NY, USA) and confirmed by RBSFinder (J. Craig Venter Institute, Rockville, MD, USA). The annotation and functional analysis of the predicted ORFs were performed using the BLASTP (1) and InterProScan (26) programs. The comparative genome analysis of these phages was conducted using the BLASTN (1) and Easyfig (24) programs. The phylogenetic analysis was conducted using MEGA5 with the neighbor-joining method (15).

IV-5-2-3-8. Nucleotide sequence accession number

The complete genome sequences of *B. cereus*-infecting phages BPS10C and BPS13 are available in GenBank database under accession number KC430106 and JN654439, respectively.

IV-5-2-4. Results and Discussion

To further develop this novel type of biocontrol agents, phages BPS10C and BPS13 were isolated in this study from food waste samples using *B. cereus* ATCC 10876 as a host strain due to the high lytic activities of this phages against *B. cereus* (see the supplementary methods). An analysis of these phages using an energy-filtered transmission electron microscope (EF-TEM) was conducted as previously described (23) . These phages are quite similar to each other and have heads and contractile tails, which suggests that these phages belong to the *Myoviridae* family (Fig. 4.10). The diameters of the heads and tails were approximately 79.9 nm and 18.7 nm, respectively, and the non-contracted and contracted tail lengths were approximately 193.5 nm and 177 nm, respectively (Fig. 4.10). In addition, the analysis of their range of hosts revealed that these phages can inhibit *B. cereus*, *B. thuringiensis*, and *B. mycoides* but cannot inhibit *Listeria monocytogenes*, *Staphylococcus aureus*, and *S. epidermis*, which indicates their host specificity at the genus level (Table 4.5).

To understand the inhibitory effect of phages BPS10C and BPS13 against *B. cereus*, a bacterial challenge test was conducted in liquid culture, as described in the supplementary methods. Interestingly, the initial

inhibition of phage BPS13 against the *B. cereus* host strain was slightly higher than that of the BPS10C phage at an MOI of 1 (Fig. 4.11a). However, at an MOI of 10, the inhibition of both BPS13 and BPS10C against the host strain was similar (Fig. 4.11b). This result suggests that phage BPS13 may exhibit slightly higher host inhibition at the initial inhibition step against *B. cereus* compared with phage BPS10C. In addition, the phage stability test of phages BPS10C and BPS13 revealed that phage BPS13 is more stable at a higher range of pH and temperature conditions than BPS10C, which suggests that phage BPS13 is a better candidate than phage BPS10C for *B. cereus* inhibition applications (Fig. 4.11c and 11d).

The genome of phage BPS10C contains 159,590-bp DNA with G+C contents of 38.74% and 271 ORFs, whereas the genome of phage BPS13 exhibits 158,305-bp DNA with G+C contents of 38.75% and 268 ORFs. In addition, neither of these genomes contains the tRNA gene (Table 4.6). The functional ORFs of these two phages were classified into seven groups: structure, packaging, host lysis, DNA manipulation, host interaction, regulation, and additional functions (Fig. 4.12). The functionally classified genes in each group are listed in Table 4.7.

The phylogenetic analysis of the isolated phages using the major capsid proteins of several phages suggested that these two phages belong to

the *Spounavirinae* subfamily. However, these two phages did not belong to any known genera (Spo1-like virus and Twort-like virus), similarly to the previously reported phage Bc431v3 (9) (Fig. 4.13). To predict the lifestyle of the BPS10C and BPS13 phages, the amino acid sequences of predicted ORFs were analyzed using the Phage Classification Tool Set (PHACTS) program (17). However, this program was unable to predict whether the lifestyle of these two phages was virulent or temperate, which suggests that these phages have genomes that are significantly different compared with the other phage genomes in the GenBank database. The prediction of the packaging type (6) showed that the packaging strategies of these two phages were not belong to any known packaging strategies of other phages (Fig. 4.14).

Interestingly, these two phages have two tail lysins (BPS10C_247 and BPS10C_248 in phage BPS10C and BPS13_0246 and BPS13_0247 in phage BPS13) within the structure group, which are most likely involved in the additional host lytic activity against *B. cereus* (Table 4.7). A previous report showed that the endolysin of phage BPS13 (LysBPS13, BPS13_0008) exhibits effective lytic activity and remarkable thermostability in the presence of glycerol, which suggests that LysBPS13 exhibits high potential for the development of a new biocontrol agent (19). Interestingly, due to the

high similarity of the endolysin of phage BPS10C (BPS10C_008) to LysBPS13, phage BPS10C is predicted to have a host lysis mechanism against *B. cereus* that is similar to that of phage BPS13. Interestingly, the ORFs encoding holins (BPS10C_172 and BPS13_0174) are located far from the ORFs encoding endolysins in these two phage genomes, which is different from the results obtained with other general phages. Thus, this finding suggests that their functions as holin proteins are not clearly understood and need to be experimentally confirmed. Although tail lysins may be associated with endolysin for host lysis, their inhibitory mechanisms should be experimentally confirmed.

The analysis of the DNA manipulation group showed that these phages may replicate their own DNA with help from the host DNA replication proteins. Interestingly, each phage genome has two transcription sigma factors (BPS10C_136 and BPS10C_175 in phage BPS10C BPS13_0137 and BPS13_0176 in phage BPS13) in the host interaction group, which suggests that these phages may have transcription regulation mechanisms that are different from those of the host (Table 4.7). The analysis of these two phage genomes revealed the presence of host lysis proteins and the absence of toxin-associated genes, which indicates their

potential usefulness as a novel biocontrol agent for the control of pathogenic

B. cereus.

Table 4.5. Host range of *B. cereus* bacteriophage BPS10C and BPS13

Bacterial isolate	Plaque formation ^a		Source ^b
	BPS10C	BPS13	
<i>Bacillus cereus</i> ATCC 10876	CC	CC	ATCC
<i>Bacillus cereus</i> ATCC 13061	C	C	ATCC
<i>Bacillus cereus</i> ATCC 14579	C	C	ATCC
<i>Bacillus thuringiensis</i> ATCC 29730	CC	CC	ATCC
<i>Bacillus thuringiensis</i> subsp. <i>kurstaki</i> ATCC 35866	CC	CC	ATCC
<i>Bacillus mycoides</i> ATCC 6462	T	T	ATCC
<i>Listeria monocytogenes</i> ATCC 19115	-	-	ATCC
<i>Staphylococcus aureus</i> ATCC 29213	-	-	ATCC
<i>Staphylococcus epidermis</i> ATCC 35983	-	-	ATCC

^a, CC, EOP 1 to 0.1 with clear plaque; C, EOP 0.1 to 0.001 with clear plaque; T, turbid plaque; -, not susceptible to phage BPS10C or BPS13.

^b, ATCC, American Type Culture Collection.

Table 4.6. General genomic features of bacteriophage BPS10C and BPS13

Characteristics	<i>B. cereus</i> bacteriophage	
	BPS10C	BPS13
Length (bp)	159,590	158,305
Overall G+C content (%)	38.74	38.75
No. of annotated genes	271	269
Avg gene length (bp)	539	530
Gene density (no. of genes/kb)	1.698	1.699
Gene coding content (%)	91.6	90.1
Gene GC content (%)	39.11	39.13
No. of tRNAs	0	0
No. of putative endolysin protein	1	1
No. of putative tail lysin protein	2	2

Table 4.7. Functional grouping of predicted ORFs in bacteriophage BPS10C and BPS13

Functional groups	Predicted function	Locus_tag	
		BPS10C	BPS13
Structure	adsorption associated tail protein	BPS10C_237	BPS13_0236
	baseplate J protein	BPS10C_239	BPS13_0238
	baseplate protein	BPS10C_240	BPS13_0239
	minor structural protein	BPS10C_245	BPS13_0244
	putative tail fiber	BPS10C_246	BPS13_0245
	tail lysine 1	BPS10C_247	BPS13_0246
	tail lysine 2	BPS10C_248	BPS13_0247
	tail sheath protein	BPS10C_255	BPS13_0254
	major capsid protein	BPS10C_263	BPS13_0261
Packaging	terminase large subunit	BPS10C_006	BPS13_0006
	portal protein	BPS10C_266	BPS13_0264
Host lysis	endolysin	BPS10C_008	BPS13_0008
	putative holin	BPS10C_172	BPS13_0174
DNA manipulation	DNA helicase 1	BPS10C_231	BPS13_0230
	DNA helicase 2	BPS10C_235	BPS13_0234
	primase	BPS10C_224	BPS13_0223
	possible DNA polymerase	BPS10C_167	BPS13_0169
	putative DNA polymerase	BPS10C_193	BPS13_0194
	exonuclease	BPS10C_226	BPS13_0226
	recombination/repair protein	BPS10C_178	BPS13_0179

Table 4.7. Functional grouping of predicted ORFs in bacteriophage BPS10C and BPS13 (continued)

Functional groups	Predicted function	Locus tag	
		BPS10C	BPS13
Host interaction	RNA polymerase sigma factor	BPS10C_136	BPS13_0137
	integration host factor	BPS10C_175	BPS13_0176
Regulation	transcriptional regulator	BPS10C_233	BPS13_0232
Additional function	ribose-phosphate pyrophosphokinase	BPS10C_009	BPS13_0009
	nicotinate phosphoribosyltransferase	BPS10C_010	BPS13_0010
	thymidylate synthase	BPS10C_018	BPS13_0018
	dephospho-CoA kinase	BPS10C_020	BPS13_0020
	dihydrofolate reductase	BPS10C_023	BPS13_0023
	metal-dependent hydrolase	BPS10C_130	BPS13_0131
	MazG nucleotide pyrophosphohydrolase	BPS10C_156	BPS13_0157
	thioredoxin	BPS10C_207	BPS13_0206
	flavodoxin	BPS10C_211	BPS13_0210
	ribonucleoside-diphosphate reductase subunit alpha	BPS10C_215	BPS13_0214
	ribonucleoside-diphosphate reductase subunit beta	BPS10C_212	BPS13_0211
	deoxyuridine 5'-triphosphate nucleotidohydrolase	BPS10C_222	BPS13_0221

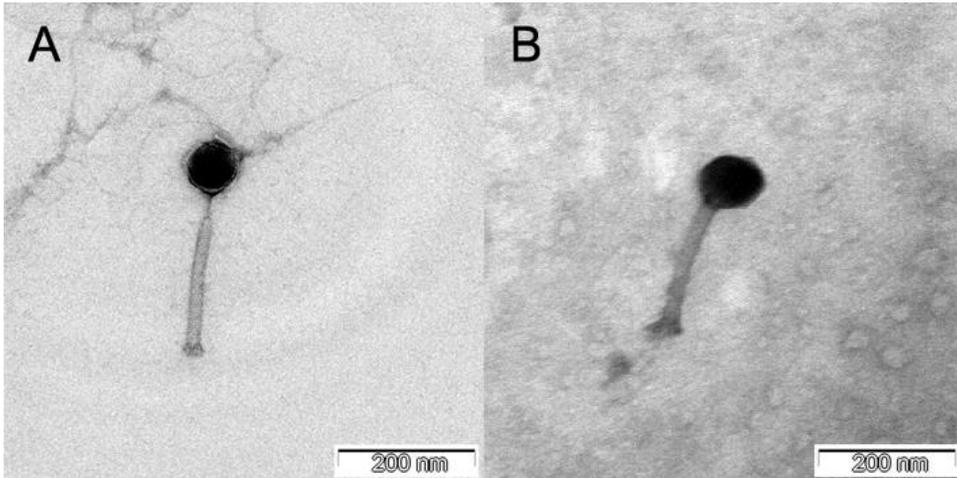


Figure 4.10. Electron microscopy images of phages BPS10C (A) and BPS13 (B).

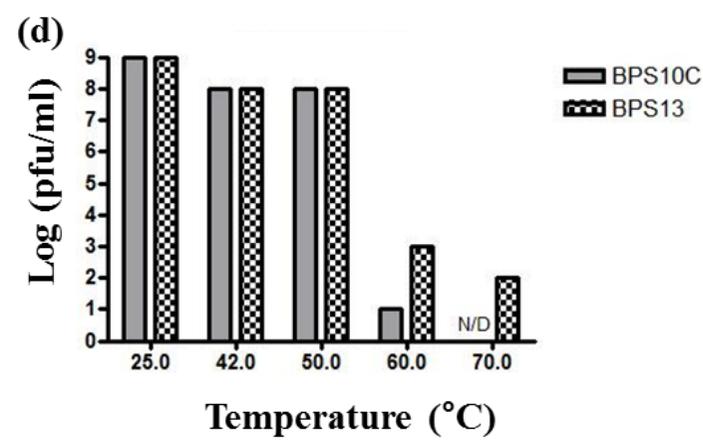
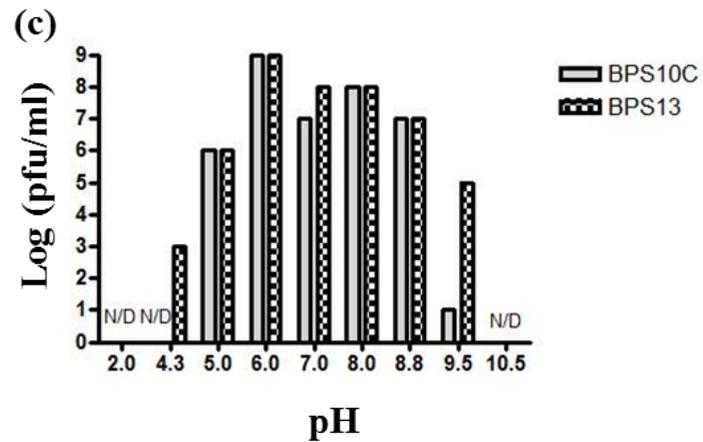
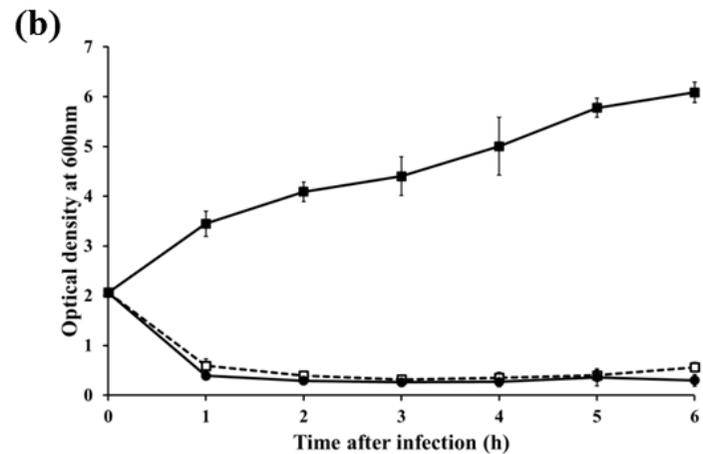
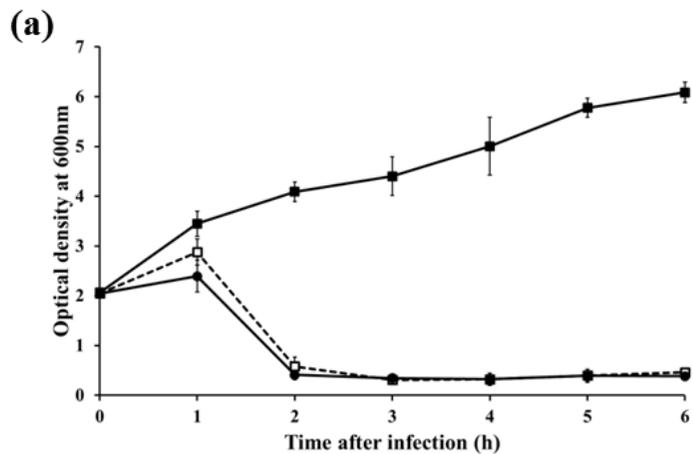


Figure 4.11. A-B. Bacterial challenge assay of phages BPS10C and BPS13 against *B. cereus* ATCC 10876 at two different multiplicities of infection (MOI): 1.0 (A) and 10 (B). Uninfected sample, filled square; phage BPS10C, open square; phage BPS13, filled circle.

C-D. Test of the pH (C) and thermal (D) stability of phages BPS10C and BPS13. N/D, not detected.

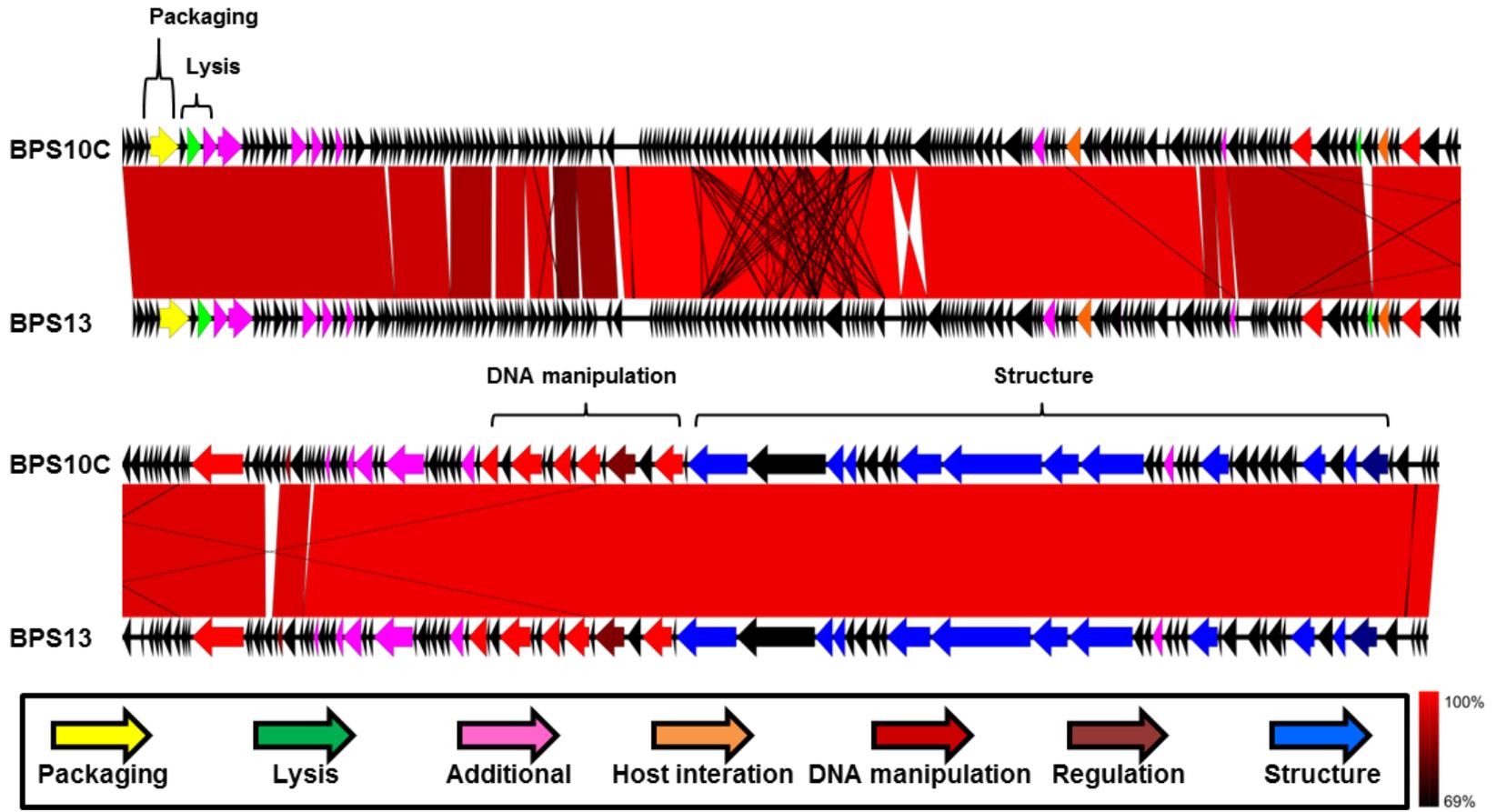


Figure 4.12. Comparative genome map of bacteriophages BPS10C (above) and BPS13 (bottom). The similarities between the two genomes at the DNA level were determined using the Easyfig program and are represented through gradations in color from red (100% similarity) to black (69% similarity).

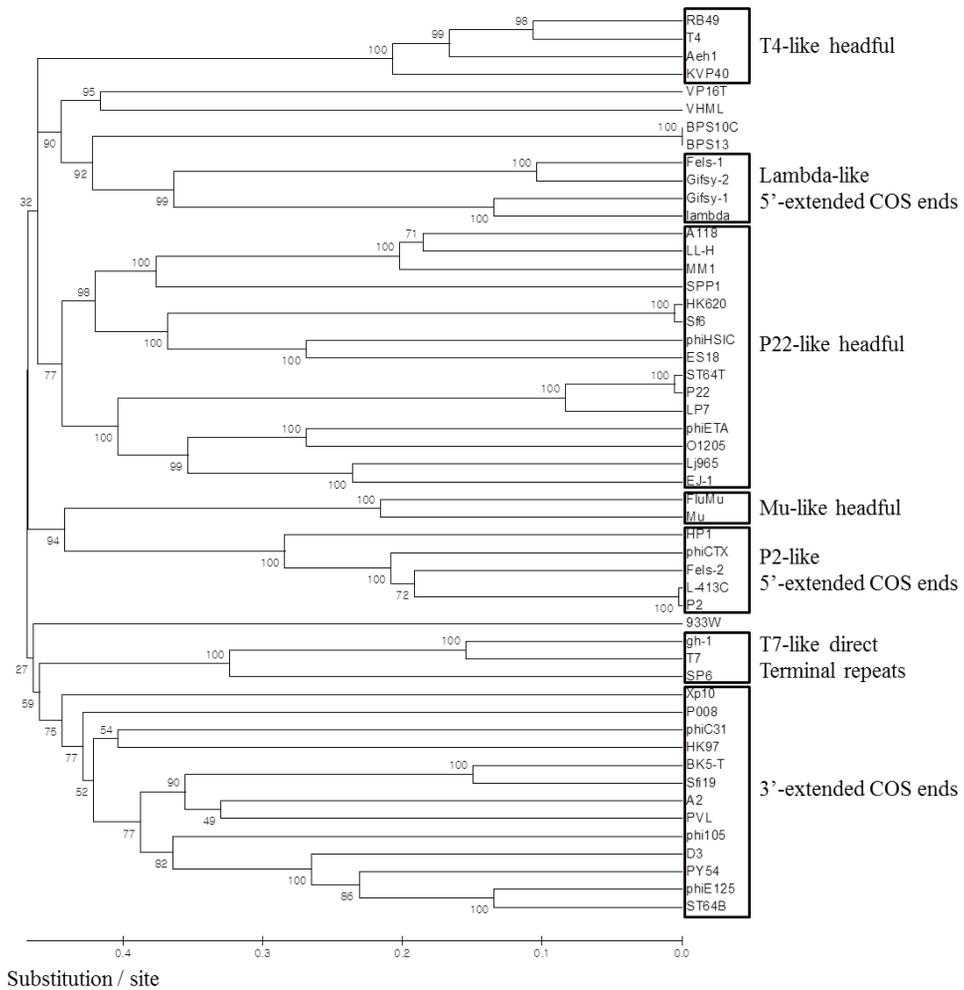


Figure 4.14. Phylogenetic analysis of the terminase large subunits in several bacteriophages. The terminase large subunits were compared using the ClustalW program, and the phylogenetic tree was generated through the neighbor-joining method with *P* distance values using the MEGA5 program.

IV-5-2-5. References

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Chapter V. Overall conclusion

V-1. Receptor Diversity and Host Interaction of Bacteriophages Infecting *Salmonella enterica* serovar Typhimurium

Twenty-five *Salmonella* phages were isolated and their receptors were identified by screening several mutants of *S. Typhimurium* SL1344. Three receptor groups of 25 newly isolated *S. Typhimurium*-targeting phages were determined. Among them, two subgroups of group F phages interact with their host receptors in different manners. In addition, the host receptors of group B or group L SPN9TCW phages hinder infection by phages in another group, probably due to interaction between receptors of their groups. This study provides novel insights into phage-host receptor interaction for *Salmonella* phages and will aid development of optimal phage therapy for protection against *Salmonella*.

1. Three types of receptors were identified: flagella (11 phages), vitamin B12 uptake outer membrane protein, BtuB (7 phages) and lipopolysaccharide-related O-antigen (7 phages)
2. All 25 phages could be categorized into three morphological groups. The phages using flagella (group F) or BtuB (group B) as a

receptor belong to the *Siphoviridae* family, and the phages using O-antigen of LPS as a receptor (group L) belong to the *Podoviridae* family.

3. SPN bacteriophages infect *S. Typhimurium* strains but not other Gram-negative and Gram-positive bacteria. Exceptively, the group B phages infect *E. coli* and *Shigella flexneri* strains as well as *Salmonella*.

4. While some of group F phages (F-I) target FliC host receptor, others (F-II) target both FliC and FljB receptors, suggesting that two subgroups are present in group F phages.

5. Group B phage-resistant *Salmonella* were transiently resistant to re-infection with group B phages, and most group F phage-resistant and group L SPN9TCW phage-resistant strains also showed transient resistance to re-infection with phages from their own group. All group L phage-resistant strains, except for the strain resistant to phage SPN9TCW, showed stable phage resistance to group L phages.

6. Cross-infection of group F phages on other phage-resistant strains showed sensitivity to these phages, suggesting no mutual influence between flagellin and other phage receptors on the

sensitivity to the phages

7. Group L phages could not infect group B phage-resistant strains and reversely group B phages could not infect group L SPN9TCW-resistant strain.

V-2. Genomic Investigation of Lysogen Formation and Host Lysis Systems of the *Salmonella* Temperate Bacteriophage SPN9CC

A novel *Salmonella* Typhimurium-targeting bacteriophage SPN9CC, belonging to *Podoviridae* family, was isolated and characterized. Phage SPN9CC showed distinct clear plaques with cloudy centers and was able to infect *S. Typhimurium* via O-antigen of lipopolysaccharide (LPS). Phylogenetic analysis revealed that this phage is a member of P22-like phage group. However, their lysogeny control regions and host lysis gene clusters share very low identities, suggesting that lysogen formation and host lysis mechanisms may be diverse among phages in this group. Expression of SPN9CC host cell lysis genes encoding holin, endolysin, and Rz/Rz1-like proteins revealed that collaboration of these lysis proteins is important for host lysis and holin is a key protein. To further investigate the role of lysogeny control region in phage SPN9CC, the ΔcI mutant (SPN9CCM) of phage SPN9CC was constructed and subsequent comparative one-step growth analysis and challenge assay were performed. The present work would give temperate phages the possibility of being engineered as promising biocontrol agents similar to the virulent phages.

1. *Salmonella*-targeting phage SPN9CC, belonging to the *Podoviridae* family, was isolated from a commercially processed broiler skin sample and showed distinct clear plaques with cloudy centers.
2. Phage SPN9CC specifically infects certain strains of *Salmonella* and O-antigen of LPS is a host receptor for phage infection.
3. The genome of phage SPN9CC has 40,128-bp length dsDNA with GC content of 47.33%, 63 putative ORFs, and 2 tRNAs. Comparative phylogenetic analysis revealed that phage SPN9CC is closely related to *Salmonella*-targeting P22-like phages.
4. Lysogeny control region (Cro, CI, and CII) of phage SPN9CC is different from phage P22 but similar to ϵ 34 phage, suggesting that SPN9CC and ϵ 34 may share lytic/lysogenic decision and lysogen formation mechanisms.
5. The host lysis gene clusters in phages SPN9CC, P22, and ϵ 34 are not conserved among them, suggesting that they probably lyse the host strains in different manners. Interestingly, the host lysis gene clusters of phage SPN9CC are similar to those of ST104 and even *E. coli* K-12 prophage DLP12, suggesting that they may share the same mechanism for host cell lysis.

6. To elucidate the host cell lysis mechanism of this phage, each gene in this cluster was cloned in pBAD18 and transformed into *S. Typhimurium* and *E. coli* hosts, respectively. In *S. Typhimurium*, the holin is a key protein for lysis. However, expression of these genes in *E. coli* host showed different host cell lysis patterns. The main difference between *E. coli* and *Salmonella* in terms of the SPC9CC lysis gene cluster is the role of Rz/Rz1-like proteins, which functions only in *E. coli*.

7. Effects of *cI* gene deletion on the life cycle of phage SPN9CC was studied by constructing the ΔcI mutant phage (SPN9CCM) with BRED method. SPN9CCM does not make cloudy centers in the plaques, suggesting that the phenotype of ΔcI mutant phage may be converted from temperate to virulent. In a comparative one-step growth analysis, while phage SPN9CC showed relatively long eclipse/latent periods and small burst size, phage SPN9CCM exhibited much shorter eclipse/latent periods and bigger burst sizes. Furthermore, bacterial challenge assays of phages SPN9CC and SPN9CCM with *S. Typhimurium* SL1344 showed that inhibition activity of phage SPN9CCM is much higher than that of phage SPN9CC.

V-3. Characterization and Comparative Genomic Analysis of Bacteriophages infecting the *Bacillus cereus* Group

According to recent development in genome sequencing and bioinformatics technologies, bacteriophage studies at the genomic level investigating possible applications of phages for phage therapy and as novel biocontrol agents have boomed. To date, 30 complete genome sequences of *B. cereus* group bacteriophages (18 for *B. cereus* phages, four for *B. anthracis*, and eight for *B. thuringiensis*) are available in the GenBank database. The general features of *B. cereus* group phages as well as comparative and functional genomic analyses and insights are described. This genomic information is useful for extending our understanding of their general genomic characteristics and their various applications in the control of bacterial pathogens and for phage therapy.

1. The comparative phylogenetic analyses of 30 *B. cereus* group bacteriophages revealed that they are categorized into three different phage groups (Group I, Group II, and Group III) with different morphology (*Myoviridae* for group I, *Siphoviridae* for group II, and *Tectiviridae* for group III), genome size (group I >

group II > group III), and life style (virulent phenotype for group I and temperate phenotype for group II and III). Subsequent phage genome comparison using dot plot analysis showed that phages in each phage group are highly homologous, substantiating the grouping of *B. cereus* phages.

2. The functions of proteins encoded by 34.9 to 93.8% of the genes in the phage genomes are still unknown. Especially, more than 69% of the genes in the *Myoviridae* phage genomes are hypothetical, probably due to insufficient annotation information for their genomes.

3. The annotation information about core genes of the *B. cereus sensu lato* phage group I, generally involved in host infection/interaction and phage replication/reconstruction, is available, and the core genes are shared in all phages in group I, suggesting that they may have been evolved from a common ancestor

4. In *B. cereus sensu lato* phage group I, four different endolysin groups were detected, according to combinations of two types of cell wall binding domain (CBD) and four types of enzymatic activity domain (EAD).

5. More than 36% of the *B. cereus* group phages are not assigned in this grouping, based on the comparative phylogenetic and dot plot analyses. When more phage genome sequences are available in the GenBank database, new phage groups could be generated from a further comparative phylogenetic analysis, and the phages that are not assigned to a group may belong to these new phage groups.

Chapter VI. Appendix :
Genomic Study of Other Bacteria and Bacteriophages

**VI-1. Complete Genome Sequence of the Opportunistic Food-borne Pathogen, *Cronobacter sakazakii* ES15
(Published in Journal of Bacteriology, 2012)**

VI-1-1. Abstract

Cronobacter sakazakii has recently been issued due to its high risk to powdered milk formula-fed infants. To characterize its physiology and pathogenicity in molecular level, *C. sakazakii* ES15 was isolated in South Korea and its genome was completely sequenced and analyzed. Here, the complete genome sequence and its analysis result of *C. sakazakii* ES15 are announced and major findings from its annotation data are reported.

VI-1-2. Main text

Cronobacter sakazakii is a Gram-negative opportunistic food-borne pathogen especially contaminating powdered milk formula for infants (4, 9). Recently, it has come into the spotlight due to the incredibly high risk to powdered formula-fed infants with 50 to 80% mortality (6, 13). Interestingly, its production of capsular material was reported (7), suggesting that this capsule formation may contribute to the host strain with high survival rate in extremely dried conditions. In addition, it causes meningitis, bacteremia, and necrotizing enterocolitis in infants probably due to its effective invasion into intestinal epithelial cells and brain microvascular endothelial cells (BMEC) (15). To further understand the physiology and pathogenicity of this pathogen in molecular level, its genome was completely sequenced and analyzed.

C. sakazakii ES15 was originally isolated from the ground whole grains and the genomic DNA was sequenced using GS-FLX pyrosequencer (Macrogen, South Korea). Prediction of the open reading frames (ORFs) was first performed using GeneMarkS (2) and Glimmer3 (3). The functional analyses of ORFs were conducted using BLASTP and InterProScan (1, 17). Transfer RNA and CRISPR repeat regions were predicted using tRNAscan-SE (12) and CRISPR finder (5). The functional categorization and metabolic

pathway analyses were carried out using COG and KEGG databases (10, 16), respectively.

The complete genome of *C. sakazakii* ES15 revealed 4,268,675-bp containing 3,916 ORFs, seven rRNA operons, and 80 tRNAs with GC content of 57.11%. In addition, this genome has two prophages and two CRISPR loci containing 9 and 16 CRISPR repeats, respectively.

Interestingly, one of the prophages, phiES15 is UV-inducible and its genome sequence was recently analyzed to elucidate the interaction between the host strain and this phage. The metabolic/biosynthetic pathway analysis using KEGG database showed that this genome has complete set of genes for glycolysis and TCA cycle as well as for flagella assembly, substantiating that this bacterium is really facultative aerobic and motile (8). In addition, it also has essential genes for biosynthesis of 20 amino acids. However, two aminoacyl-tRNA synthetases, such as glutaminyl-tRNA and asparaginyl-tRNA synthetases, are missing, suggesting that *C. sakazakii* may have alternative routes for successful translations of glutamine and asparagine (14). Interestingly, this genome has relatively many ABC transport systems and phosphotransferase systems (PTS), suggesting that *C. sakazakii* could uptake nutrients efficiently in the extremely dried conditions. It is intriguing that *C. sakazakii* ES15 genome encodes an outer membrane protein A

(OmpA, ES15_2832), probably involved in its invasion into BMEC, suggesting its pathogenicity. However, IbeB, a component of the copper/silver resistance cation efflux system, was not detected in this genome, which is different from *C. sakazakii* BAA-894 (11). While the complete genome sequence analysis of *C. sakazakii* increases our knowledge on the characteristics of this pathogenic bacterium in the extreme condition, further study of its pathogenicity in molecular level with this complete genome annotation needs to be elucidated in near future.

Nucleotide sequence accession number. The complete genome sequence of *Cronobacter sakazakii* ES15 is available in GenBank under the accession number CP003312.

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**VI-2. Complete Genome Sequence of *Cronobacter sakazakii*
Temperate Bacteriophage phiES15
(Published in Journal of Virology, 2012)**

VI-2-1. Abstract

While most of phage genome studies have been focused on the virulent phages, the inducible temperate bacteriophage genome study provides more detailed information about interaction between the host strain and the phage. To study this detailed interaction, UV-induced phiES15 bacteriophage was isolated from the host strain *Cronobacter sakazakii* ES15 and its genome was completely sequenced. Here we announce the genome sequence of phiES15 and report major findings from the annotation.

VI-2-2. Main text

Cronobacter sakazakii has been recognized as critical pathogenic bacteria especially for powdered formula-fed infants, due to high mortality up to 80% (6, 7, 12). While various antibiotics have been used to control this pathogen, recent studies reported emergence of antibiotic-resistant strains (14). Therefore, bacteriophage therapy using *C. sakazakii*-targeting phages has been suggested as alternative treatment to control this pathogen (8, 15). To optimize this phage therapy, the molecular interaction study between *C. sakazakii* host and its specific phages needs to be done. Furthermore, unlike virulent phages targeting *C. sakazakii*, the interaction study between the *C. sakazakii* host and its temperate phage will provide more detailed information about interaction between them. To study this detailed interaction, prophage-harboring *C. sakazakii* ES15 was isolated from the powder of ground whole grains and its temperate phage, designated phiES15, was also isolated from the host strain after UV induction. Here the genome of phage phiES15 was completely sequenced and analyzed.

Isolated and purified genomic DNA of phiES15 was sequenced using GS-FLX 454 pyrosequencer by Macrogen, Seoul, South Korea and the qualified reads were assembled using GS De Novo Assembler 2.3. The

prediction of open reading frames (ORFs) were conducted using three major gene prediction programs, GeneMarkS (2), Glimmer3 (5), and FgenesB (Softberry, Inc., Mount Kisco, NY) and confirmed by their ribosomal binding site analyses using RBS finder (J. Craig Venter Institute, Rockville, MD). The functions of ORFs were predicted by BLASTP analyses using Conserved Domain Database (CDD) (1, 10) and conserved protein motif analyses using InterProScan (13). Transfer RNA was predicted by tRNAscan-SE program (9) and the annotated genome information was handled using Artemis14 (3).

The phiES15 genome has 39,974-bp containing 52 ORFs and no tRNA with GC content of 53.54%. While twenty-three ORFs among them (43.3%) were hypothetical proteins, other ORFs were predicted to be functional and they were categorized into seven groups such as integration (integrase and excisionase), DNA modification & recombination (adenine-specific DNA methyltransferase and crossover junction endodeoxyribonuclease RusA), host interaction (host-nuclease inhibitor protein Gam and host division inhibitor protein Kil), transcription regulation (transcriptional repressor DicA, CII and antitermination protein Q), replication (replication protein O & P), host lysis (endolysin and Rz/Rz1), phage packaging (terminase small & large subunits). It is intriguing that the

phiES15 genome encodes host interaction proteins such as Gam (probably involved in protection of phiES15 phage genome from the foreign DNA degradation by host nucleases) (11) and Kil (probably involved in utilization of the host resources for phage reconstruction by inhibition of the host cell division) (4). In addition, 26-bp *attP* sequence is shared between the phage phiES15 and the host strain ES15, substantiating that this phiES15 is a temperate phage and it serves as phage integration site into the host genome. The complete genome study of this phage phiES15 would provide further information about the interaction between *C. sakazakii* host and its temperate phage.

Nucleotide sequence accession number. The complete genome sequence of *Cronobacter sakazakii* phage phiES15 is available in GenBank under the accession number JQ780327.

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VI-3. Complete Genome Sequence of *Cronobacter sakazakii*

Bacteriophage CR3

(Published in Journal of Virology, 2012)

VI-3-1. Abstract

Due to high risk of *Cronobacter sakazakii* to powdered milk formula-fed infants and emergence of antibiotic-resistant strains, an alternative biocontrol agent using bacteriophage is needed to control this pathogen. To further develop this, *C. sakazakii*-targeting bacteriophage CR3 was isolated and its genome was completely sequenced. Here we announce the genome analysis result of this largest genome sequence among *C. sakazakii* phages to date and report major findings from the genome annotation.

VI-3-2. Main text

Cronobacter sakazakii is an opportunistic food-borne pathogen often contaminating powdered infant milk formula, vegetables, and fruits and causes septicemia, meningitis and necrotizing enterocolitis in neonates (3, 6, 9). It has recently attracted public attention due to extremely high risk with 50-80% fatality rates to contaminated formula-fed infants (5, 9). High resistance of *C. sakazakii* to unusual dry condition supports high survival rate in the powdered infant formula (4). However, emergence of antibiotic-resistant *C. sakazakii* has limited antibiotic usage to control this pathogen (12), suggesting that development of alternative biocontrol agents like bacteriophage is urgently needed. Here, a novel *C. sakazakii* bacteriophage CR3 was isolated from an environmental sample and its genome was completely sequenced.

Genomic DNA was isolated using standard alkaline lysis method (10) and was sequenced using the GS-FLX Titanium by Macrogen, Seoul, South Korea. The quality-filtered reads were assembled using Newbler 2.3 and the prediction of open reading frames (ORFs) was performed using GeneMarkS (1), Glimmer3 (2), and FgenesV (Softberry, Inc., Mount Kisco, NY). Transfer RNAs were predicted using tRNAscan-SE (8) and conserved protein motif analyses of the predicted ORFs were conducted using

InterProScan (11). Comparative codon preference analyses of the *C. sakazakii* BAA-894 (7) and phage CR3 genomes were carried out using CodonW 1.4.4 in MOBYLE portal website (Pasteur Institute, Paris, France).

Complete genome of *C. sakazakii* phage CR3 belonging to *Myoviridae* family consists of 149,273-bp length with a GC content of 50.95%, 265 ORFs, and 18 tRNAs, indicating the largest genome among *C. sakazakii* bacteriophages to date. Interestingly, this phage genome contains many tRNAs and comparative codon preference analyses between the phage and *C. sakazakii* BAA-894 showed different codon preference of valine, serine, alanine, lysine, asparagine, arginine, and glycine, suggesting that these extra phage tRNAs may play a role in translation of phage mRNA, not host mRNA (data not shown). The genome of phage CR3 encodes structure/packaging proteins (major capsid protein, head stabilization/decoration protein, tail fiber proteins, tail fiber assembly protein, tape measure protein, and terminase), DNA manipulation proteins (polymerases, methylases, primase, helicase, ligase, methyltransferase, and endonucleases), and many additional functional proteins such as a thymidylate synthase and a cell wall hydrolase SleB. Although this genome encodes many ORFs, most of them are hypothetical proteins (84.5%), probably due to insufficient information about *C. sakazakii* phage genes in

GenBank database. This phage CR3 has two copies of tail fiber proteins targeting flagella of *C. sakazakii*, experimentally confirmed by non-infection of CR3 to the flagella-deletion mutant (data not shown). While this phage genome does not encode endolysin for host lysis, it encodes a cell wall hydrolase SleB, suggesting that this protein may be involved in the host lysis. The complete genome analysis of *C. sakazakii* phage CR3 provides further information of *C. sakazakii* phages and extends its application as a natural biocontrol agent to control *C. sakazakii*.

Nucleotide sequence accession number. The complete genome sequence of *C. sakazakii* bacteriophage CR3 is available in GenBank under accession number JQ691612.

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VI-4. Complete Genome Sequence of Phytopathogen

Pectobacterium carotovorum subsp. *carotovorum*

Bacteriophage PP1

(Published in Journal of Virology, 2012)

VI-4-1. Abstract

Pectobacterium carotovorum subsp. *carotovorum* is a phytopathogen causing soft rot and blackleg of potatoes. To control this plant pathogen, *P. carotovorum*-targeting bacteriophage PP1 was isolated and its genome was completely sequenced to develop a novel biocontrol agent. Interestingly, the 44,400-bp genome sequence does not encode any gene involved in the formation of lysogen, suggesting that this phage may be very useful as a biocontrol agent because it does not make lysogen after host infection. This is the first report on the complete genome sequence of *P. carotovorum*-targeting bacteriophage and it would increase our knowledge about the interaction between phytopathogens and their targeting bacteriophages.

VI-4-2. Main text

Pectobacterium carotovorum subsp. *carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora*) is a phytopathogenic Gram-negative bacterium, causing soft rot and blackleg of various potato and Chinese cabbages (5, 8, 10). This bacterial soft-rot causes serious loss of produce quality during their growth, transit and even storage. Recently, a novel bacteriocin, carocin D was isolated from *P. carotovorum* Pcc21 to develop a biocontrol agent to control this phytopathogen (9). Furthermore, to increase this antibacterial activity, additional biocontrol agent such as bacteriophage treatment is needed. This approach could be one of the best biocontrol agents to maximize the growth inhibition of this pathogen in plants. Here, we isolated *P. carotovorum*-targeting bacteriophage PP1 and fully sequenced its genome to understand its inhibition mechanism against this pathogen.

The genome of phage PP1 was isolated using alkaline lysis and phenol extraction method (11) and completely sequenced using GS-FLX pyrosequencing technology (Macrogen, Seoul, Korea). The open reading frames (ORFs) were bioinformatically predicted using Glimmer3 (4), GeneMarkS (2), and FgenesB (Softberry, Inc., Mount Kisco, NY) and confirmed by RBS finder (J. Craig Venter Institute, Rockville, MD). The

functions of ORFs were predicted by BLASTP (1) and InterProScan with protein motif databases (12). The complete genome sequence and annotation information was edited and handled using Artemis14 (3).

The genome of *P. carotovorum*-targeting phage PP1 consists of 44,400-bp length with 49.74% GC content, encoding 48 ORFs with no tRNA. Although six phage genomes are available in GenBank database infecting *Erwinia amylovora*, *E. tasmaniensis*, and *E. pyrifoliae* to date (6, 7), there is no report on the complete genome sequence of *P. carotovorum*-infecting phage. Therefore, more than 50% of the annotated ORFs (26 of 48) encode hypothetical proteins due to insufficient genome annotation information on this phage. The genes encoded in this genome are categorized into five groups, DNA replication/manipulation (DNA-directed RNA polymerase, DNA primase, DNA polymerase, HNH endonuclease, 5'-3' exonuclease, and DNA ligase), phage structure (head-tail connector, scaffolding protein, major capsid protein, tail tubular proteins, and internal virion proteins), phage packaging (terminase large and small subunits), host lysis (lysozyme-domain protein and holin), and host specificity (tail fiber protein). Interestingly, this genome has only one gene encoding tail fiber protein for host specificity, suggesting that this gene may be important to recognize and to infect the host. After infection of this host, the genes

encoding lysozyme and holin may play important roles in the host lysis. However, this genome does not encode proteins involved in lysogen formation, suggesting that this phage probably does not make lysogen after phage infection. The absence of genes involved in lysogen formation may be very useful to develop an effective biocontrol agent with this phage. This is the first report on the complete genome sequence of *P. carotovorum*-targeting bacteriophage and it would extend our understanding about bacterial pathogenesis to plants and its control between *P. carotovorum* and its infecting phages.

Nucleotide sequence accession number. The complete genome sequence of *P. carotovorum*-targeting bacteriophage PP1 is available under GenBank accession number JQ837901.

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VI-5. Complete Genome Sequence of Marine Bacterium

***Pseudoalteromonas phenolica* Bacteriophage TW1**

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VI-5-1. Abstract

For molecular study of marine bacteria *Pseudoalteromonas phenolica* using bacteriophage, a novel bacteriophage TW1 belonging to the *Siphoviridae* family was isolated, and its genome was completely sequenced and analyzed. The phage TW1 genome consists of 39,940-bp length double-stranded DNA with GC content of 40.19% and it was predicted to have 62 open reading frames (ORFs) and they were classified into functional groups such as phage structure, packaging, DNA metabolism, regulation, and additional function. The phage life style prediction using PHACTS showed that it may be a temperate phage. However, genes related to lysogeny and host lysis were not detected in phage TW1 genome, indicating that annotation information about *P. phenolica* phages in the genome databases may not be sufficient for the functional prediction of encoded proteins. This is the first report of *P. phenolica*-infecting phage and this phage genome

study would provide useful information for further molecular research of *P. phenolica* host and its phage as well as their interactions.

VI-5-2. Main text

The genus *Pseudoalteromonas* is generally found in eukaryotic hosts (8) associated with marine animals (i.e. tunicates and mussels) (9, 11) and marine algae (6). This genus was firstly suggested after division of *Alteromonas* into *Alteromonas* and *Pseudoalteromonas* by Gauthier *et al.* (7) with comparative analysis of 16S rRNA sequences. This genus is aerobic, Gram-negative and motile with a single polar flagellum. Among this genus, *P. phenolica* attracted the public attention because the strain *P. phenolica* O-BC30^T produces the phenolic antimicrobial compound against methicillin-resistant *Staphylococcus aureus* (MRSA) (10). In addition, a novel agarase to digest agarose for DNA gel extraction was recently found in *P. phenolica* JYBCL1 (14). Therefore, due to its usefulness in various applications, further molecular study and application of this species would be required.

Bacteriophage is a bacterial virus to infect specific bacterial host strain. After host infection, it lyses the host strain as a virulent phage or integrates its phage genome into the host genome as a temperate phage. Because of this genome integration, phage has been considered as a useful gene delivery carrier or a molecular tool for functional study of the host bacterial genome. The *P. phenolica*-infecting bacteriophage TW1 was isolated from the sea water in Taepyeong salt pond of Shinan, South Korea.

This phage TW1 (multiplicity of infection (MOI) at 1) was enriched with the culture of *P. phenolica* CL-TW1 at 30°C for 24 h with vigorous shaking. To obtain the phage solution, the mixture was centrifuged at $1,036 \times g$ for 20 min and filtered with 0.22 μm pore size filters (Millipore, Billerica, MA, USA). To concentrate and purify the phage from the filtrate containing the phage TW1, it was centrifuged with Amicon Ultra-15 centrifugal filter units (Millipore) and extracted using CsCl gradient ultracentrifugation from 1.2 to 1.55 g/ml densities at $25,000 \times g$, 4°C for 2.5 h. The genomic DNA was isolated following the procedure by Wilcox *et al.* (17). The phage particle was lysed using the phage standard lysis buffer containing 0.5% SDS and 100 $\mu\text{g/ml}$ of proteinase K and the mixture was incubated at 37°C for 30 min. To purify the phage genomic DNA, subsequent phenol-chloroform extraction and ethanol precipitation were conducted using the standard protocols by Sambrook *et al.* (16). The purified phage genomic DNA was sheared and randomly sequenced using GS-FLX pyrosequencer at Macrogen, Seoul, South Korea, and the qualified sequence reads were assembled using Newbler v2.3. The open reading frames (ORFs) in the genome were predicted using GeneMarkS (2), Glimmer3 (5), and FgenesB (Softberry, Inc., Mount Kisco, NY, USA) and confirmed using RBSFinder (J. Craig Venter Institute, Rockville, MD, USA). Annotation and functional

analysis of the predicted ORFs were performed using BLASTP (1) and InterProScan (18) programs. The genomic DNA sequence and annotation result were edited using Artemis14 (3), and the lifestyle of the phage TW1 for virulent or temperate phage was predicted using PHACTS program with all protein sequences from Artemis14 (13). Phylogenetic analysis of terminase large subunit of bacteriophages including phage TW1 was conducted using MEGA5 based on the neighbor-joining method with *P* distance values (12).

Morphological observation of phage TW1 using transmission electron microscopy (TEM) revealed that it has 103 ± 7 nm non-contractile tail and 73 ± 2 nm capsid, belonging to the *Siphoviridae* family (Fig. 6.1A). Pedulla *et al.* previously suggested that phage tail length is proportional to the size of gene encoding tail measure protein only in *Siphoviridae*-family phages (15). Based on this theory, the tail length of phage TW1 containing 2,028-bp tail measure protein gene (TW1_014) is predicted to be 101.4 nm. Consistently, the actual tail length of phage TW1 was observed to be 103 ± 7 nm, supporting this idea.

The complete genome sequence analysis of phage TW1 showed that the double-stranded genomic DNA consists of 39,940-bp length (GC content of 40.19%) with 62 predicted ORFs and no tRNA gene (Fig. 6.1B).

The average gene length is 586-bp and the gene coding percentage is 91.0%. The annotation results of all predicted ORFs were listed in Table 6.1. The complete genome sequence analysis showed that the predicted functions of all ORFs in phage TW1 was classified into five functional groups such as phage structure, packaging, DNA metabolism, regulation, and additional function. These functional groups and conserved protein domain analysis of predicted ORFs in phage TW1 were listed in Table 6.2 and Table 6.3, respectively. However, even though some core genes are functionally predicted, functions of more than 77% predicted ORFs are still unknown and host-lysis-related genes are undetected, probably due to insufficient annotation data of *P. phenolica*-infecting phages in the sequence databases.

Interestingly, BLASTP analysis result showed that about 65% of functionally predicted ORFs are slightly similar to the proteins encoded in various bacterial genomes, not in phage genomes, supporting this insufficient sequence data. To predict the lifestyle of phage TW1, PHACTS analysis was conducted with amino acid sequences of all predicted ORFs. While phage TW1 genome does not have genes encoding integrase or recombinase for phage genome integration into the host genome, the analysis result showed that phage TW1 may be a temperate phage (data not shown). These core genes for phage genome integration may be not properly

annotated, probably due to absence of similar integrase or recombinase genes in the sequence databases. An additional experiment to confirm the lifestyle of phage TW1 may be required. To further elucidate type of phage TW1, additional comparative phylogenetic analysis of phage TW1 was performed with phage terminase large subunits according to Casjens and Gilcrease's method (4). The result showed that phage TW1 may belong to Mu-like headful group (Fig. 6.2).

To our knowledge, this is the first report of a *P. phenolica*-infecting bacteriophage and its complete genome sequence. This phage genome sequence analysis result would be useful basic information about further molecular research of *P. phenolica* host and its phage as well as their infection/interaction mechanisms.

Nucleotide sequence accession number. The complete genome sequence of bacteriophage TW1 is available in GenBank database under accession number KC542353.

Table 6.1. List of all predicted ORFs in the genome of phage TW1

Locus_tag	Start	End	Strand	Predicted function
TW1_001	1	558	+	hypothetical protein
TW1_002	574	816	+	hypothetical protein
TW1_003	869	1102	+	hypothetical protein
TW1_004	1084	1428	+	hypothetical protein
TW1_005	1429	1821	+	hypothetical protein
TW1_006	1818	2180	+	hypothetical protein
TW1_007	2180	2464	+	hypothetical protein
TW1_008	2469	3002	+	hypothetical protein
TW1_009	2999	3439	+	hypothetical protein
TW1_010	3436	3873	+	hypothetical protein
TW1_011	4133	4435	+	hypothetical protein
TW1_012	4909	5415	+	hypothetical protein
TW1_013	5417	6058	+	hypothetical protein
TW1_014	6059	8086	+	tail tape measure domain-containing protein
TW1_015	8086	8556	+	hypothetical protein
TW1_016	8559	9059	+	hypothetical protein
TW1_017	9056	9463	+	hypothetical protein
TW1_018	9463	11964	+	phage tail domain-containing protein
TW1_019	11993	13969	+	hypothetical protein
TW1_020	13966	14166	-	hypothetical protein
TW1_021	14249	14515	+	hypothetical protein
TW1_022	14717	15430	-	HNH endonuclease domain-containing protein
TW1_023	15530	16561	-	possible head morphogenesis protein
TW1_024	16551	17930	-	possible portal protein
TW1_025	17989	18285	-	hypothetical protein
TW1_026	18443	18640	-	hypothetical protein
TW1_027	18640	19143	-	hypothetical protein
TW1_028	19140	19583	-	possible phosphoribosyl-ATP pyrophosphohydrolase
TW1_029	19585	20043	-	hypothetical protein
TW1_030	20040	20210	-	hypothetical protein
TW1_031	20207	20677	-	hypothetical protein
TW1_032	20844	21317	-	hypothetical protein
TW1_033	21350	21916	-	single-stranded DNA-binding protein
TW1_034	21957	22361	-	hypothetical protein
TW1_035	22358	23218	-	exodeoxyribonuclease 8 domain-containing protein

Table 6.1. List of all predicted ORFs in the genome of phage TW1**(continued)**

Locus_tag	Start	End	Strand	Predicted function
TW1_036	23281	25092	-	possible ATP-dependent helicase
TW1_037	25085	25864	-	possible DNA primase
TW1_038	25868	27403	-	hypothetical protein
TW1_039	27403	27651	-	hypothetical protein
TW1_040	28170	28349	+	hypothetical protein
TW1_041	28352	28549	+	hypothetical protein
TW1_042	28759	29295	+	putative N-6-adenine-methyltransferase
TW1_043	29285	29467	+	hypothetical protein
TW1_044	29670	30245	+	hypothetical protein
TW1_045	30462	30725	+	hypothetical protein
TW1_046	30729	31217	+	possible terminase small subunit
TW1_047	31204	32814	+	possible terminase large subunit
TW1_048	32959	33129	-	hypothetical protein
TW1_049	33163	33333	-	hypothetical protein
TW1_050	33320	34228	-	hypothetical protein
TW1_051	34647	34931	+	hypothetical protein
TW1_052	34928	35131	+	hypothetical protein
TW1_053	35131	35304	+	hypothetical protein
TW1_054	35301	35921	+	hypothetical protein
TW1_055	35978	37120	+	hypothetical protein
TW1_056	37123	37569	+	hypothetical protein
TW1_057	37583	38641	+	possible phage coat protein
TW1_058	38641	38808	+	hypothetical protein
TW1_059	38821	39018	-	hypothetical protein
TW1_060	39008	39202	-	hypothetical protein
TW1_061	39252	39578	-	hypothetical protein
TW1_062	39589	39837	-	hypothetical protein

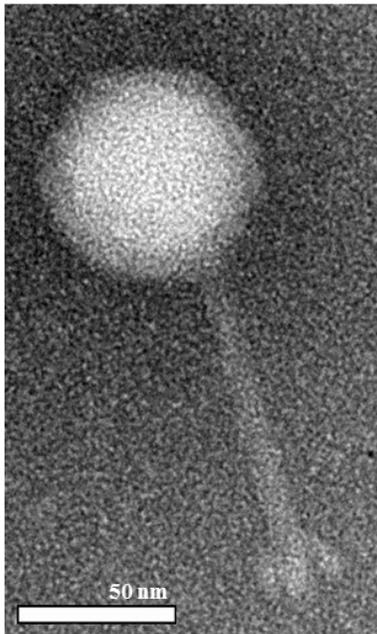
Table 6.2. Functional grouping of predicted ORFs in phage TW1

Functional group	Locus_tag	Predicted function	BLASTP best match	Identity
Structure	TW1_014	tail tape measure domain-containing protein	hypothetical protein EcolC_1230 [Escherichia coli ATCC 8739]	28%
	TW1_018	phage tail domain-containing protein	phage tail protein [Cronobacter sakazakii ES15]	27%
	TW1_023	possible head morphogenesis protein	head morphogenesis protein [Salmonella phage vB_SosS_Oslo]	28%
	TW1_057	possible phage coat protein	putative coat protein [Salmonella phage E1]	28%
Packaging	TW1_024	possible portal protein	unnamed protein product [Pseudomonas phage phi297]	25%
	TW1_046	possible terminase small subunit	terminase small subunit [Escherichia coli O83:H1]	40%
	TW1_047	possible terminase large subunit	putative TerL [Burkholderia phage Bups phi1]	44%
DNA manipulation	TW1_022	HNH endonuclease domain-containing protein	HNH endonuclease [Cronobacter phage vB_CsaM_GAP32]	27%
	TW1_035	exodeoxyribonuclease 8 domain-containing protein	recE protein [Enterobacteriaceae bacterium 9_2_54FAA]	29%
	TW1_036	possible ATP-dependent helicase	putative ATP-dependent helicase [Salmonella phage E1]	43%
	TW1_037	possible DNA primase	DNA primase [Pseudogulbenkiania ferrooxidans 2002]	22%
	TW1_042	putative N-6-adenine-methyltransferase	phage DNA methyltransferase [Alteromonas macleodii AltDE1]	43%
Regulation	TW1_033	single-stranded DNA-binding protein	ssDNA-binding protein [Glaciecola arctica BSs20135]	55%
			ssDNA-binding protein [Pseudoalteromonas sp. BSi20652]	48%
Additional Function	TW1_028	possible phosphoribosyl-ATP pyrophosphohydrolase	Phosphoribosyl-ATP pyrophosphohydrolase [Gallibacterium anatis]	48%

Table 6.3. Functional analysis of predicted ORFs using InterProScan program

Locug_tag	Predicted fuction	Pfam accession number	Description
TW1_010	hypothetical protein	PF11351	DUF3154 : Protein of unknown function
TW1_014	tail tape measure domain-containing protein	PF06791	TMP_2 : Bacteriophage lambda, GpH, tail tape measure, N-terminal
TW1_017	hypothetical protein	PF00877	NLPC_P60 : NLPC/P60 domain
TW1_018	phage tail domain-containing protein	PF13550	Phage-tail_3
TW1_022	HNH endonuclease domain-containing protein	PF13392	HNH_3
TW1_023	possible head morphogenesis protein	PF04233	Phage_Mu_F : Phage head morphogenesis domain
TW1_024	possible portal protein	PF06381	DUF1073 : Protein of unknown function
TW1_028	possible phosphoribosyl-ATP pyrophosphohydrolase	PF01503	PRA-PH : Phosphoribosyl-ATP pyrophosphohydrolase-like
TW1_033	single-stranded DNA-binding protein	PF00436	SSB : Primosome PriB/single-strand DNA-binding
TW1_035	exodeoxyribonuclease 8 domain-containing protein	PF12684	DUF3799 : Putative exodeoxyribonuclease 8, PDDEXK-like domain
TW1_036	possible ATP-dependent helicase	PF00271	Helicase_C : Helicase, C-terminal
TW1_036	possible ATP-dependent helicase	PF04851	ResIII : Helicase/UvrB domain
TW1_037	possible DNA primase	PF01807	zf-CHC2 : Zinc finger, CHC2-type
TW1_038	hypothetical protein	PF13148	DUF3987 : Protein of unknown function
TW1_042	putative N-6-adenine-methyltransferase	PF05869	Dam : DNA N-6-adenine-methyltransferase
TW1_047	possible terminase large subunit	PF04466	Terminase_3 : Caudovirales, terminase large subunit

(A)



(B)

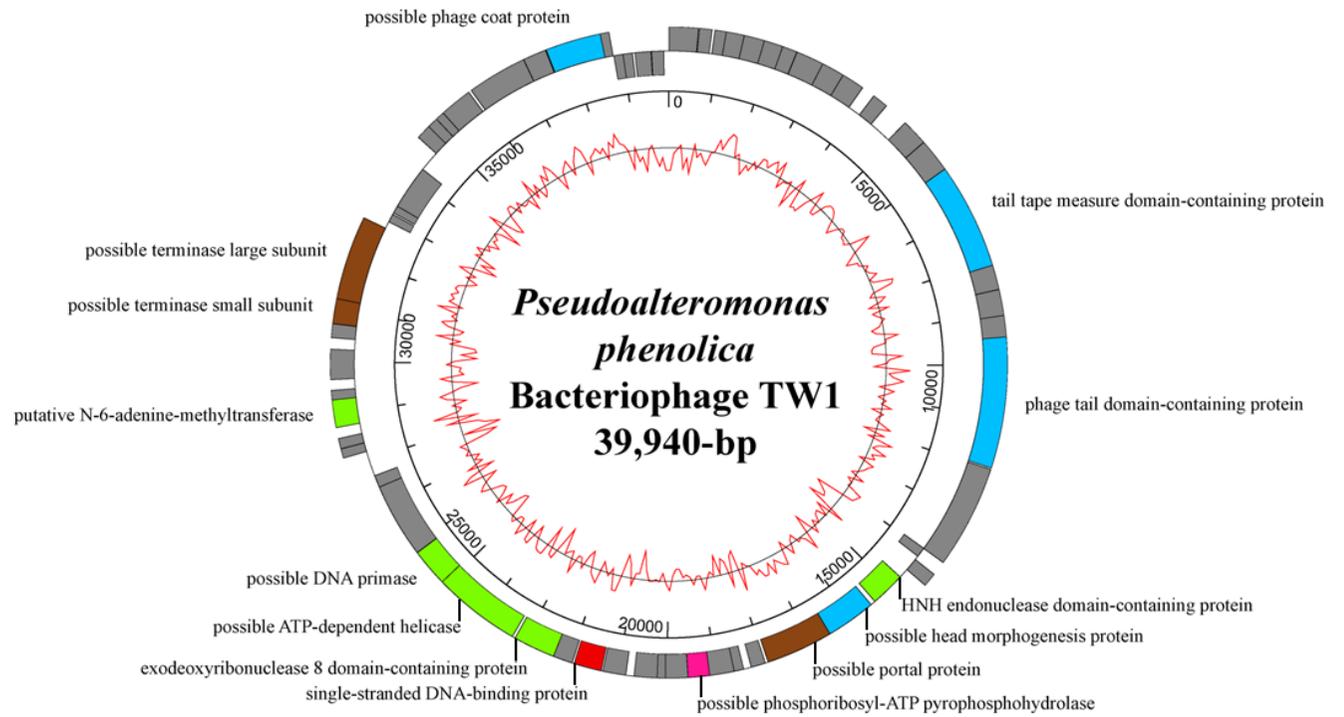


Figure 6.1. TEM morphological observation and genome map of *P. phenolica*-infecting phage TW1. (A) TEM morphological observation of *P. phenolica*-infecting phage TW1. Size bar is 50 nm. (B) The inner circle with red line indicates the GC content. The outer circle indicates predicted ORFs by strand. The categories of functional ORFs were indicated by the following colors. Blue, structure; green, DNA manipulation; brown, packaging; red, regulation; pink, additional function.

VI-5-3. References

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국문 초록

Salmonella enterica subspecies *enterica* serovar

Typhimurium 균은 그람 음성의 병원균으로 살모넬라증을 유발한다. 이에 *S. Typhimurium* 균을 특이적으로 감염하는 박테리오파지는 항생제 대체제로서 주목받고 있다. 이러한 *S. Typhimurium* 균의 저해제로서 박테리오파지 사용 가능성을 규명하기 위해, 파지와 숙주 사이의 감염 기작 및 상호 작용 기작에 대한 분자적 수준의 이해가 필수적이다. 이에 본 연구에서는 25 종의 *S. Typhimurium* 균 특이성 파지를 새롭게 분리하고, 다양한 유전자 결여 *S. Typhimurium* SL1344 균주를 이용하여 각 파지들의 수용체를 파악하였다. 그 결과, 총 세가지 종류의 수용체 (flagella, vitamin B₁₂ uptake outer membrane protein, BtuB 및 lipopolysaccharide (LPS) O-antigen)로 구분할 수 있었다. TEM 관찰을 통한 morphology 연구 결과, flagella 를 수용체로 사용하는 group F 파지들과 BtuB 를 수용체로 사용하는 group B 파지들은 *Siphoviridae* family 에 속함을 파악하였고, LPS 의 O-antigen 을 수용체로 사용하는 파지들은 *Podoviridae* family 에 속함을 알 수 있었다. 더불어 group F 파지들의 경우, FliC 만을 수용체로하는 subgroup F-I 파

FliC 및 FljB 를 모두 수용체로 사용하는 subgroup F-II 로 구분할 수 있었다. Cross-resistance assay 를 통해, group B 파지 저항성을 가지는 균주에 group L 파지가 감염을 하지 못함과 반대로 group B 파지가 group L 의 SPN9TCW 파지 저항성을 가지는 균주에 감염을 하지 못함 파악하였다. 이는 BtuB 및 LPS 의 O-antigen, 두 수용체 사이에 특수한 상호작용 존재 가능성을 시사하였다. 본 수용체 다양성에 대한 연구는, 파지와 숙주 사이의 수용체 상호작용에 대한 정보를 제공하였고, *Salmonella* 균을 목표로 하는 최적의 파지 콕테일 개발에 기반이 되는 정보를 제공하였다.

더불어 파지의 감염 및 숙주 용해 기작을 연구하기 위하여, *S. Typhimurium* 균을 숙주로 하는 새로운 파지 SPN9CC 를 분리 및 동정하였다. 이 파지는 *Podoviridae* family 에 속하며, LPS 의 O-antigen 을 수용체로 사용함을 확인하였다. 또한 SPN9CC 는 투명한 plaque 을 형성하지만, plaque 내부에 불투명한 환을 형성하는 독특한 plaque morphology 를 보였으며, 이는 SPN9CC 가 lysogen 을 형성할 수 있음을 시사하였다. 파지의 major capsid protein 의 아미노산 서열을 이용한 phylogenetic analysis 를 통해 SPN9CC 가 lysogen 을 형성하는 P22-like 파지에 속함을 파악하였다. 하지만, SPN9CC 와 P22-

like 파지 사이의 comparative genomic analysis 결과, lysogeny control region 및 host lysis gene cluster 의 유사성이 거의 없음을 확인하였다. 이 결과는, P22-like 파지 사이의 lysogeny 형성 기작 및 숙주 용해 기작이 다양할 수 있음을 시사하였다. SPN9CC 의 host lysis cluster (holin, endolysin, Rz/Rz1) 유전자들을 각각 혹은 여러 조합으로 *S. Typhimurium* 및 *E. coli*에서 발현을 시켜본 결과, 이 lysis protein 들 사이의 협동 작용이 균주 용해에 필수적임을 확인하였고, 이 협동 작용에 holin 이 중추적인 역할을 함을 파악하였다. SPN9CC 의 lysogeny control region 에 대한 후속 연구를 위해서, SPN9CC 의 ΔcI 뮤타트 (SPN9CCM)를 구축 하였다. SPN9CCM 는 plaque 내부에 불투명한 환을 형성하지 않았고, 이는 SPN9CCM 가 용원성 파지에서 용균성 파지로 전환 되었음을 시사하였다. 추가적으로 one-step growth 분석 및 challenge assay 를 통해, SPN9CCM 가 기존의 SPN9CC 보다 eclipse/latent period 가 감소하고 burst size 가 증가함을 확인하였고, 균 저해 능력 또한 높아졌음을 파악하였다. 본 연구 결과를 통해, engineering 용원성 파지의 항균 물질 개발 가능성을 시사하였다.

Bacillus cereus, *Bacillus anthracis*, 및 *Bacillus thurigiensis*(Bt) 균을 감염시키는 *Bacillus cereus* group

파지들에 대한 연구는, 병원성 균주인 *B. cereus* 및 *B. anthracis* 를 제어하는 목적과 천연 살충제로 사용되는 Bt 의 생산 중 파지 오염을 방지하는 목적으로 분자적인 수준의 연구 및 최근들어 유전체학적인 연구가 진행되고 있다. 모든 *Bacillus cereus* group 파지들의 genome 에 대한 comparative phylogenetic 분석을 진행한 결과, 세가지의 group 으로 구분할 수 있었다. 이러한 구분은 morphology 의 차이 (*Myoviridae* 은 group I, *Siphoviridae* 은 group II, *Tectiviridae* 는 group III), genome size 의 차이 (group I > group II > group III) 및 생활사의 종류 (용균성은 group I, 용원성은 group II 및 group III)에도 연관이 있음을 알 수 있었다. 파지들의 genome 에 대한 추가적인 dot plot analysis 를 통한 비교 분석 결과, 각각 group 의 파지들 사이에 DNA 수준에서 상동성이 큼을 알 수 있었다. 또한 group I 파지들에 대한 endolysin 비교 분석 결과, 두 종류의 cell wall binding domain 과 네 종류의 enzymatic activity domain 의 조합으로 구성됨을 파악하였다. Group II 파지에 대한 연구는 *B. anthracis* 특이적인 파지에 대해 집중되어 진행되었으며, typing 및 신속 검출 방법으로 응용이 시도되고 있다. Group III 파지의 경우, Bam35 및 GIL01 파지들에 대한 연구가 대표적이며 파지의 entry 기작 및 lytic switch regulation

기작에 대한 연구가 진행되었다. 본 연구를 통해, *Bacillus cereus* group 파지들에 대한 추후 연구는, *B. cereus* 및 *B. anthracis*에 대한 제어, typing 및 신속 검출, Bt 생산 중 파지 오염을 막는 방안 등 실질적인 적용에 유용한 정보를 제공할 것임을 시사하였다.

핵심어: 박테리오파지, 살모넬라 티피뮤리움 (*Salmonella* Typhimurium), 바실러스 시리우스 (*Bacillus cereus*), Phage-host 상호관계, phage 유전체학

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