A Thesis for the Degree of Doctor of Philosophy

Characterization, Genomic Analysis, and Application of *Salmonella* Typhimurium-targeting Bacteriophages

Salmonella Typhimurium을 숙주로 하는 박테리오파지의 특성 분석, 유전체분석과 생물방제제 활용에 관한 연구

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Abstract

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Salmonella is a Gram-negative, rod-shaped, and flagellated bacteria which can be found in all warm-blooded animals including human and in the environment. Up to date, more than 2,500 serotypes of *Salmonella* have been discovered and it is noticed to be one of the most important pathogens associated with various foodborne illnesses all over the world. *Salmonella* infections can cause gastroenteritis with symptoms including nausea, vomiting, abdominal cramps, diarrhea, fever, and headache. In most cases, symptoms of salmonellosis are relatively mild to healthy people and recovered in a few days without specific treatments. However, in some cases of young and elderly patients, *Salmonella* infection can become severe and even cause death. Recently, infections by antibiotics resistant non-typhoid *Salmonella* have emerged as one of the important problems by pathogenic bacteria. Due to the emergence of multidrug-resistant *Salmonella*, *Salmonella*-infecting bacteriophages have been considered as promising alternative biocontrol agents to antibiotics.

To develop a novel biocontrol agent against *S*. Typhimurium, 26 new bacteriophages targeting *S*. Typhimurium were newly isolated and characterized. Host receptor analysis identified five different cell wall receptors including flagella,

O-antigen, BtuB, LPS core oligosaccharide (OS) region, and OmpC which are utilized by *S*. Typhimurium phages. For further understanding of the host-phage interactions, whole genomes of selected phages were sequenced and analyzed. Comparative genomic analysis among the phages showed that phage tail and tail fiber structures are important to determine the host ranges as well as the host receptor.

Based on the receptor study, three phages (BSPM4, BSP101 and BSP22A) target different receptors including flagella, O-antigen, and BtuB, respectively were selected. Genome sequence analysis results revealed that all three phages neither have lysogen module nor toxin genes, supporting that they are strictly virulent and safe to be developed as biocontrol agents. A phage cocktail comprised of three phages was designed and its antimicrobial efficiency was evaluated. *In-vitro* treatment of the phage cocktail showed a significant reduction in the development of bacterial resistance to phage infection. Since a significant number of foodborne outbreaks and sporadic infections of *Salmonella* are mediated by contaminated fresh produces, the antimicrobial efficiency of the phage cocktail was evaluated using two fresh produces, lettuce and cucumber, as food models. The phage cocktail significantly inhibited *S*. Typhimurium growth in fresh produces for 12 h. These results suggest that the phage cocktail composed of phages targeting three different host receptors would be a useful material for developing a novel biocontrol agent against *S*. Typhimurium to ensure the safety of fresh produces.

Bacteriophage endolysin, a peptidoglycan hydrolase encoded by phage genomes, are synthesized at the end of the phage life cycle and play important roles in the host cell lysis after phage replication and propagation. Since the endolysins show specific

activities only to the peptidoglycan layer generally found in bacteria, they have been considered as safe to humans. Therefore, they have also been suggested as a novel biocontrol agent as well as a natural food preservative to control food-borne pathogens in foods. However, the use of endolysins are still limited to control of Gram-positive bacteria because of the presence of the outer membrane in Gram-negative bacteria which prevent endolysin assessment to the peptidoglycan substrate. On this account, studies of endolysins targeting Gram-negative bacteria are still in the beginning stage. Therefore, further studies of endolysins from Gram-negative bacteria targeting phages are required to develop endolysin-based novel antimicrobial agents against Gram-negative bacteria.

For this purpose, a novel endolysin designated M4LysA was newly identified from the phage BSPM4 genome and characterized. Bioinformatics analysis revealed that M4LysA was not homologous to the previously known endolysins. However, when M4LysA was induced in *E. coli* cell, rapid cell lysis was observed, suggesting that M4LysA is a host cell lysis protein. Indeed, colorimetric assay revealed that M4LysA have endopeptidase activity. Domain analysis results showed that M4LysA is a membrane protein having an apparent transmembrane domain (TMD). By deletion of the C-terminal TMD from M4LysA, solubility was increased while the peptidoglycan lysis activity still remained, suggesting that M4LysA cause cell lysis by degrading the peptidoglycan. Since M4LysA contains unusual membrane domain in C-terminal region, it was revealed to be secreted Sec-translocase pathway independently. Instead, TMD of C-terminal region seemed to be important for its translocation to the periplasm. In addition, the host ranges of M4LysA were broader

than those of the parental phage BSPM4, supporting its potential use as a novel antimicrobial agent against Gram-negative bacteria.

Despite the advantages of endolysins as biocontrol agents, their applications to the Gram-negative bacteria still have limitations because of the outer membrane barrier. In order to overcome this problem, newly purified endolysin BSP16Lys which is revealed to have *N*-acetylmuramonyl-L-alanine amidase activity was encapsulated into the lipid vesicles and its antimicrobial activity was evaluated. Without outer membrane permeabilizers addition, the amount of *S*. Typhimurium was successfully reduced (3-log CFU/mL) within 1 h at room temperature (25ºC) by treating BSP16Lys endolysin-encapsulated liposome. In addition, a liposome containing commercial lysozyme also showed antimicrobial activity without any other membrane permeabilizers. The results suggested the promising use of peptidoglycan hydrolases-encapsulated liposomes as antimicrobial agents against Gram-negative bacteria.

In this study, I suggested novel approaches to control *S*. Typhimurium by utilizing and maximizing the advantage of bacteriophages and endolysins as biocontrol agents. These results will provide not only deep insight into the phage biology but also advanced application strategies of the phages and endolysins as novel antimicrobial agents.

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Chapter I.

General Introduction

I-1. Bacteriophage

I-1-1. General features and phylogeny

Bacteriophages (phages) are the most abundant microorganisms on Earth, and also have the ability to infect bacteria. Basic structure of phages in the order *Caudovirales* consists of two parts: phage head and its tail. The phage head contains a genetic material in a form of DNA or RNA [1]. Linked to the phage head, the phage tail generally plays roles in recognition and adsorption of the specific bacterial host receptor [2]. After binding to the host bacterium, phage injects its genetic material into the host cytosol via tail structure by diffusion, osmotic pressure, or transport by specific protein [3, 4]. The injected genetic material undergoes host genome integration for lysogenic cycle or replication for lytic cycle. During the lytic cycle, structural proteins are produced from encoded genes in the phage genome. After replication of the genetic material and production of structural proteins, progeny phages are assembled with them and released from the host bacterium [5, 6].

Since the first phage was discovered and characterized by Felix d'Herelle in 1917 [7], tailed phages in the order *Caudovirales* are the most abundant (about 96% of all phages). This order consists of three major families including *Siphoviridae, Myoviridae,* and *Podoviridae* with different morphological characteristics. Among the reported phages to date, phages in the *Siphoviridae* family are the most abundant (61.6% of all phages) with long flexible noncontractile tails ranged from 79 to 539 nm. The phages in the *Myoviridae* family are the second most abundant (24.5%) and they have larger heads ranged from 53 to 160 nm in

comparison to those of other two families. Moreover, contractile tails give *Myoviridae* its unique characteristics. The phages in the *Podoviridae* family (13.9%) have a distinctly short non-contractile tail ranged from 3 to 40 nm [8].

I-1-2. Phage therapy

The first clinical study using phages was a direct phage injection in six patients with staphylococcal boils in 1921 [9]. Since then, phages have been used to cure various diseases caused by bacterial infections for several decades in Eastern Europe. However, antibiotics have been widely used for the same purpose in other parts of the world and this resulted in the emergence of antibiotic-resistant bacteria. Therefore, it has been a big issue how to control these antibiotic-resistant bacteria. Because recently, phage has attracted the public attention due to its high host specificity and efficient host lysis, phage therapy has been revisited to control these problematic bacteria in Western Europe [1].

To date, numerous clinical phage trials have been reported against various pathogens including *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeroginosa,* and *Salmonella* Typhimurium. As an example, in Poland, 550 patients with gastrointestinal, head, neck, and skin infections caused by these pathogens were successfully treated and symptoms of 506 patients (92%) were relieved [6]. In addition, in Russia, 1,340 patients with conjunctivitis, dermatitis, pharyngitis, and rhinitis were divided into three groups for different treatment regimens: phage treatment (360 patients), antibiotics treatment (404 patients), and combination (576 patients) [6]. Interestingly, the phage-treated and the combination

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groups were clinically improved up to 86% and 83%, respectively. However, the antibiotics-treated group showed minor improvement up to 48%, suggesting that phage therapy may be effective to control these pathogens but combination of phages and antibiotics did not show synergistic effect. In Ireland, ten methicillin-resistant *S. aureus* (MRSA) DPC5246-infected human hands were soaked in a solution containing 10^8 PFU/ml of a single phage K, revealing 2 log reduction, suggesting that MRSA can be controlled using a specific phage [10, 11]. Based on the clinical studies, many commercial phage therapy products were developed and produced in Eastern Europe, including "Phagestaph" (JSC Biochimpharm, Tbilisi, Georgia), "*E. coli* bacteriophage" (Microgen, Moscow, Russia), and "Complex pyobacteriophage" (Microgen). In addition, other countries have many phage therapy-related companies producing commercial products: USA (Elanco Food Solutions, Gangagen Inc., Intralytix, Neurophage Pharmaceuticals, New Horizons Diagnostics, OmniLytics Inc., Phage International, Targanta Therapeutics, Viridax), UK (AmpliPhi Biosciences Corporation, Blaze Venture Technologies, BigDNA, Novolytics, Phico), Georgia (Biopharm Ltd., JSC Biochimpharm, Phage Therapy Center), Australia (Special Phage Services Pty, Ltd.), Canada (Biophage Pharma Inc.), Germany (Hexal Genentech), India (Gangagen Biotechnologies PVT Ltd.), Ireland (Phage Works), Israel (Phage Biotech Ltd.), Portugal (Innophage), South Korea (CJ CheilJedang Corporation) and the Netherlands (EBI Food Safety) [12]. Consequently, phage therapy would provide novel insights and approaches to overcome the limitations of antibiotics and biocontrol of various antibiotics-resistant bacteria without any side effect in humans.

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I-1-3. Phages as biocontrol agents

Food safety issues has become one of the major concerns for consumers because it threatens human health. Every year in the United States, about 9.4 million cases of foodborne illness with about 56,000 hospitalizations and 1,300 deaths caused by major food-borne pathogens including *Salmonella*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Campylobacter* have been reported [13]. Because of food contaminations by pathogens, about 25% of their food productions were lost in food industries every year [14]. In general, control of these food-borne pathogens has been continuing by using various natural or chemical food preservatives. Natural preservatives such as organic acids, bacteriocins, chitosan, and lactoferrin have tendency to exhibit weak and limited antimicrobial activities [15]. Meanwhile, consumers generally do not prefer chemical preservatives due to their side effects [16]. Furthermore, while antibiotics have strong and stable antimicrobial activities, they are not allowed to use in foods.

Phages are bacterial viruses which have host specificity and lysis activities, indicating that they can infect and lyse the specific host bacteria for their replication and propagation [5]. Phages have been suggested as natural biocontrol agents against food-borne pathogens because phages are specific to bacteria cell and safe to human [17]. Therefore, phages could be applied as promising alternative biocontrol agents, especially in food industry. ListSheild (Intralytix, Inc., Baltimore, MD, USA), a cocktail of six phages targeting *L. monocytogenes*, was first approved by the United States Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA)'s Food Safety and Inspection Service (FSIS) for food additives in 2006 and

re-approved as GRAS status by FDA in 2014. In addition, EcoShield (Intralytix), a cocktail of three phages (ECP-100) targeting *E. coli* O157:H7, was also approved by FDA and FSIS for food applications in 2011. Listex P100 (Micreos Food Safety, Wageningen, The Netherlands), a single phage targeting *L. monocytogenes*, was also approved as GRAS status by FDA in 2006. Recently, SalmoFresh (Intralytix), a cocktail consisted of six *Salmonella*-targeting phages, was also approved as GRAS status by FDA in 2013 [18]. These phage products were allowed to use as food preservatives to control specific food-borne pathogens in foods. Furthermore, phages can be also used as sanitizers to prevent cross contamination in food-contact materials as well as food processing facilities [19].

I-1-4. Challenges in phage application

Compare to antibiotics, the therapeutic use of phages have some advantages as follows; i) natural product, ii) high host specificity, iii) low inherent toxicity, iv) relatively low cost, etc [20]. However, though phages have a number of advantages as antibacterial agents those can be used as alternatives to chemical antibiotics, there still remain some problems and concerns associated with phage application.

First, not all phages are able to be used as biocontrol agents. To be used as a biocontrol agent, phage should be obligatory lytic without any transduction of bacterial toxin genes or virulence factors after the host cell lysis. Since not all isolated phages are totally lytic but have properties of temperate phages, it is essential to check their life cycles after the phage isolation. However, since these selection processes need too much time and labor, new methods for selecting useful

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phages need to be developed. It has become much easier to select virulent phages based on genomic data due to the bioinformatics tools and genome sequencing analysis have been developed. However, most open reading frames (ORFs) from phages are predicted as hypothetical proteins with unknown functions and has not yet fully studied. Therefore, more studies are required for better understanding the phage genomes.

Second, bacteria have developed various strategies against phage infection; alterations or modifications of host cell receptors against phage adsorption, prevention of phage DNA entry by cutting or blocking, and defense systems by abortive phage infection targeting critical steps of phage multiplication [21]. Among them, prevention of phage adsorption by altering or modifying the host receptors is one of the common strategies developed by host bacteria. To date, many studies about modifications or hiding of host receptors such as LPS, membrane proteins, and flagella against phages infection have been reported [22-24]. Although many approaches to construct phage cocktails have been studied and suggested, only a few have been approved to be used [25]. Therefore, more studies are needed to develop efficient phage cocktails and their safety verifications are also required.

Third, unfamiliarity of the Western medical establishment with therapeutic phage applications is one of the greatest hurdles to overcome. Moreover, though some phage products have been approved by Food and Drug Administration (FDA) as GRAS (generally recognized as safe) or allowed by United States Department of Agriculture (USDA), more efforts are still required for the public acceptance [26]. Since phages are one of "virus" particles, they can be misinterpreted by the public

with confusion equivalent to "human viruses". Therefore, researches about phage applications as well as educations and promotions to the public are need to be invigorated.

I-1-5. Phage endolysins

Endolysins are phage-encoded enzymes which are synthesized at the final step of the phage multiplication cycle. After replication and propagation of the *Caudovirales* phages in the host bacterial cells, assembled phages are released upon breakdown of bacterial cell wall caused by phage encoded endolysins. Additionally, endolysin has a specific activity to hydrolyze peptidoglycan of the cell wall and holin are known to help endolysin to cross the bacterial membrane to reach cell wall [27]. Therefore, endolysins are promising biocontrol agents which could be applied in food biotechnology. Especially, endolysin can lyse Gram-positive food-borne pathogens from outside of the cells as they do not have outer membrane in their cell wall structure. In contrast, Gram-negative bacterial cells which possess the outer membrane that prevents access of hydrophilic enzymes become insensitive to external peptidoglycan hydrolase treatment.

Generally, endolysins from Gram-positive bacteria-targeting phages have two conserved protein domains, enzymatic activity domain (EAD, also called as catalytic domain, located in N-terminal region) and cell wall binding domain (CBD, located in C-terminal region) [28]. Meanwhile, the majority of endolysins (~ 94%) purified from Gram-negative bacteria-targeting phages contain one catalytic domain and form a globular structure [29]. It has been reported that there are five types of

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EADs according to the peptidoglycan cleavage sites: *N*-acetylmuramidases (lysozymes), *N*-acetyl-*β*-D-glucosaminidases (glycosidases), *N*-acetylmuramoyl-L-alanine amidases, L-alanoyl-D-glutamate endopeptidases, and interpeptide bridge-specific endopeptidases [30].

Since the endolysins specifically target the peptidoglycan layer in the bacteria, they have been considered safe for humans without any immunological responses [31]. Furthermore, no studies on the emergence of endolysin resistance strains have been reported up to date [32-35]. Therefore, endolysins could be good candidates for biocontrol agents to control food-borne pathogens in foods without harming humans. However, most of endolysins control bacteria limited to Gram-positives and the studies for Gram-negative bacteria-targeting endolysins are still at the preliminary stage. Therefore, further studies are needed to develop Gram-negative bacteria-targeting endolysins as novel antibacterial agents.

I-2. *Salmonella*, a foodborne pathogen

Salmonella is a Gram-negative, rod-shaped, facultative anaerobic, and flagellated food-borne pathogen causing illness called salmonellosis. Salmonellosis is one of major causes of bacterial enteric disease with symptoms, mild diarrhea, common gastroenteritis, enteric fever, and ulceration in both humans and warm-blooded animals. Up to date, more than 2,600 serotypes (serovars) of *Salmonella* have been isolated and reported [36].

According to the current Centers for Disease Control and Prevention (CDC) system, the *Salmonella* genus contains two distinct species: *Salmonella enterica* and *Salmonella bongori*. Based on biochemical properties and genomic relations, *S. enterica* is divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica* [37, 38]. All isolated serotypes are considered as potential pathogens and the majority serotypes can infect different hosts.

Depend on the symptoms and target hosts, *Salmonella* serovars can be divided into two groups: invasive typhoidal serotypes and non-typhoidal *Salmonella* (NTS) serovars. Despite they have high genetic similarity, two serovars trigger different illnesses and distinct immune responses in humans. The former group comprised of *Salmonella* Typhi, *Salmonella* Paratyphi, and *Salmonella* Sendai and they are highly adapted to human host in specific. They usually cause enteric fever especially in the developing country where clean water and adequate sanitation are

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limited. Enteric fever is a systemic disease with high a mortality, resulting in more than 200,000 deaths annually over the world [39, 40]. During recent years, the occurrences of serovar Paratyphi A infections have risen, particularly in South–East Asia [41]. The latter group includes *Salmonella* Enteritidis and *Salmonella* Typhimurium. In contrast to typhoidal infection which is common in the developing countries, NTS salmonellosis outbreaks are worldwide. Every year, approximately 93.8 million cases of gastroenteritis are caused by NTS infection with more than 155,000 deaths and about 80.3 million cases were foodborne [42]. Food-mediated NTS infection is generally caused by contaminated animal-derived products including poultry, eggs, and dairy products. In addition, NTS infection is also caused by the consumption of contaminated fresh produces such as sprouts, tomatoes, peanuts, and spinach. Moreover, NTS transmission also can be resulted by person to person contact or by contact with animals like cats, dogs, reptiles, or amphibians [43]. In general, NTS mediated infection predominantly causes self-limiting diarrheal illness, bloodstream, or focal infection. Actually, it is rarely shown in high-income countries, however, in Africa, bloodstream infection caused by NTS are commonly shown to both adults and children, presenting fever, and about 25% of them are associated with a case fatality [36]. Moreover, two major changes in the epidemiology of non-typhoid salmonellosis occurred in the 20th century in Europe and the United States: one is the emergence of foodborne human infections by *S.* Enteritidis and the other one is emergence of multidrug-resistant strains of *S.* Typhimurium. Moreover, non-typhoid *Salmonella* resistant strains against fluoroquinolones and third-generation cephalosporin were increased in the 21th

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century [44, 45]. As described above, *Salmonella* infection has been a notable burden in both developing and developed countries. Although various food preservatives have been used to prevent *Salmonella* contaminations, they are not specific for *Salmonella* and even worse not that effective. Accordingly, efforts to reduce the spread of *Salmonella* by food and other routes must be carried out all over the world. In addition, better strategies to prevent the emergence of antibiotics-resistant *Salmonella* also have to be developed.

I-3. Purpose of this study

S. Typhimurium is a common intracellular pathogen that cause a number of foodborne outbreaks all over the world. Because of its strong persistence and survival in the environment such as food matrices, it is urgent to control *Salmonella* in foods. Because of the increasing concern about the possible hazards and limitations of chemical and physical treatments, bacteriophages and endolysins have been considered to be developed as novel and safe biocontrol agents. However, studies about phage receptors, phage genome, and biological applications of phages and endolysins are not sufficient yet. In this respect, it would be important not only to isolate novel phages but also to characterize them in detail from biology to genomes and apply them to the foods in order to examine their capabilities in field test. Moreover, there is pressing need to purify and characterize new endolysins, and their novel application method are needed to be developed.

Therefore, this study aims to (i) isolate and characterize *S*. Typhimurium-targeting phages, (ii) construct a phage cocktail which is composed of various phages using different host receptors to enhance its antibacterial activity, and (iii) purify, characterize new endolysins, and develop a novel endolysin application method to control *S*. Typhimurium.

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Chapter II.

Characterization of *S*. Typhimurium-infecting bacteriophages and their genomes

II-1. Introduction

Salmonella can cause non-typhoidal salmonellosis, the most common food-borne disease with symptoms like common gastroenteritis, enteric fever, and ulceration (1). Even though Salmonella has been widely detected in various animal-based foods (2), it is very difficult to control in the foods. Natural and chemical food preservatives have been used for prevention of food-borne pathogen contaminations, however, they are not specific for Salmonella. Therefore, the development of novel type antimicrobial agents has been required all over the world.

Bacteriophages (also called as phages), natural antibacterial agents, are bacterial viruses that lyse specific bacterial host strains (3). Phages are the most numerous particles (approximately ~10^{32} particles) on earth, outnumbering their host bacteria by about 10-fold (4). They are considered to coexist with their host bacterial cells and consequently they have been expected to play an important role in microbial ecology (5). The initial step of phage infection begins with recognition and adsorption, the step binding to a specific target site on the host bacterial surface. A phage adsorption is mediated by receptor-binding proteins (RBPs) and tail fibers those are localized on the tip of phage tail structure. Phages recognize their hosts by interacting with surface molecules on host bacteria using those structures. Many studies have revealed that various surface structures of Gram-negative and Gram-positive bacteria are used as phage receptors, including outer membrane proteins such as OmpA, OmpC, OmpF, OmpT, OmpX, LamB, FhuA, BtuB, lipopolisaccharides (LPS), capsular and slime polysaccharides, flagella, pili, and

wall teichoic acids (6, 7). Since phages utilize diverse surface molecules on host cell as receptors, adsorption step renders high host specificity to individual phages.

Since phages have been approved as novel type of food preservatives by the US Food and Drug Administration (FDA) (8), they have been recognized as interesting subjects for biocontrol agents of pathogenic bacteria including *Salmonella* (9-12). Therefore, more detailed characterizations of newly isolated phages are required to understand phage biology and their genomic features for the development and application of phage-based biocontrol agents near future.

In this study, 23 new bacteriophages targeting *S.* Typhimurium were isolated and characterized using various *Salmonella* mutant strains as hosts. Host receptor analysis results revealed five different cell wall receptors including BtuB, flagella, O-antigen, outer-core region of LPS, and OmpC. Comparative genomic analysis between phages within the similar morphology group revealed that phage tail compositions are important to determine the host ranges as well as host receptors. These results will provide useful information for further understanding about the *S.* Typhimurium targeting phages including general features, genomic characteristics and phage-host interactions.

II-2. Materials and Methods

II-2-1. Bacterial strains, growth conditions, and mutant construction

The bacterial strains used in this study are listed in Table 2.1. All of the strains were aerobically grown in Luria-Bertani (LB) broth or LB agar medium supplemented with 1.5% Bacto agar (Difco, Detroit, MI, USA) at 37°C or 30°C. Soft top agar containing LB was prepared with 0.4% agar for phage plaque confirmation. For phage isolation, prophage-cured *S.* Typhimurium LT2 strain (designated as LT2C) from Cancer Research Center and its various mutants were used as host bacterial strains. LT2C-derived mutants were constructed by specific gene knock-out method as previously described (13) and primers used for mutant construction are listed in Table 2.2. When appropriate, antibiotics were added as follow (final concentrations): kanamycin (50 μg/ml), ampicillin (50 μg/ml), and chloramphenicol (25 μg/ml).

Table 2.1. Bacterial strains and plasmids used in chapter II

Strains (description)	Source or references[a]	Strains (description)	Source or references[a]
Samonella enterica		***Salmonella* Typhimurium LT2C mutants**	
serovar Typhimurium		Δ*rfaC*	(14)
SL1344	NTCT	Δ*rfaCbtuB*	This study
UK1	(15)	Δ*btuB*	(16)
LT2	(17)	Δ*rfbP*	(16)
LT2C [prophage-cured LT2 strain]	(18)	Δ*flgK*	Park (2014)
ATCC14028	ATCC	Δ*btuBrfbP*	(16)
ATCC19586	ATCC	Δ*flgKrfbP*	(14)
ATCC43147	ATCC	Δ*flgKbtuB*	This study
ATCC13076	ATCC	Δ*btuBrfbPflgK*	Park (2014)
DT104		Δ*ompC*	Kim (2010)
serovar Typhi Ty 2-b	IVI	Δ*ompCrfbP*	(14)
serovar Parathyphi			
A IB 211	IVI		
B IB 231	IVI	***Escherichia coli* O157:H7**	
C IB 2973	IVI	ATCC35150	ATCC
serovar Dublin IB 2973	IVI	ATCC43890	ATCC
		ATCC43894	ATCC
Escherichia coli		ATCC43895	ATCC
K-12 MG1655	Wild type	O157:NM 3204-92	IVI
DH5α	Invitrogen	O157:NM H-0482	IVI

[a]ATCC, American Type Culture Collection; NTCT, National Collection of Type Cultures; IVI, International Vaccine Institute.

Table 2.1. Bacterial strains and plasmids used in chapter II (continued)

Strains (description)	Source or references[a]	Strains (description)	Source or references[a]
Gram-negative bacteria		**Plasmid**	
Shigella flexineri 2a strain 2457T	IVI	pKD46	(13)
Shigella boydii IB 2474	IVI	[P$_{BAD}$-*gam-beta-exo oriR101 repA101^{ts}*; Amp^R]	
Vibrio fischeri ES-114 ATCC 700601	IVI	pKD13	(13)
Pseudomonas aeruginosa ATCC 27853	ATCC	[R6Kγ *ori*, Amp^R-FRT, and Km^R-FRT]	
Cronobacter Sakazakii ATCC29544	ATCC	pCP20	(13)
		[pSC101 (Ts) *ori*, Amp^R, Cm^R, *cI857*, and λP$_R$*flp*]	
Gram-positive bacteria		pUHE21-2 *lacI^q*	(19)
Listeria monocytogenes ATCC 19114	ATCC	[*reppMB1 lacI^q*; Amp^R]	
Staphylococcus aureus ATCC 29213	ATCC	pACYC184	(20)
Staphylococcus epidermis ATCC 35983	ATCC	[*repp15A* Cm^R Tet^R]	
Bacillus subtilis ATCC 23857	ATCC	pCas9	(21)
Bacillus cereus ATCC 14579	ATCC	pCRISPR	(21)

[a]ATCC, American Type Culture Collection; NTCT, National Collection of Type Cultures; IVI, International Vaccine Institute.

Table 2.2. Primers used in chapter II

Primer	Nucleotide sequences [5`→ 3`][a]
btuB-RED-F-LT2	ATG ATT AAA AAA GCT ACG CTG CTG ACG GCG TTC TCC GTC ATG TAG GCT GGA GCT GCT TCG
btuB-RED-R-LT2	TAA TGG CGT ATC GGT AAT CGC ATT ACG CGC ATC AAC GTA A AT TCC GGG GAT CCG TCG ACC
btuB-Di-F	CAT CAT CGC GTA CTA TCG AT
btuB-Di-R	GAT GTG AGG TGA CCG GAT AT
K1	CAG TCA TAG CCG AAT AGC CT
K2	TTG TCA AGA CCG ACC TGT CC
BSPM2_158_guide1_di_F	GCA TCT ATT AAT AGC ATT AAT GAA TCT
BSPM2_158_guied2_di_F	GCT GTT GTT CAA GCT ATG AAA GTA CAG
pCRISPR_di_R	ATC AGT GAT AGA GAT TGA CAT CCC
pCas9_di_R	ATG GAC GAT CAC ACT ACT CTT C
BSPM2_158_di_F	CAG AAC TTC CAT CAC CCC ACA AAA
BSPM2_158_di_R	AGC AGA AGC TGA TGC TGA TGT TGG

II-2-2. Bacteriophage isolation and propagation

Twenty-three samples were collected from diverse environments including sewage, sludge, soil, pond water, fountain water, animal feces, swine mucin samples, and etc. as summarized in Table 2.3. All samples were used for bacteriophage isolation as described previously with slight modifications (22). Briefly, 25 g of each sample was mixed with 225 ml of sterile Butterfield's phosphate-buffered dilution water (0.25 M KH_2PO_4 adjusted to pH 7.2 with NaOH) in sterile bags and then homogenized for 90 s using a BagMixer 400 blender (Interscience Laboratory Inc., St. Nom, France). Twenty-five milliliter of each homogenized sample was then mixed with same volume of 2X LB broth and incubated at 37°C for 12 h with vigorous shaking (220 rpm). After incubation, the samples were centrifuged at 10,000 x g for 10 min and filtrated using 0.22-µm pore sized filter (Millipore, Billerica, MA) to remove residual bacterial cells. Ten-milliliter of each supernatant was added to 40 ml LB broth containing proper *Salmonella* strains (1%, final concentration) and then incubated at 37°C for 12 h with shaking (220 rpm). The culture was centrifuged at $10,000 \times g$ for 10 min and the supernatant was filtrated using 0.22-µm pore sized filter (Millipore). To confirm the presence of phages, 10 µL of each serially diluted phage was spotted onto LB plates covered with 0.4% soft agar containing 1% (final concentration) *Salmonella* strain. Plates were incubated at 37°C for 12 h and plaque formation was observed.

To purify the phage, a single plaque was picked with sterile tip and eluted in sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl, pH 7.5, 100

mM NaCl, 10 mM $MgSO_4$) and the overlaid onto LB plate. Purification step was repeated at least three times for purification.

For phage propagation, the lysate of a single phage was added at a multiplicity of infection (MOI) of 1 to the culture of host *Salmonella* strain (2 h, OD_{600} = 0.5) and the mixture was incubated at 37°C for 3 h. This phage propagation steps were serially performed with three different culture volumes (3, 50, and 200 ml culture) to obtain enough phages, accompanied with centrifugation and filtration as described above. To prepare a high-titer phage, the phage particles were precipitated using polyethylene glycol (PEG) 6000 (Junsei, Japan) and concentrated using CsCl gradient (step density = 1.3, 1.45, 1.5 and 1.7 g/ml) ultracentrifugation (himac CP 100β, Hitachi, Japan) at 78,500 × g for 2 h at 4°C. Finally, the phage band fraction was collected and purified with dialysis for 1 h, repeated twice in standard dialysis buffer (5 M NaCl, 1 M $MgCl_2$ and 1 M Tris·HCl at pH 8.0) using Spectra/Por dialysis membrane (molecular weight cutoff, 12,000 to 14,000; Spectrum Laboratories, Inc.). Purified phage was stored in glass vial at 4°C.

Table 2.3. Environmental sample lists used in chapter II

Designation of phages	Plaque morphology	Sources	Bacterial host
BSP1	Turbid, small	Sewage1	LT2C
BSP2	Clear, small	Sewage2	LT2C
BSP3	Clear, small	Sludge1	*ΔbtuBrfbPflgK*
BSP4	Clear, small	Sludge2	*ΔbtuBrfbPflgK*
BSP5	Clear, small	Sewage3	*ΔbtuBrfbPflgK*
BSP9	Clear, small	Soil1	LT2C
BSP11	Turbid, small	Soil3	LT2C
BSP12	Turbid, small	Soil4	LT2C
BSP14	Clear, small	Soil5	LT2C
BSP15	Clear, small	Soil6	LT2C
BSP16	Clear, big	Pond water1	*ΔbtuBrfbPflgK*
BSP17	Clear, small	Pond water2	*ΔbtuBrfbPflgK*
BSP18	Clear, small	Pond water3	*ΔbtuBrfbPflgK*
BSP19	Clear, small	Fountain water1	LT2C
BSP20	Clear, big	Fountain water2	*ΔbtuBrfbPflgK*
BSP21	Clear, small	Fountain water3	LT2C
BSP25	Clear, small	Swine feces1	*ΔrfaCbtuB*
BSP64	Clear, small	Swine feed	*ΔrfaCbtuB*
BSPM1	Clear, small	Swine intestine	*ΔrfaC*
BSPM2	Clear, small	Swine intestine	*ΔrfaC*
BSPM3	Turbid, small	Swine intestine	*ΔrfaC*
BSPM8	Clear, small	Swine intestine	LT2C
BSPM9	Clear, small	Swine intestine	LT2C

II-2-3. Bacterial challenge assay

Fifty milliliters of LB broth was sub-inoculated with appropriate *S.* Typhimurium strains (1%, final concentration) and the culture was incubated at 37°C with shaking (220 rpm) until it reached the early exponential phase ($OD_{600} = 0.5$). At that time, the culture was infected with the phage at a MOI of 1. Only SM buffer was added into the bacterial culture as a negative control. After phage infection, bacterial samples were collected every 1 h and OD_{600} was measured. Samples were serially diluted if necessary. All experiments were performed in triplicate.

II-2-4. Bacteriophage host range determination

Bacterial strains used for phage host range analysis were listed in Table 2.1. Each bacterial strain was incubated overnight at 37°C with shaking and then a 100 μl of each bacterial culture was added to 5 mL of the 0.4% molten LB soft agar and mixed by slight vortexing. Then the mixture was overlaid on the 1.5% LB agar plate. Subsequently, serially diluted phage lysates were spotted onto the prepared bacterial lawns and incubated at 37°C for 12 h. After incubation, the formation of a single phage plaque or the inhibition zone was observed to determine the sensitivity of each bacterium.

II-2-5. Transmission electron microscopy (TEM)

Morphological analysis of the phages was performed with transmission electron microscopy (TEM). Each phage stock diluent (approximately 10^9 to 10^{10}

PFU/ml) was placed on a carbon-coated copper grid and negatively stained with aqueous 2% uranyl-acetate (pH 4.0) for 30 s. Phage sample was observed by an energy-filtering transmission electron microscope (LIBRA 120, Carl Zeiss) at an operating voltage of 80 kV at the National Academy of Agricultural Science (Suwon, South Korea). Morphology of phages were identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses (23).

II-2-6. Receptor analysis using various mutants

The LT2C-derived mutant strains used for phage receptor analysis were listed in Table 2.1. In brief, overnight culture of various LT2C mutants were mixed with 0.4% molten LB soft agar to make the bacterial lawn as described above. Each phage lysate was serially diluted and spotted onto the bacterial lawns. After overnight incubation, the formation of single plaque was examined to determine their phage receptors.

II-2-7. Adsorption assay

The adsorption abilities of phages BSPM1 and BSPM2 were examined as previously described with slight modification (24). Overnight cultures of the bacterial strains were diluted 1:100 in LB broth and incubated until the OD_{600} reached 1.0. One milliliter of the culture was serially diluted in 9 ml of fresh LB in order to make $OD_{600} = 0.1$. Each phage was added to the diluted culture at an MOI of 0.001. The cultures were incubated at 37°C for 15 min and samples were collected

at indicated time points. Collected samples were immediately centrifuged at 14,000 x g at 4ºC for 1 min and filtrated using 0.22-µm filters. Subsequently, the obtained filtrates were serially diluted and overlaid on LB agar plates to determine the numbers of unabsorbed phages.

II-2-8. Extraction of bacteriophage genomic DNA

Bacteriophage genomic DNA was purified from concentrated phage high titer stock (about 10^9 to 10^{10}) as previously described (25). Prior to extraction of the phage DNA, the phage lysate was treated with DNase and RNaseA at 37°C for 30 min in order to remove bacterial DNA or RNA contaminants, respectively. After then, the phage lysate was treated with 20 mM of EDTA (pH 8.0), 50 µg/mL proteinase K, and 0.5% sodium dodecyl sulfate (SDS) for 15 min at 65°C. Finally, standard phenol-chloroform DNA purification with ethanol precipitation was carried out to get purified phage genomic DNA.

II-2-9. Full-genome sequencing and bioinformatics analysis

The extracted phage genomic DNA was subjected to be sequenced by a Genome Sequencer 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 performed by using FGENESB (Softberry, Inc., Mount Kisco NY), Glimmer v3.02 (26), and GeneMarkS (27) softwares. Predicted ORFs were annotated based on the results of the

InterProScan (28) and BLASTP (29) analysis. For comparative genome analysis of the phages, artemis ACT (30) and Easyfig (31) were used.

II-2-10. Proteomic analysis of phage BSPM1 and BSPM2

To analyze and compare the total phage protein profiles of phages BSPM1 and BSPM2, each purified phage (10^{11} PFU/ml) was 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). Then samples were boiled for 7 min, and the denatured phage proteins were subsequently separated using a 12% SDS-polyacrylamide gel. After SDS-PAGE analysis, 13 major visible bands were excised from the gel. Then, each band was digested with trypsin and peptide mapping was processed by Yonsei Proteome Research Center (Seoul, Korea).

II-2-11. LC-MS/MS analysis

For peptide analysis, Nano LC-MS/MS analysis was performed with an Easy n-LC (Thermo Fisher San Jose, CA, USA) and a LTQ Orbitrap XL mass spectrometer (Thermo Fisher, San Jose, CA, USA) equipped with a nanoelectrospray source. Samples were separated on A C18 nanobore column (150 mm × 0.1 mm, 3 μm pore size; Agilent). The mobile phase A for LC separation was 0.1% formic acid, 3% acetonitrile in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 5% B to 30% B in 30min, 30% B to 60% B in 4 min, 95% B in 4 min, and 3% B in

6 min. The flow rate was maintained at 1500 nL/min. Mass spectra were acquired using data-dependent acquisition with a full mass scan (350–1200 m/z) followed by 10 MS/MS scans. For MS1 full scans, the orbitrap resolution was 15,000 and the AGC was 2×10^5. For MS/MS in the LTQ, the AGC was 1×10^4.

II-2-12. Database searching

The mascot algorithm (Matrixscience, USA) was used to identify peptide sequences present in a protein sequence database. Database search criteria were, taxonomy; phage BSPM1 and BSPM2, fixed modification; carbamidomethylated at cysteine residues; variable modification; oxidized at methionine residues, maximum allowed missed cleavage; 2, MS tolerance; 10 ppm, MS/MS tolerance; 0.8 Da. The peptides were filtered with a significance threshold of $P < 0.05$.

II-2-13. Isolation of BSPM2 tail fiber mutant phage

Previously, the type-II-A CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and Cas9 system from *Streptococcus thermophiles* had been recently applied for genome editing of virulent phage (32). Therefore, in this study, RNA-guided targeting of the tail fiber region of BSPM2 (OFR_158) was performed using a CRISPR-Cas9 system of *Streptococcus pneumoniae*. In this system, a guide RNA (gRNA) containing a crRNA fused with the tracrRNA and additional expression of the Cas9 protein will bring the specific cleavage of target DNA region.

A pCRISPR plasmid (Addgene reference number: 42875) containing the guide RNA 1 for ORF_158 (5`- AAACTGCATCTATTAATAGCATTAATGAATCTAT G-3`) and a pCas9 plasmid (Addgene reference number: 42876) containing the guide RNA 2 for ORF_158 (5`- AAACGCTGTTGTTCAAGCTATGAAAGTACAGTTTG-3`) were designed and co-transformed into a *E. coli* DH5α strain. Then, BSPM2 phage was infected to the *E. coli* DH5α expressing the guide RNA. Consequently, two guide RNAs will cleave two different regions of the tail fiber genes and make mutation of the tail fiber gene during phage infection. The resultant plaques on the lawn of *E. coli* DH5α cell were picked and spotted onto a *E. coli* DH5α and *E. coli* O157:H7 ATCC 43890 strains in parallel. Finally, phages   forming clear plaques on the *E. coli* DH5α only but not   on the *E. coli* O157:H ATCC 43890 strain were selected and purified for further analysis.

II-3. Results and Discussion

II-3-1. Isolation of bacteriophages and their receptor determination

Twenty-three phages infecting *S*. Typhimurium were newly isolated from various environmental samples and they were listed in Table 2.2. Receptor of each phage was determined by spotting assay against various mutants lacking desired receptors of *S*. Typhimurium LT2C strain (listed in Table 2.3). Phage receptors were predicted based on the results of Table 2.3. Three phages (BSP1, BSP11, and BSP12) used bacterial O-antigen, seven phages (BSP3, BSP4, BSP5, BSP16, BSP17, BSP18, and BSP20) used outer-core region of LPS and five phages (BSP9, BSP14, BSP15, BSPM8, and BSPM9) used bacterial flagella as a receptor. Also, other phages used outer membrane protein as receptors including BtuB (BSP2, BSP19, BSP21, BSPM1, BSPM2, and BSPM3) and OmpC (BSP25 and BSP64), respectively. Interestingly, all seven phages (BSP3, BSP4, BSP5, BSP16, BSP17, BSP18, and BSP20) isolated using the *ΔbtuBrfbPflgK* strains used LPS-outer core as a receptor. Since the host strain lacks in three major receptors of *S*. Typhimurium (33), those phages may select and interact with a new type receptor for infection. Meanwhile, all the phages (BSP25, BSP64, BSPM1, BSPM2, and BSPM3) isolated using LPS-outer core mutant (*ΔrfaC* strain) used BtuB protein as a receptor. Previous study have revealed the cross-resistance between LPS-phages and BtuB-phages (33). In addition, *S*. Typhimurium can develop resistance against a BtuB-phage by phase-variable glucosylation of O-antigen (33). Moreover, *rfaC* mutant strain showed lack in motility because of the inhibited flagella formation (34). Therefore, it can be thought

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that BtuB-targeting phages could easily recognize the host in the absence of other two major receptors of *S*. Typhimurium.

Interestingly, other known *Salmonella* phage receptors such as FhuA, TolC, and OmpC have not be found by our group previously (14, 33). Therefore, a new strain which lacks in previously identified receptors including BtuB, Flagella, O-antigen, and LPS-outer core was constructed and used for phage isolation (Fig. 2.1) in order to find phages using novel receptors. When the mutant (*ΔrfaCbtuB*) lacking BtuB, Flagella, O-antigen, and LPS-outer core was used as a host, two phages were newly isolated. Following receptor identification results revealed that those two phages commonly used OmpC as a receptor. Surprisingly, it had been reported that outer membrane OmpC porin is present about 1,000 times greater than the BtuB protein in *E. coli* (35). Since BtuB protein is not a major membrane protein, it has still remained as question why the majority of phages isolated in this study prefer BtuB to other outer membrane receptors. And this phenomenon is worth to be studied in near future to understand the ecology of *Salmonella*-infecting phages.

Table 2.4. Receptor determination of isolated phages in chapter II

Designation of phages	LT2C	ΔrfaC	ΔompC	ΔbtuB	ΔrfbP	ΔflgK	ΔbtuBrfbP	ΔrfbPflgK	ΔbtuBrfbPflgK	ΔompCrfbP	Receptors expected
BSP1	T	-	T	T	-	C	-	-	-	N/D	O-antigen
BSP2	C	T	C	-	C	C	-	C	-	N/D	BtuB
BSP3	-	-	-	-	C	-	C	C	C	N/D	LPS-outer core
BSP4	-	-	-	-	C	-	C	C	C	N/D	LPS-outer core
BSP5	-	-	-	-	C	-	C	T	T	N/D	LPS-outer core
BSP9	C	-	C	C	C	-	C	-	-	N/D	Flagella
BSP11	T	-	T	T	-	C	-	-	-	N/D	O-antigen
BSP12	T	-	T	T	-	C	-	-	-	N/D	O-antigen
BSP14	C	-	C	C	C	-	C	-	-	N/D	Flagella
BSP15	C	-	C	C	C	-	C	-	-	N/D	Flagella
BSP16	-	-	-	-	C	-	C	C	C	N/D	LPS-outer core
BSP17	-	-	-	-	C	-	C	C	C	N/D	LPS-outer core
BSP18	-	-	-	-	C	-	C	C	C	N/D	LPS-outer core
BSP19	C	C	C	-	C	C	-	C	-	N/D	BtuB
BSP20	-	-	-	-	C	-	C	C	C	N/D	LPS-outer core
BSP21	C	C	C	-	C	C	-	C	-	N/D	BtuB
BSP55	-	C	-	C	C	C	C	C	C	-	OmpC
BSP64	C	C	-	C	C	C	C	C	C	-	OmpC
BSPM1	-	C	-	-	C	-	-	C	-	N/D	BtuB
BSPM2	-	C	-	-	C	-	-	C	-	N/D	BtuB
BSPM3	-	T	-	-	C	-	-	C	-	N/D	BtuB
BSPM8	C	-	C	C	C	-	C	-	-	N/D	Flagella
BSPM9	C	-	C	C	C	-	C	-	-	N/D	Flagella

C, clear plaques; T, turbid plaques; -, no plaque; N/D, not determined.

A

L   1   C   L : 1kb ladder
1 : *ΔrfaF,btuB::kan* + pcp20
C : *ΔrfaC,btuB::kan*

B
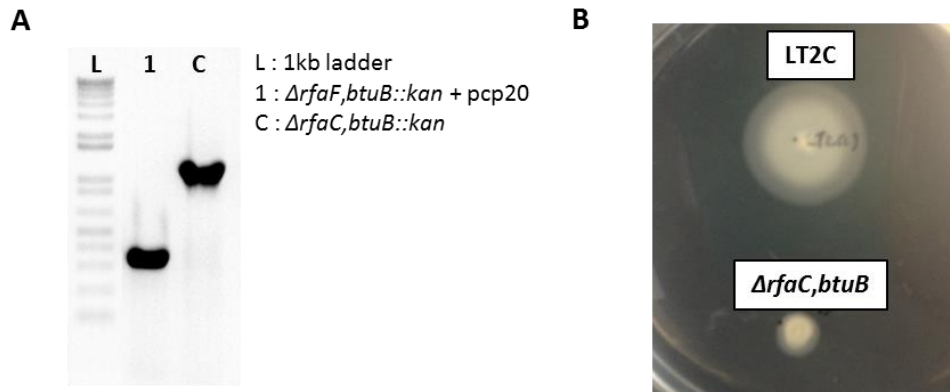
LT2C

ΔrfaC,btuB

Fig. 2.1. Construction of LT2C-*ΔrfaCbtuB* mutant and phenotype confirmation.
(A) A gene encoding *btuB* was deleted by one-step gene inactivation method and specific deletion of *btuB* gene was confirmed by PCR. (B) Motility of *ΔrfaCbtuB* mutant was tested on 0.3% LB soft agar.

II-3-2. Host range determination

The host specificities of the 23 isolated phages were determined using 13 *Salmonella* strains, seven *E. coli* strains and other strains including both Gram-positive and Gram-negative bacteria (summarized in Table 2.5). Generally, phages isolated by using the wild type host (BSP1, BSP2, BSP9, BSP11, BSP12, BSP14, BSP15, BSP19, BSP21, BSP64, BSPM8 and BSPM9) could infect a wide range of *Salmonella* serovars including Typhimurium, Typhi, Paratyphi, and Dublin. In contrast, some phages isolated using the rough type host (BSP3, BSP4, BSP16, and BSPM2) could infect the pathogenic *E. coli* O157:H7 strains rather than *Salmonella* strains tested, suggesting O-antigen of *Salmonella* may inhibit their adsorption. Also, among rough type specific phages, BSP3, BSP4, BSP16, BSPM1, and BSPM2 phages could infect some *Shigella* strains tested. Meanwhile, three phages including BSP17, BSP18, and BSP25 could only infect the O-antigen mutant *Salmonella*, suggesting that some strains with structural similarity with the rough type *Salmonella* may be abundant in the environment. Meanwhile, none of the other Gram-negative and Gram-positive strains tested are susceptible to all phages. Therefore, 23 phages isolated in this study are highly specific to the family Enterobacteriaceae.

Table 2.5. The host range of 23 phages

Bacterial strain	BSP1	BSP2	BSP3	BSP4	BSP5	BSP9	BSP11	BSP12
Samonella enterica								
serovar Typhimurium								
LT2C	C	C	-	-	-	-	C	C
LT2C (*ΔrfbP*)	-	C	C	C	C	C	-	-
SL1344	C	T	-	-	-	T	C	C
UK1	C	C	-	-	-	T	C	C
ATCC14028	C	C	-	-	-	-	C	C
ATCC19586	C	C	-	-	-	T	C	C
ATCC43147	C	C	-	-	-	T	C	C
ATCC13076	C	C	-	-	-	-	C	C
DT104	T	-	-	-	-	C	C	C
serovar Typhi Ty 2-b	T	T	-	-	-	-	T	T
serovar Parathyphi								
A IB 211	-	C	C	C	T	I	T	-
B IB 231	C	C	-	C	-	-	-	
C IB 216	C	C	-	C	-	C	C	C
serovar Dublin IB 2973	C	C	-	-	-	-	C	C
Escherichia coli								
MG1655	-	C	-	-	-	-	-	-
Escherichia coli **O157:H7**								
ATCC35150	-	-	C	T	-	-	-	-
ATCC43890	-	-	C	C	-	-	-	-
ATCC43894	-	-	C	T	-	-	-	-
ATCC43895	-	-	C	T	-	-	-	-
O157:NM 3204-92	-	-	C	C	-	-	-	-
O157:NM H-0482	-	-	C	C	-	-	-	-
Gram-negative bacteria								
Shigella flexineri 2a strain 2457T	-	-	C	C	-	-	-	-
Shigella boydii IB 2474	-	-	-	-	-	-	-	-
Vibrio fischeri ES-114 ATCC 700601	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	-	-	-
Cronobacter Sakazakii ATCC29544	-	-	-	-	-	-	-	-
Gram-positive bacteria	-	-	-	-	-	-	-	-
Listeria monocytogenes ATCC 19114	-	-	-	-	-	-	-	-
Staphylococcus aureus ATCC 29213	-	-	-	-	-	-	-	-
Staphylococcus epidermis ATCC 35983	-	-	-	-	-	-	-	-
Bacillus subtilis ATCC 23857	-	-	-	-	-	-	-	-
Bacillus cereus ATCC 14579	-	-	-	-	-	-	-	-

* C, clear plaques; T, turbid plaques; I, inhibition zone; -, no plaque.

Table 2.5. The host range of 23 phages (continued)

Bacterial strain	BSP14	BSP15	BSP16	BSP17	BSP18	BSP19	BSP20	BSP21
Samonella enterica								
serovar Typhimurium								
LT2C	C	C	-	-	-	C	-	C
LT2C (*ΔrfbP*)	C	C	C	C	C	C	C	C
SL1344	C	C	-	-	-	C	-	C
UK1	C	C	-	-	-	C	-	C
ATCC14028	C	C	-	-	-	C	-	C
ATCC19586	C	C	-	-	-	C	-	C
ATCC43147	C	C	-	-	-	C	-	C
ATCC13076	-	-	-	-	-	C	-	C
DT104	C	C	-	-	-	-	-	-
serovar Typhi Ty 2-b	C	C	-	-	-	C	-	C
serovar Parathyphi								
A IB 211	I	I	-	-	-	C	-	C
B IB 231	-	C	-	-	-	C	-	C
C IB 216	-	C	C	-	-	C	-	C
serovar Dublin IB 2973	-	-	-	-	-	C	-	C
Escherichia coli								
MG1655	-	-	CC	-	-	C	CC	C
Escherichia coli **O157:H7**								
ATCC35150	-	-	C	-	-	-	-	-
ATCC43890	-	-	C	-	-	-	-	-
ATCC43894	-	-	C	-	-	-	-	-
ATCC43895	-	-	C	-	-	-	-	-
O157:NM 3204-92	-	-	C	-	-	-	-	-
O157:NM H-0482	-	-	C	-	-	-	-	-
Gram-negative bacteria								
Shigella flexineri 2a strain 2457T	-	-	-	-	-	-	-	-
Shigella boydii IB 2474	-	-	C	-	-	-	I	-
Vibrio fischeri ES-114 ATCC 700601	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	-	-	-
Cronobacter Sakazakii ATCC29544	-	-	-	-	-	-	-	-
Gram-positive bacteria								
Listeria monocytogenes ATCC 19114	-	-	-	-	-	-	-	-
Staphylococcus aureus ATCC 29213	-	-	-	-	-	-	-	-
Staphylococcus epidermis ATCC 35983	-	-	-	-	-	-	-	-
Bacillus subtilis ATCC 23857	-	-	-	-	-	-	-	-
Bacillus cereus ATCC 14579	-	-	-	-	-	-	-	-

* CC, very clear plaques; C, clear plaques; I, inhibition zone; -, no plaque.

Table 2.5. The host range of 23 phages (continued)

Bacterial strain	BSP25	BSP64	BSPM1	BSPM2	BSPM3	BSPM8	BSPM9
Samonella enterica							
serovar Typhimurium							
LT2C	-	CC	I	I	-	C	C
LT2C (*ΔrfbP*)	C	C	C	C	C	C	C
SL1344	-	I	I	I	-	C	C
UK1	-	CC	I	I	-	C	C
ATCC14028	-	I	I	I	-	C	C
ATCC19586	-	CC	I	I	-	C	C
ATCC43147	-	CC	I	I	-	C	C
ATCC13076	-	C	I	I	-	-	-
DT104	-	CC	I	I	-	C	C
serovar Typhi Ty 2-b	-	I	CC	I	-	C	C
serovar Parathyphi							
A IB 211	-	C	C	C	C	-	-
B IB 231	-	C	C	C	-	-	-
C IB 216	-	C	C	C	-		
serovar Dublin IB 2973	-	C	C	-	-	-	-
Escherichia coli							
MG1655	-	-	CC	CC	C	-	-
Escherichia coli **O157:H7**							
ATCC35150	-	-	-	CC	-	-	-
ATCC43890	-	-	-	CC	-	-	-
ATCC43894	-	-	-	CC	-	-	-
ATCC43895	-	-	-	CC	-	-	-
O157:NM 3204-92	-	-	-	CC	-	-	-
O157:NM H-0482	-	-	-	CC	-	-	-
Gram-negative bacteria	-	-			-	-	-
Shigella flexineri 2a strain 2457T	-	-	-	-	-	-	-
Shigella boydii IB 2474	-	-	C	C	-	-	-
Vibrio fischeri ES-114 ATCC 700601	-	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	-	-
Cronobacter Sakazakii ATCC29544	-	-	-	-	-	-	-
Gram-positive bacteria							
Listeria monocytogenes ATCC 19114	-	-	-	-	-	-	-
Staphylococcus aureus ATCC 29213	-	-	-	-	-	-	-
Staphylococcus epidermis ATCC 35983	-	-	-	-	-	-	-
Bacillus subtilis ATCC 23857	-	-	-	-	-	-	-
Bacillus cereus ATCC 14579	-	-	-	-	-	-	-

* CC, very clear plaques; C, clear plaques; I, inhibition zone; -, no plaque.

II-3-3. Bacterial challenge assay

Among 23 phages isolated, seven phages (BSP2, BSP4, BSP5, BSP17, BSP18, BSPM3, and BSPM9) were not propagated well. Therefore, the rest 16 phages were subjected to bacterial growth inhibition activity test (Fig. 2.2 and Fig. 2.3). For the experiment, 16 phages were divided into two groups according to the host type including the wild type and the rough type hosts.

In the former group infecting the wild type host (Fig. 2.2), similar growth inhibitory patterns were observed in the phage groups using the same receptor. BtuB-targeting phages showed the most rapid cell lysis upon infection with MOI of 1 and the growth inhibition persisted for 1.5 h (Fig. 2.2A). Flagella-targeting phages showed similar growth inhibition patterns to those of BtuB-targeting phages (Fig. 2.2C). In contrast, both O-antigen (Fig. 2.2B)- and OmpC-targeting phages (Fig. 2.2D) showed weak growth inhibition effect compared to the BtuB- or Flagella-targeting phage groups.

Phages using rough type strain as a host (Fig. 2.3) showed much better growth inhibitory effect than the phages infecting the wild type strain. Both LPS-outer core- and BtuB-targeting phages showed rapid host cell lysis after infection with MOI of 1 and the growth inhibition persisted more than 8 h (Fig 2.3A and B). However, BSP25, a phage using OmpC as a receptor exhibited weak growth inhibition activity (Fig 2.3C). These results demonstrate that phages targeting BtuB, Flagella, and LPS-outer core possess stronger bactericidal activity than other

receptors targeting phages, suggesting that the phages using BtuB, Flagella, and LPS-outer core have potential to be used as biocontrol agents.
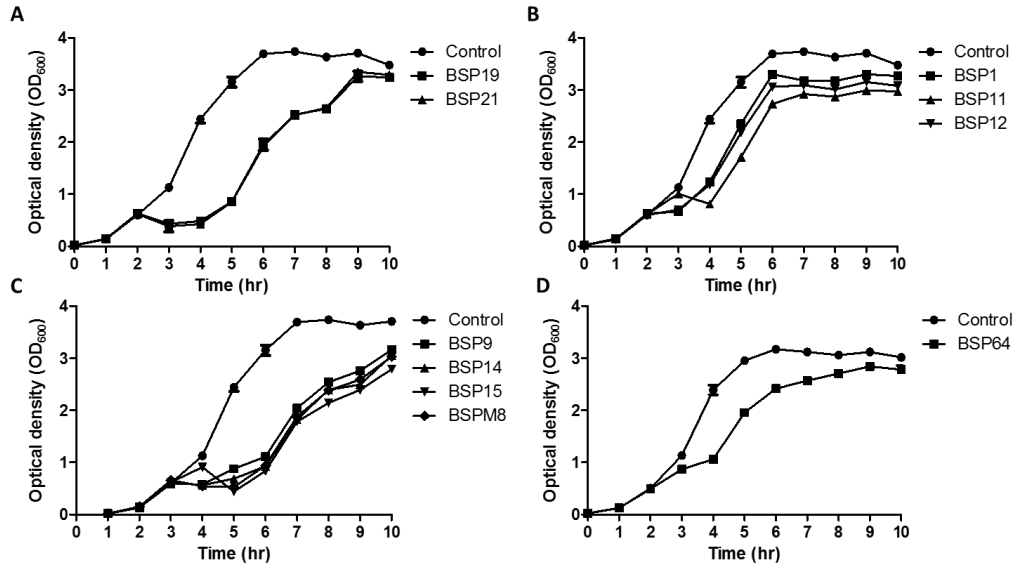
Fig. 2.2. Inhibition assays of LT2C (wild type) with each phage. A, BtuB-targeting phages; B, O-antigen-targeting phages; C, flagella-targeting phages; D, an OmpC-targeting phage. ●, control group without phage; ■, ▲, ▼, ◆, each phage treated groups with MOI of 1. The data shown are the mean values from two independent measurements and the error bars represent the standard deviations.

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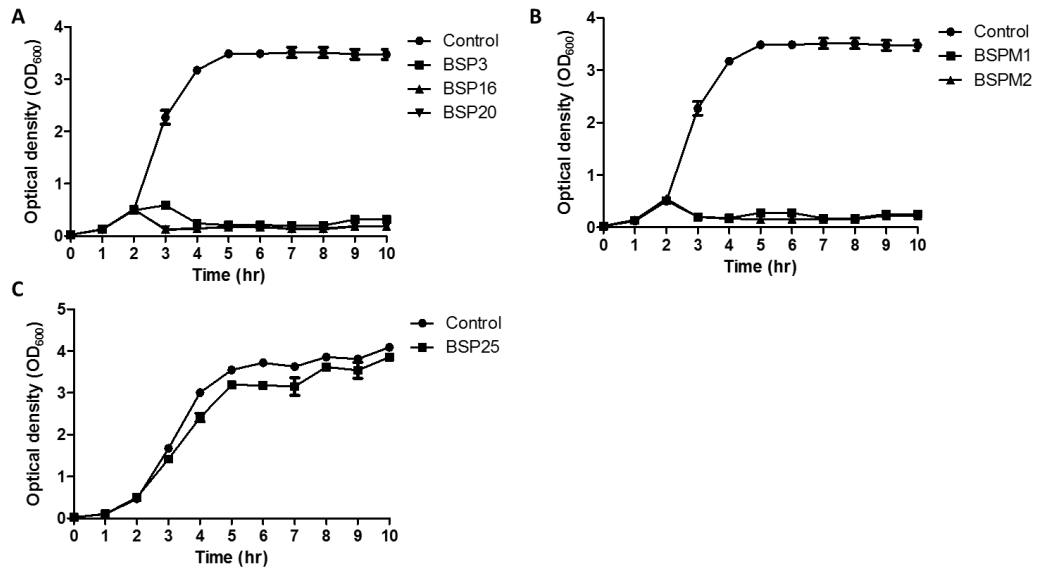
Fig. 2.3. Inhibition assays of LT2C *rfbP* mutant (rough type) with each phage.
A, LPS-outer core-targeting phages; B, BtuB-targeting phages; C, an OmpC-targeting phage.●, control group without phage; ■, ▲, ▼, each phage treated groups with MOI of 1. The data shown are the mean values from two independent measurements and the error bars represent the standard deviations.

II-3-4. Morphological analysis

Morphological analysis of by TEM revealed that the phages were classified into the *Myoviridae* (BSP3, BSP25), *Podoviridae* (BSP11, BSP16), and *Sipoviridae* (BSP21, BSP64, BSPM1, BSPM2) families (Fig. 2.4). The capsid size and tail length of each phage was summarized in Table 2.5. Further full genome sequence analysis was performed using purified DNA of six phages including BSP3, BSP16, BSP25, BSP64, BSPM1, and BSPM2.
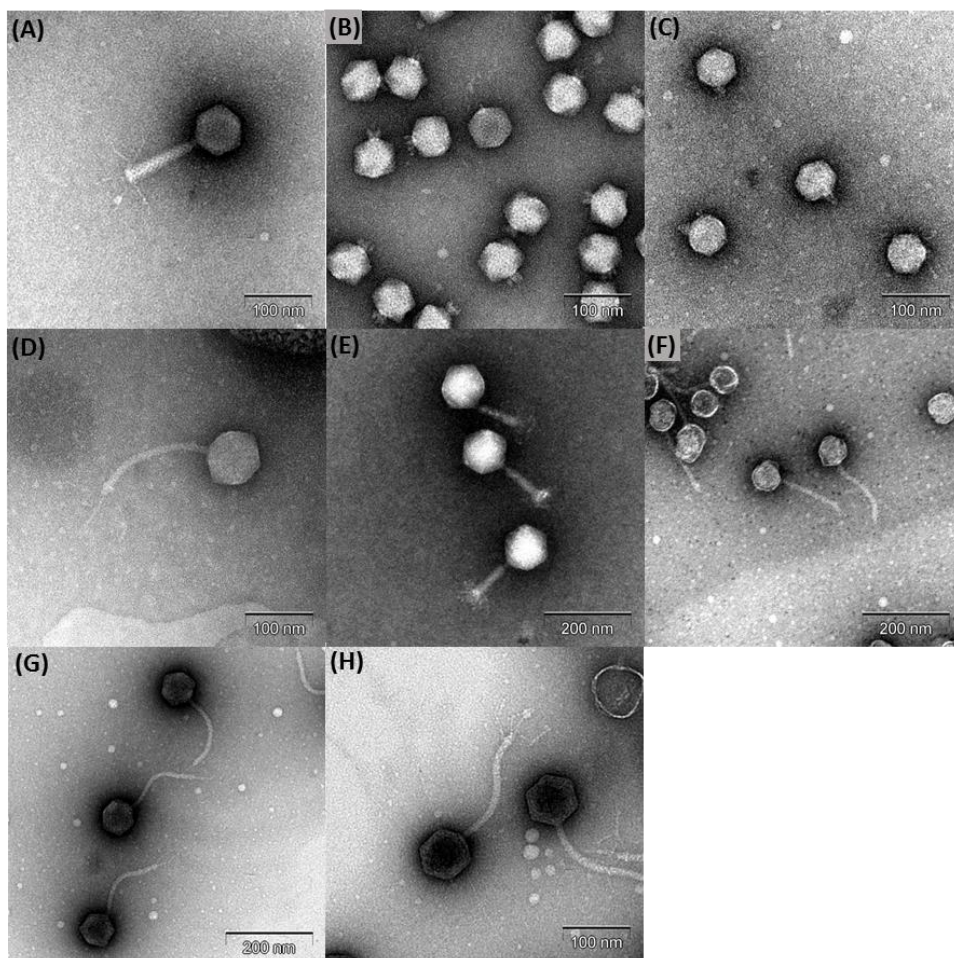
Fig. 2.4. Transmission electron micrograph images of the phages. (A) BSP3, (B) BSP11, (C) BSP16, (D) BSP21, (E) BSP25, (F) BSP64, (G) BSPM1, and (H) BSPM2.

Table 2.5. Morphological features of *S*. Typhimurium phages

Phage	Family	Capsid size (nm)	Tail length (nm)	Genome size (bp)	Predicted lifestyle
BSP3	*Myoviridae*	72	169	85,293	Virulent
BSP11	*Podoviridae*	67	15	-	-
BSP16	*Podoviridae*	56	15	39,688	Virulent
BSP21	*Siphoviridae*	84	259	-	-
BSP25	*Myoviridae*	145	178	157,280	Virulent
BSP64	*Siphoviridae*	100	245	51,587	Temperate
BSPM1	*Siphoviridae*	80	212	113,104	Virulent
BSPM2	*Siphoviridae*	79	213	112,922	Virulent

II-3-5. Full genome sequence analysis of bacteriophages

II-3-5-1. Phage BSP3

Genomic analysis of BSP3 phage was performed as it had a unique host range that strongly inhibited both *S. paratyphi* and *E. coli* O157:H7 simultaneously. The genome of phage BSP3 is a double-stranded DNA, total length of the nucleotide was 85,293-bp and the G+C content was 38.9%. Bioinformatics analysis identified that BSP3 phage was predicted to contain 128 ORFs on the genome and 24 tRNAs (Fig. 2.5). No lysogen decision cluster genes were found, indicating that BSP3 is a virulent phage having lytic life cycle. BSP3 phage has two tail fiber genes, ORF_74 and ORF_75, which corresponded to the long tail fibers observed in TEM analysis (Fig. 2.4D). However, a gene associated with the receptor binding protein was not identified in the genome (Fig. 2.5). Since phage tail fiber is regarded to be one of the important structures for the host range determination (36), the two tail fiber genes of BSP3 would be important for the phage infection.

Major tail protein
Lysis protein
Major capsid protein
Terminase large subunit
Head maturation protease
Major capsid protein
Tape measure protein
Baseplate assembly protein
Baseplate component
Tail fiber protein
Tail fiber protein
DNA adenine methylase
RIIB
RIIA
Nicotinate phosphoribosyltransferase
Ribose-phosphate pyrophosphokinase
HNH endonuclease
Anaerobic NTP reductase
Anaerobic nucleoside diphosphate reductase
Glutaredoxin
Ribonucleotide triphosphate reductase, beta chain
Ribonucleotide triphosphate reductase, alpha chain
Exodeoxyribonuclease
Thymidylate synthase
Dihydrofolate reductase
Transcriptional regulator
DNA ligase
HNH endonuclease
DNA polymerase
DNA polymerase
Deoxynucleotide monophosphate kinase
DNA primase/helicase

Salmonella Typhimurium
Bacteriophage BSP3
(85,293-bp)

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Fig. 2.5. Genome map of *S*. Typhimurium phage BSP3. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories (purple, DNA packaging; green, phage structure; orange, replication/ transcription; blue, recombination/ repair; red, host lysis). The inner circle with red line indicates the GC content.

II-3-5-2. Comparative genome analysis between BSP3 and Felix-O1 phages

Comparative genome analysis between BSP3 phage and previously reported *Salmonella* phage Felix-O1 (37) revealed that two phages share 96% average nucleotide homology. They possessed 99% identical major capsid protein and a terminase large subunit, indicating both phages are members of *Myoviridae* A1 group. In addition, BSP3 phage has 100% identical host lysis gene (endolysin) of the Felix-O1 phage. In contrast, two tail fibers of both phages showed relatively low sequence identity (Fig. 2.6). Interestingly, according to the host range analysis, BSP3 phage could not infect the most *Salmonella* strains tested (Table 2.4) while Felix-O1 phage could infect all *Salmonella* strains (Table 2.6). Instead, BSP3 phage infected *E. coli* O157:H7 strains which Felix-O1 phage could not infect. Therefore, distinct host specificities of the two phages might be attributed to the different tail fiber gene components between them. It would be interesting to prove this possibility concretely by experiments in the further study.
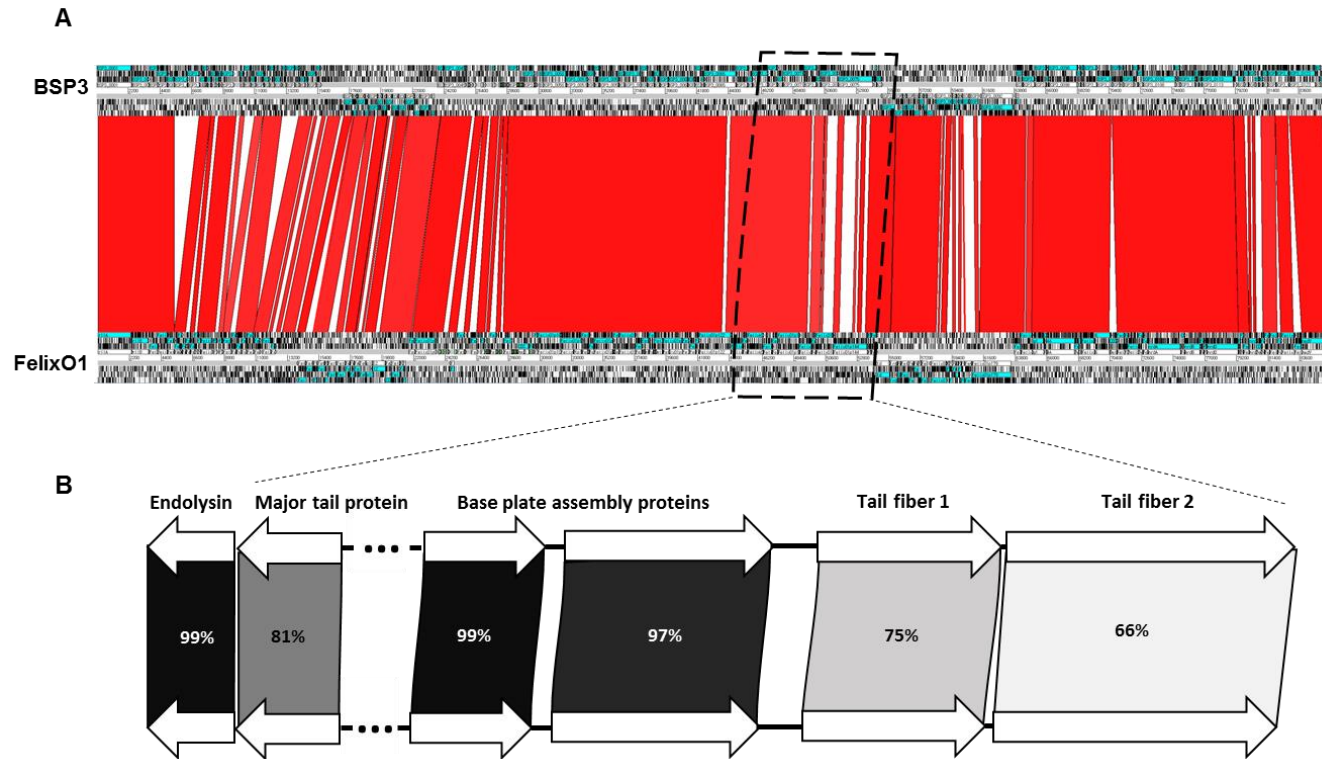
Fig. 2.6. Comparative genomic analysis of BSP3 and Felix-O1. Comparative genome analysis was performed between BSP3 and Felix-O1 phages on nucleotide sequence level (A) and host lysis and phage tail components was compared on amino acid sequence level (B).

Table 2.6. The host range comparison of BSP3 and Felix-O1 phages

Bacterial strain	BSP3	Felix-O1
Samonella enterica		
serovar Typhimurium		
SL1344	-	C
UK1	-	C
LT2	-	C
LT2C	-	C
ATCC14028	-	C
ATCC19586	-	C
ATCC43147	-	C
ATCC13076	-	-
DT104	-	C
serovar Parathyphi		
A IB 211	C	C
B IB 231	-	C
C IB 216	C	C
serovar Dublin IB 2973	-	C
Escherichia coli		
MG1655	-	-
Escherichia coli **O157:H7**		
ATCC35150	C	-
ATCC43890	C	-
ATCC43894	C	-
ATCC43895	C	-
O157:NM 3204-92	C	-
O157:NM H-0482	C	-
Gram-negative bacteria		
Shigella flexineri 2a strain 2457T	C	-
Shigella boydii IB 2474	-	-

C, clear plaques; -, no plaque.

II-3-5-3. Phage BSP16

BSP16 phage is one of the rough type *Salmonella*-infecting phages isolated in this study. Genome analysis results revealed that it had relatively a short 39,688-bp genome compared to the other phages analyzed and G+C contents was 48.7%. A total of 50 ORFs and no tRNA was predicted (Fig. 2.7). In particular, for BSP16, all predicted ORFs were aligned in the same direction and predicted to have a typical lysis system of Gram-negative bacteria-infecting phages which is comprised of endolysin, holin, and Rz-like proteins. However, different to the canonical order of lysis-related genes which gathered together, the lysis genes of BSP16 were positioned totally apart from each other, indicating these genes were obtained during the phage evolution. Interestingly, these protein homologous were also found in various Gram-negative bacteria-targeting phages including *Erwinia*, *Kluyvera*, *Yersinia*, *E. coli*, *Citrobacter*, *Krebsiella* and *Salmonella* phages. Moreover, it was found in a genome of *Clostridium difficile*, Gram-positive bacteria, suggested that the target of this endolysin is highly conserved in many bacteria. Bioinfomatics analysis revealed that the endolysin gene from BSP16 phage contains an amidase 2 domain (pfam01510) and predicted to have *N*-acetylmuramonyl-Lalanine amidase activity. Since the characteristics of this endolysin had not been studied yet, general features of this protein were characterized in chapter V.

S-adenosyl-L-methionine hydrolase
Protein kinase

RNA polymerase

Deoxyguanosine triphospho hydrolase inhibitor

DNA ligase

RNA polymerase inhibitor
Single stranded DNA binding protein

Endonuclease
N-acetylmuramonyl-Lalanine amidase

DNA primase/helicase

DNA polymerase

Exonuclease

Head-to-tail joining protein

Capsid assembly protein

Major capsid protein 10A
Major capsid protein 10B
tail tubular protein A

tail tubular protein B

Internal virion protein A
Internal virion protein B

Internal virion protein C

Internal virion protein D

Tail fiber protein

Holin
Terminase small subunit
Phage lambda Rz-like lysis protein

Terminase large subunit

Salmonella Typhimurium
Bacteriophage BSP16
(39,688-bp)

35000
30000
25000
20000
15000
10000
5000
0

Fig. 2.7. Genome map of *S*. Typhimurium phage BSP16. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories (purple, DNA packaging; green, phage structure; orange, replication/ transcription; red, host lysis) The inner circle with red line indicates the GC content.

II-3-5-4. Phage BSP25

DNA genome of a phage BSP25 was 157,280 bp in length (G+C content of 44.9%). A total of 213 ORFs and three tRNAs were found (Fig. 2.8). Annotation and functional analysis of the predicted ORFs revealed that this phage genome contains gene clusters correspond to phage structure proteins including phage head (major capsid protein, prohead core proteins, prohead protease, and head completion protein), tail structure (tail tube protein, tail sheath protein, tail sheath stabilizer protein, neck proteins, baseplate wedge subunits, tail tube-associated base plate protein, and baseplate hub subunit), and an accessory structural protein (baseplate tail tube initiator protein), suggesting that this phage contains all the genes required for the complete assembly of phage head and tail structures. Moreover, BSP25 phage contained genes related to nucleotide manipulation/repair (DNA topoisomerases, DNA helicases, DNA primase, DNA polymerase, and RNA polymerase sigma factor) and recombination/repair (DNA repair/recombination protein, UvsX RecA-like recombination protein, DNA ligase, DNA end protector protein, and recombination endonuclease subunit). The results suggested that the phage BSP25 has developed its own replication/recombination/repair systems which is distinct to the host bacterial strain. Interestingly, host lysis-related genes such as holin and endolysin was not identified in the genome of BSP25 phage. This means that the BSP25 phage might have a unique lysis system. According to annotation results, ORF_135 was predicted as a base plate hub subunit containing a tail lysozyme domain. Following, CHAP domain which is associated with autolysis of bacteria and usually found in peptidoglycan hydrolase genes of Gram positive-targeting phages (38) was predicted

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in ORF_146. Therefore, further examination for revealing the involvement of the two proteins in phage lysis would be an interesting subject in near future.

Prohead core protein
Major capsid protein
Tail tube protein
DNA repair/recombination protein
Helicase
Sliding clamp DNA polymerase accessory protein
Clamp loader small subunit DNA polymerase accessory protein
Clamp loader subunit DNA polymerase accessory protein
RegA translational repressor

Prohead protease
prohead core protein
Portal protein
Tail tube protein
Tail sheath protein
Terminase large/small subunit
Tail sheath stabilization protein
Neck protein 1
Neck protein 2

Superinfection exclusion protein
DNA-binding protein
ATP-dependent helicase
Ribonuclease H
RNA polymerase sigma factor
Recombination endonuclease subunit

Base plate wedge subunit

DNA primase subunit
Peptidoglycan binding protein
PhoH-like protein
NrdB/NrdA Ribonucleotide-diphosphate reductase
Glutaredoxin
Base plate wedge protein
Base plate hub subunit and tail lysozyme
Base plate hub subunit
T4-like late promoter transcription accessory protein
Single-stranded DNA-binding protein
Baseplate tail tube initiator protein
DNA end protector protein
thymidylate synthase
dUTP diphosphatase
Loader of T4-like helicase
Phage DNA ligase
Primase-helicase subunit
UvsX RecA-like recombination protein

putative DNA topoisomerase II large subunit
putative DNA topoisomerase II medium subunit
Exonuclease A
Serine/ threonine phosphatase
Deoxycytidylate deaminase
Head completion protein
HNH endonuclease
Baseplate tail tube cap
Baseplate wedge subunit
Tape measure protein

Salmonella Typhimurium
Bacteriophage BSP25
(157,280-bp)

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Fig. 2.8. Genome map of bacteriophage BSP25. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories (purple, DNA packaging; green, phage structure; orange, replication/transcription; blue, recombination/repair; yellow, nucleotide metabolism; pink, additional function) The inner circle with red line indicates the GC content.

II-3-5-5. Comparative genome analysis of BSP25 and related phages

BSP25 phage genome showed high nucleotide homology about 84% with two phages, BSP101 and SFP10, previously isolated by me and our group, respectively (39). Interestingly, BSP25 phage utilized outer membrane porin protein, OmpC, as a receptor, while other two phages both used BtuB protein as a receptor. Therefore, comparative genomic analysis of three phages were performed to investigate a gene which is responsible for the host receptor recognition. Genome comparison of three phages revealed that a cluster of tail structural genes of BSP25 phage have low homology with other two phages (red box in Fig. 2.9A). Specifically, a tail spike gene (tail spike 2) was absent in BSP25 phage genome and a tail fiber gene of BSP25 showed very low homology of 13% while those of two other phages showed 99% amino acid homology (Fig. 2.9B). These results suggested that a tail spike 2 gene and a tail fiber gene would be important to recognize the host receptor by BSP25 as well as by other two phages.
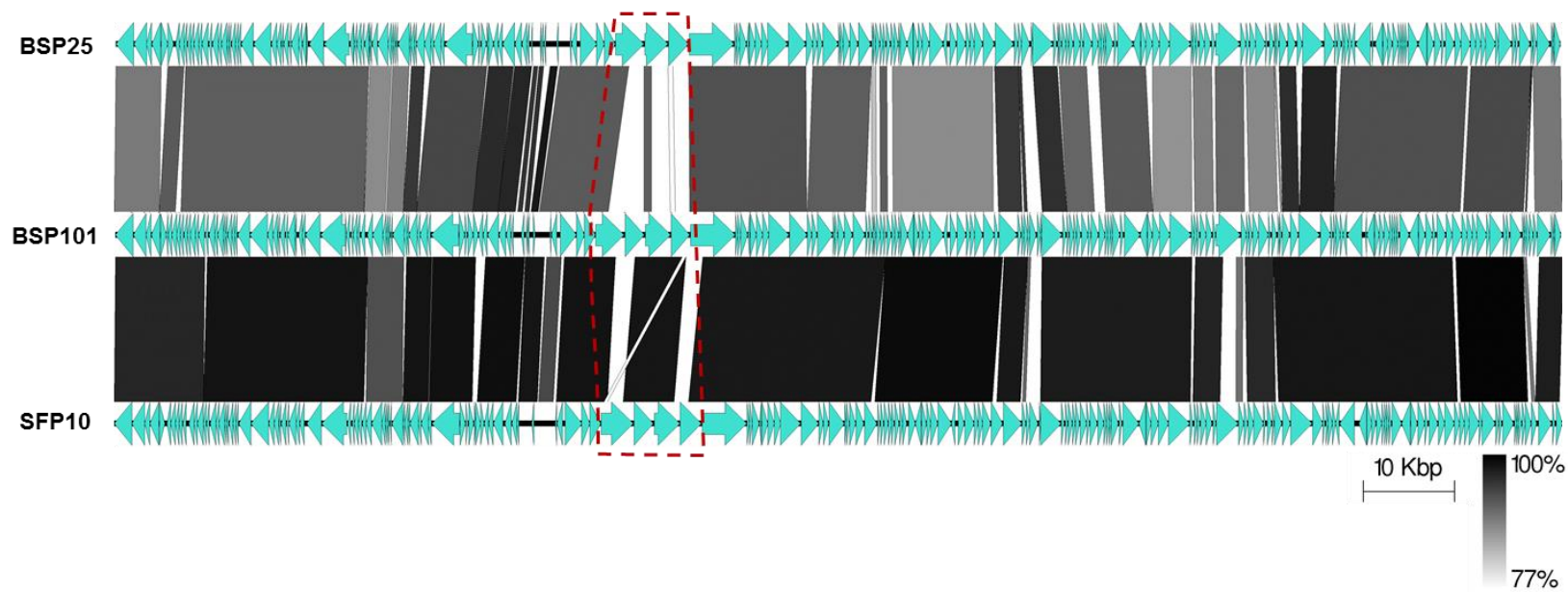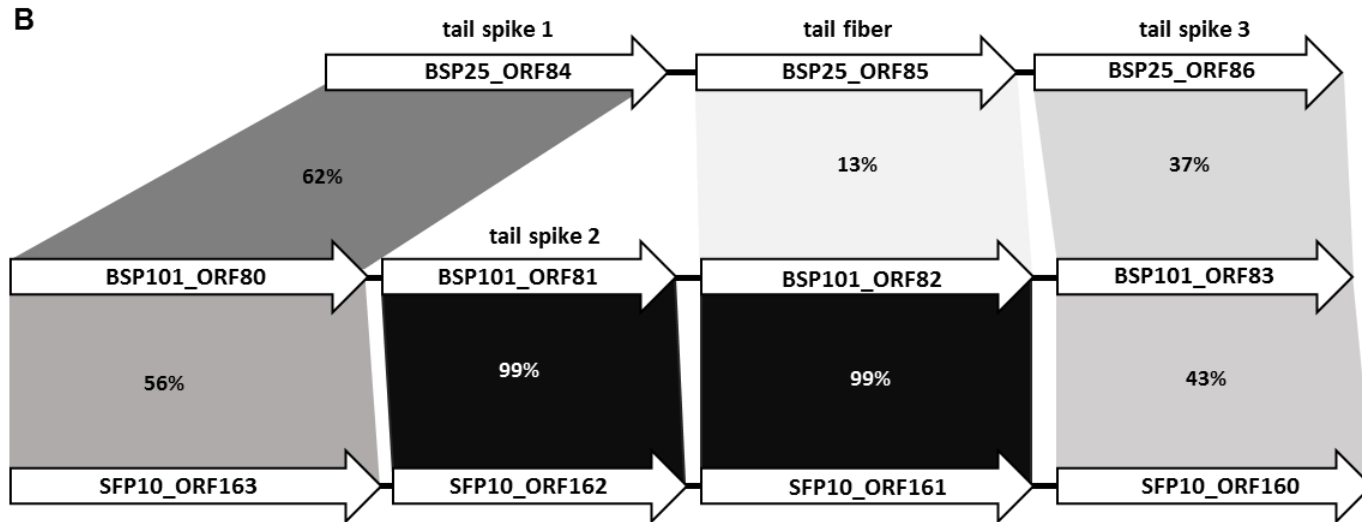
Fig. 2.9. Comparative genomic analysis of BSP25, BSP101 and SFP10 phages. Comparative genome analysis was performed between BSP25, BSP101, and SFP10 phages on nucleotide sequence level (A) and sequences of phage tail components including tail spikes and a tail fiber were compared on amino acid sequence level (B). The genome of SFP10 phage was rearranged for better visualization.

II-3-5-6. Phage BSP64

BSP64 phage has a genome of 51,587 bp and the G+C content was 51%. A total of 81 ORFs and one tRNA were predicted (Fig. 2.10). Bioinformatics analysis showed that BSP64 phage had very high nucleotide homology (over 90%) with two lambdoid *Salmonella* prophages, Gifsy-1 and Gifsy-2, assuming that the phage BSP64 is a temperate one. Actually, BSP64 phage genome contained various genes originated from Gifsy-1 and Gifsy-2 phages such as Gifsy-1 prophage RecE and Gifsy-2 prophage RecT. In addition, a terminase large protein, a DNA packaging protein, and a major capsid protein of BSP25 were very similar to a gifsy-1 prophage terminase large chain gp2, a gifsy-1 prophage head protein gp7, and a gifsy-1 prophage DNA packaging protein gp9, respectively, indicating that they may share the same DNA packaging system each other. Moreover, lysogen decision gene cluster (integrase, exicionase, *cI*-like repressor, and anti-repressor) genes were present in its genome, supporting that BSP64 phage will have both lysogenic and lytic life cycles. Indeed, following experiment revealed to form a stable lysogen, meanwhile deletion of integrase gene prevented further induction of BSP64 prophage by mitomycin C (data not shown). BSP64 phage genome contained a canonical host lysis system containing a holin, muramidase (pfam00959), and a putative Rz-protein (pfam03245). Usually the putative Rz-protein mis-annotated as a peptidase, however, this protein family is not considered to be a peptidase according to the MEROPs database. BSP64 phage encode a GogA protein (ORF_6) which is homologue to another gene known to be one of *Salmonella* effector proteins

important for virulence. In addition, BSP64 phage encoded a PagK protein (ORF_52) known as a phage-encoded virulence factor activated by the PhoP/Q system (IPR029335). Therefore, it is seemed that the proteins encoded by phage BSP64 may be important for virulence of the host bacterial strain.

Host specificity protein J (partial)
Terminase small subunit
Terminase large subunit
Head-to-tail joining protein
Portal protein

Head-tail precconnector protein
Head decoration protein
Major capsid protein E
DNA packaging protein
Minor capsid protein FII
Minor tail protein Z

Minor tail protein U
Major tail protein
Minor tail protein G
Minor tail protein T

Minor tail protein H

Minor tail protein M
Major tail protein L
Minor tail protein L
Tail assembly protein
Tail assembly protein

Host specificity protein J

Tail fiber protein

Tail assembly protein
Tail assembly protein

Putative Rz-protein
Muramidase
Holin

Antirepressor
Antiterminator protein
DNA breaking-rejoining protein

DNA damage-inducible protein I
Antiterminator protein
Methyltransferase
Ead-like protein
Phage replication protein P
Phage replication protein O
cI protein
prophage anti-repressor
DNA binding protein
Nucleotide-bindign protein

DNA breaking-rejoining protein

Gifsy-1 prophage RecE

Gifsy-2 prophage RecT
Exicionase

Integrase

Salmonella Typhimurium
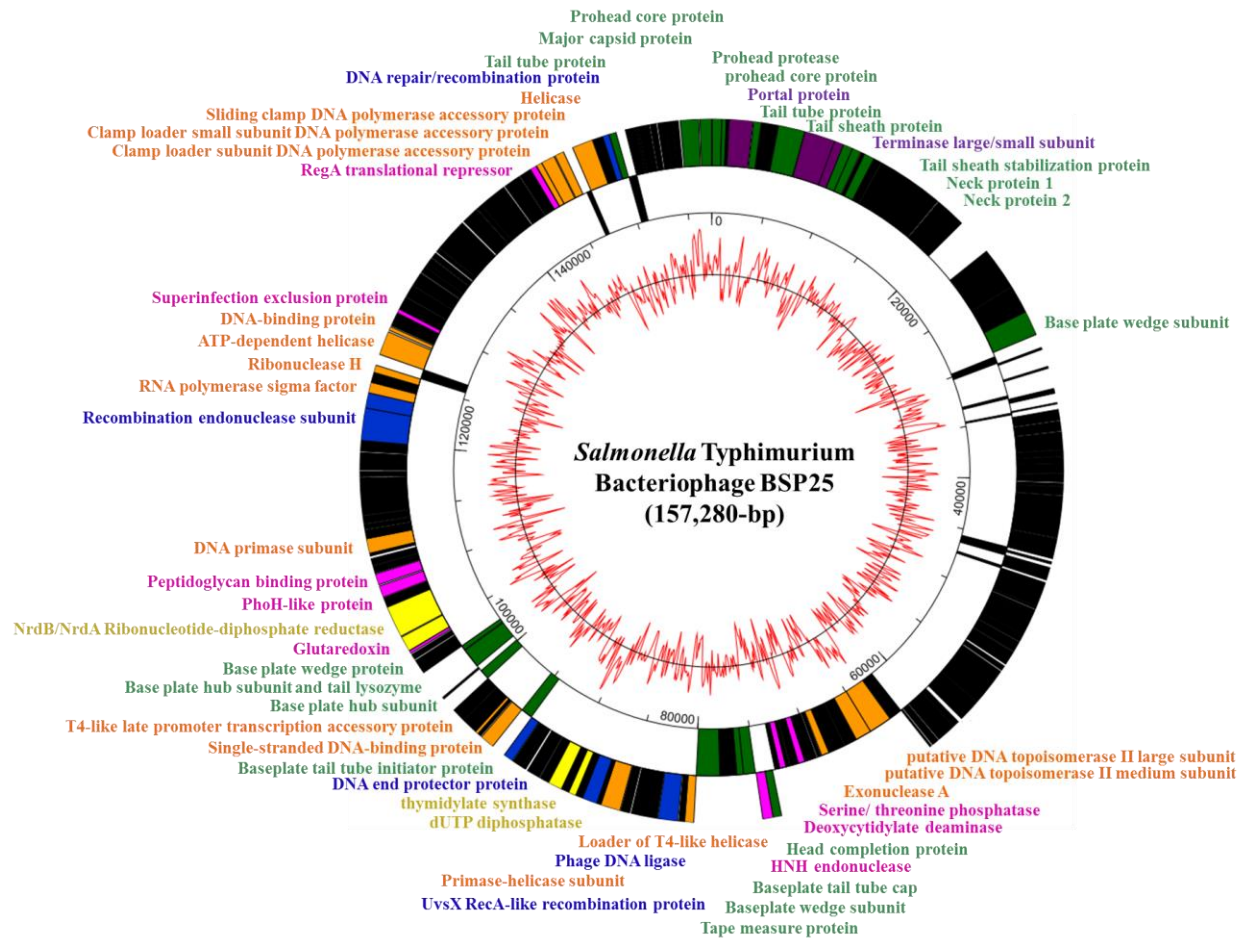Bacteriophage BSP64
(51,587-bp)

50000   0
10000
20000
30000
40000

69

Fig. 2.10. Genome map of the BSP64 phage. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories (purple, DNA packaging; green, phage structure; orange, replication/ transcription; red, host lysis; sky blue, host interaction; pink, additional function). The inner circle with red line indicates the GC content.

II-3-5-7. BSPM1 and BSPM2 phages

BSPM1 phage has a genome of 113,104 bp and contained 28 tRNAs. BSPM2 phage has a genome of 112,922 bp with 29 tRNA genes. The G+C contents of both phages were 40% and a total of 188 ORFs and 180 ORFs were predicted on the genome of BSPM1 and BSPM2, respectively (Fig. 11A and 11B). Phages BSPM1 and BSPM2 showed high nucleotide homology of 82% and 83%, respectively, when compared to a phage EPS7, indicating they are belonged to T5-like phage family. The composition of the genomes of both phages could be divided into pre-early, early, and late regions. The pre-early gene cluster contains the A1 protein that blocks host DNA, RNA, and protein synthesis, the A2-3 protein that is important for second step transfer (FST), and deoxyribonucleoside-5'-monophosphatase, an enzyme that converts deoxyribonucleotides to deoxyribonucleosides. Early gene products consist of genes involved in replication (primase, helicase, and endonuclease), recombination, DNA repair, transcription (D5 protein), signal transduction (serine/threonine phosphatase), metabolism, and cell lysis (endolysin and holin). The late region gene cluster is composed of phage structural and morphogenesis proteins, including major capsid protein, major tail protein, and long-tail fibers. A comparative genomic analysis was performed since two phages showed high similarity both at the phenotypic and genotypic levels.

A

Receptor-blocking protein
Receptor-binding protein
Terminase large subunit
Portal protein
Tail protein
Prohead protease
Major head protein precursor
Major tail protein
Minor tail protein
Pore-forming tail tip protein pb2
Tail protein Pb3
Tail protein Pb4
Phage tail protein
Phage tail fiber protein
Phage tail fiber protein
deoxyUTP pyrophosphatase
Flap endonuclease
D14 protein
Exonuclease subunit 2
D12 protein
D11 protein
ATP-dependent helicase
DNA polymerase
Primase
Helicase
D5 protein
NAD-dependent DNA ligase subunit A/B
D2, D3 protein
Replication origin binding protein
Sir2-like protein
Anaerobic ribonucleoside triphosphate reductase
PhoH-like protein

Deoxynucleoside-5'-momophosphatase
A1, A2-A3 protein

Salmonella Typhimurium
Bacteriophage BSPM1
(113,104-bp)

Protein 2C
Serine/threonine protein phosphatase
Serine/threonine protein phosphatase
Thioredoxin
Lysozyme
Holin
ATP-dependent Clp protease
Deoxynucleoside-5'-momophosphate kinase
Putative acetyltransferase-related protein

metallopeptidase
Ribonuclease H
Thymidylate synthase
Dihydrofolate reductase
Aerobic ribonucleoside diphosphate reductase small subunit
Aerobic ribonucleoside diphosphate reductase large subunit

B

Receptor-blocking protein
Receptor-binding protein
Terminase large subunit
Portal protein
Tail protein
Prohead protease
Major head protein precursor
Major tail protein
Minor tail protein
Pore-forming tail tip protein pb2
Tail protein Pb3
Tail protein Pb4
Phage tail protein
Phage tail fiber protein
Phage tail protein
deoxyUTP pyrophosphatase
Flap endonuclease
D14 protein
Exonuclease subunit 2
D12 protein
D11 protein
ATP-dependent helicase
DNA polymerase
Primase
Helicase
D5 protein
NAD-dependent DNA ligase subunit A/B
D2, D3 protein
Replication origin binding protein
Sir2-like protein
Anaerobic ribonucleoside triphosphate reductase
PhoH-like protein

Deoxynucleoside-5'-momophosphatase
A1, A2-A3 protein

Salmonella Typhimurium
Bacteriophage BSPM2
(112,922-bp)

Protein 2C
Serine/threonine protein phosphatase
Serine/threonine protein phosphatase
Thioredoxin
Lysozyme
Holin
ATP-dependent Clp protease
Deoxynucleoside-5'-momophosphate kinase
Putative acetyltransferase-related protein

metallopeptidase
Ribonuclease H
Thymidylate synthase
Dihydrofolate reductase
Aerobic ribonucleoside diphosphate reductase small subunit
Aerobic ribonucleoside diphosphate reductase large subunit

72

Fig. 2.11. Genome maps of (A) BSPM1 and (B) BSPM2 phages. Outer circle indicates the gene coding regions by strand. The color of each gene refers to the functional categories (purple, DNA packaging; green, phage structure; orange, replication/transcription; red, host lysis; sky blue, host interaction; pink, additional function). The inner circle with red line indicates the G+C content.
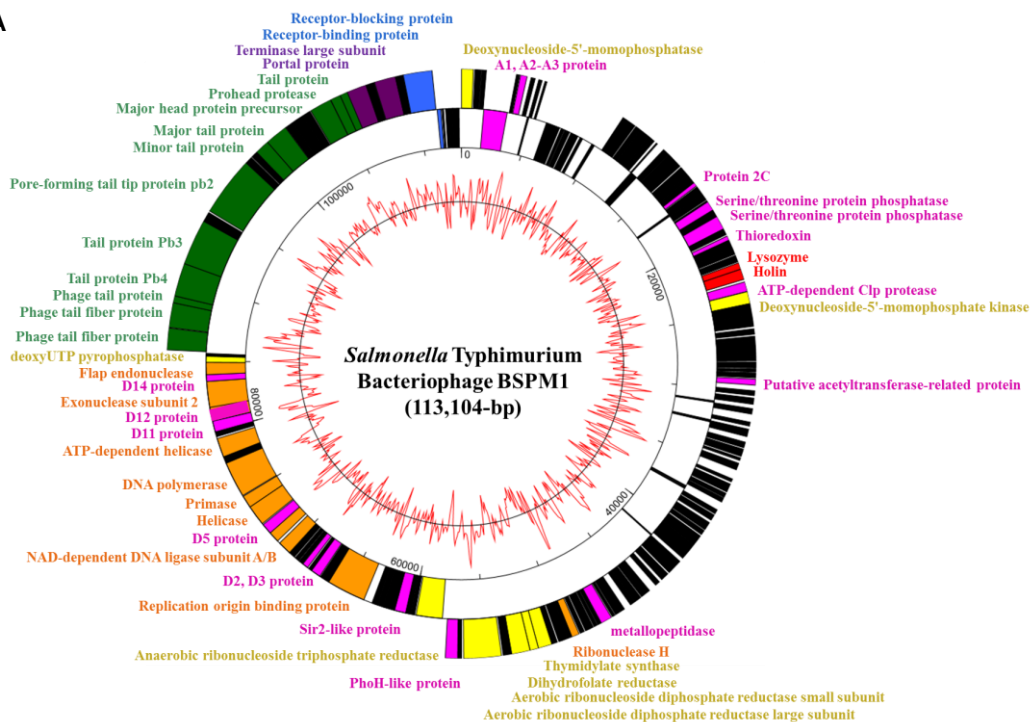
II-3-5-8. Comparative genome analysis of BSPM1 and BSPM2 phages

Both two phages, BSPM1 and BSPM2, were isolated from a same swine intestine sample. Both two phages infected O-antigen-lacking rough type *Salmonella* (Table 2.3) and utilized BtuB as a host receptor (Table 2.4). However, interestingly, host ranges of two phages were totally different from each other. That is, only BSPM2 phage could infect *E. coli* O157:H7 strains tested (Table 2.5) even though two phages showed high homology of 98% at the nucleotide level (Fig. 2.12A). Comparative genomic analysis revealed that the phage lysis-related genes (holin and endolysin) were 100% homologous to each other. In addition, two phages share highly similar receptor binding proteins of homology of 99% at amino acid sequence level. However, it was noticeable that the tail structural gene cluster showed very low homology with each other, two tail fiber genes with 16% and 13% amino acid sequence homology and a putative tail gene with 32% homology. On the other hand, all other genes related to phage tail structure showed high homology of over 95% (Fig. 2.12B). Adsorption assay of BSPM1 and BSPM2 showed that both phages rapidly bound to the rough type *Salmonella*, more than 50% of phages were adsorbed within 3 min (Fig. 2.13). Therefore, it is seemed that tail fiber differences may not affect to the phage adsorption rate. Considering that the host ranges of BSPM1 and BSPM2 phages are very different (Table 2.5), these three tail genes might be important for determining the host specificity of two phages.

A

B

Tail protein Pb4 Pb3 Pb2 Tail protein RBP

72% 16% 13% 95% 99% 99% 99% 88% 32% 99%

Tail protein Tail protein

75
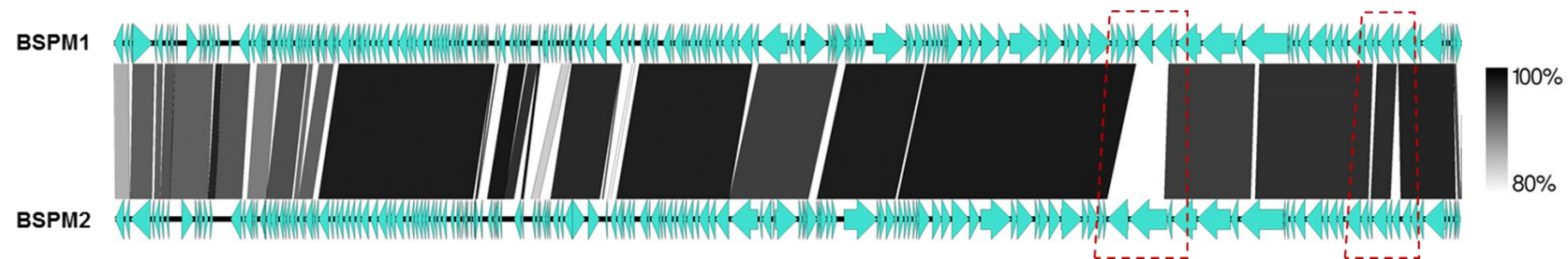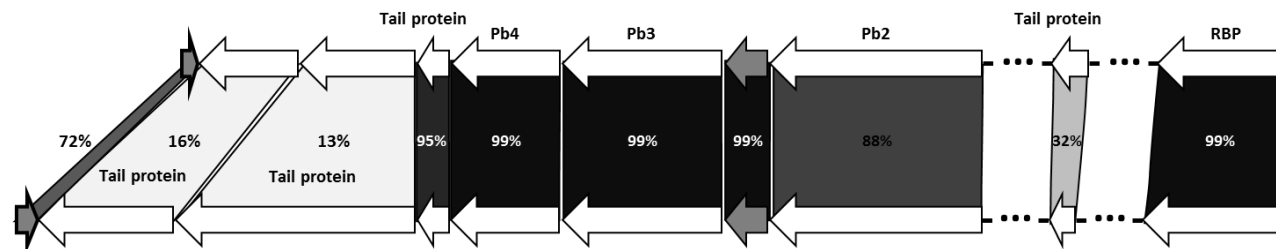
Fig. 2.12. Comparative genomic analysis of BSPM1 and BSPM2 phages. (A) Whole nucleotide sequences of BSPM1 and BSPM2 phages were compared using BLASTN and the result was drawn by Easyfig (ver. 2.1) program. Percent of nucleotide homology was indicated with gray scale. Regions with low homology include putative phage tail fiber proteins and other tail components were indicated with red dashed box. (B) The regions containing tail structural proteins and a RBP (receptor binding protein) were expanded for better visualization. The predicted protein functions of each ORFs were indicated and percent identity was estimated in amino acid level using BLASTP.
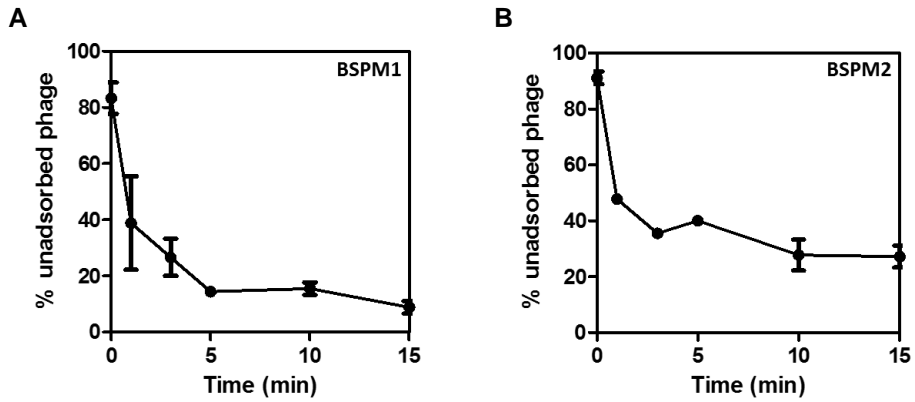
Fig. 2.13. *In vitro* **adsorption assay of BSPM1 and BSPM2 phages.** The adsorption efficiency of BSPM1 and BSPM2 phages were shown. The data shown are the mean values from two independent measurements and the error bars represent the standard deviations.

II-3-5-9. Comparative proteomic analysis of BSPM1 and BSPM2 phages

Given that phages BSPM1 and BSPM2 showed distinct tail morphologies (Fig. 2.4G and H) and the genes encoding the tail proteins were not homologous to each other (Fig. 2.12), I hypothesized that the difference in tail structure may responsible for the host specificity of two phages. To confirm this, structural proteins of two phages were analyzed and compared by LC-MS/MS (Fig. 2.14)

As expected, two phages showed similar band patterns except some protein bands. Surprisingly, two bands (bands 8 and 11) which corresponded to the tail fiber proteins (ORF_0160 and ORF_0158 of BSPM2 phage, respectively), were additionally detected in BSPM2 phage. Related to this, an interesting thing was revealed in TEM images (Fig. 2.4G and H) that long side tail fibers were observed only in BSPM2 phage, however, there were no ones in BSPM1. Previously, there was a reported that six long tail fibers in T7 phage mediated the interaction between LPS and host bacteria, suggesting the important role of tail fibers in T7 infection (40). In addition, *E. coli* and *Salmonella* showed different profiles of LPS structure (41). Considering that BSPM2 phage contains six long tail fibers, these tail proteins have an important role for host-range determination, presumably by binding to a specific LPS region that differs between *E. coli* O157:H7 and *S.* Typhimurium strains. Therefore, further studies were performed to understand the exact role of these tail fiber genes in phage BSPM2.

A

BSPM1   M.W   BSPM2
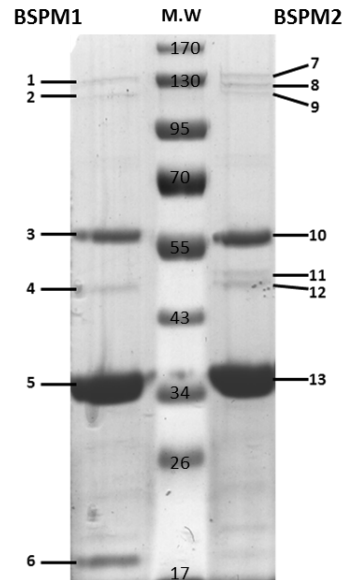
B

Bands	Locus_tag	Predicted M.W. (kDa)[a]	Predicted pI	Identified Peptides	Sequence Coverage (%)	Predicted functions
1	BSPM1_0162	132.7	5.57	94	61	Pore-forming tail tip protein Pb2
2	BSPM1_0160	107.5	5.01	14	16	Tail protein Pb3
3	BSPM1_0167	50.3	4.72	22	47	Major tail protein
4	BSPM1_0174	45.5	5.55	17	33	Portal protein
5	BSPM1_0171	50.6	5.18	34	57	Major head protein precursor
6	BSPM1_0173	17	8.61	29	63	Putative tail protein
7	BSPM2_0165	133.2	5.88	78	56	Pore-forming tail tip protein Pb2
8	BSPM2_0160	113.5	4.71	39	32	Side tail fiber
9	BSPM2_0163	107	5.05	19	23	Tail protein Pb3
10	BSPM2_0170	50.2	4.92	22	44	Major tail protein
11	BSPM2_0158	62.5	5.18	38	23	Side tail fiber
12	BSPM2_0177	45.5	5.55	13	25	Portal protein
13	BSPM2_0174	50.6	5.18	38	63	Major head protein precursor

[a], molecular weight

Fig. 2.14. Comparison of phage structural proteins by LC-MS/MS analysis. (A) Each phage protein was separated by SDS-PAGE and the visible bands were excised for in-gel digestion. (B) Protein bands from BSPM1 and BSPM2 phages were predicted using each phage sequence as subjects.

II-3-5-10. Tail fiber mutant construction in phage BSPM2

To investigate the exact role of tail fiber genes in host range determination of BSPM2 phage, tail fiber regions were truncated using a pCRISPR-Cas9 system. As a result, three phages which could not infect one *E. coli* O157:H7 strain (ATCC 43890) but the infection activity still remained against *E. coli* DH5α. The phenomenon was identified by spotting assay, single plaque was purified, and designated as phages m7, m21, and m34, respectively (Fig. 2.15). Interestingly, PCR confirmation and sequence analysis results revealed that all mutant phages have wide range of gene deletion inside two tail fiber genes (Fig. 2.16A). Same regions correspond to 3,161 bp were deleted in phages m7 and m21 while m34 have shorter nucleotide deletions of 1,860 bp (Fig. 2.16B). As a result, two types of tail fiber mutant phages were obtained and the loss of tail fibers did not affect infectivity against a non-pathogenic *E. coli* DH5α. In contrast, these phages lost their infection ability against *E. coli* O157:H7. Therefore, these results supported that truncation of tail fiber genes of BSPM2 phage changed the host specificity, confirming the role of tail fibers in host range determination. However, the deletion of tail fiber genes was not specific to each single tail fiber gene. Previous study showed that recombination of phage DNA can occur between phage DNAs (42). As CRISPR targeting experiments used in this study was performed without providing the template DNA for the recombination of phage tail fiber genes, thus the mutation may occurred between phage DNAs. Therefore, further experiments for specific deletion of each tail fiber gene with addition of appropriated template will be needed to obtain single gene deletion for better understanding the specific roles of each tail fiber gene in host

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range determination.
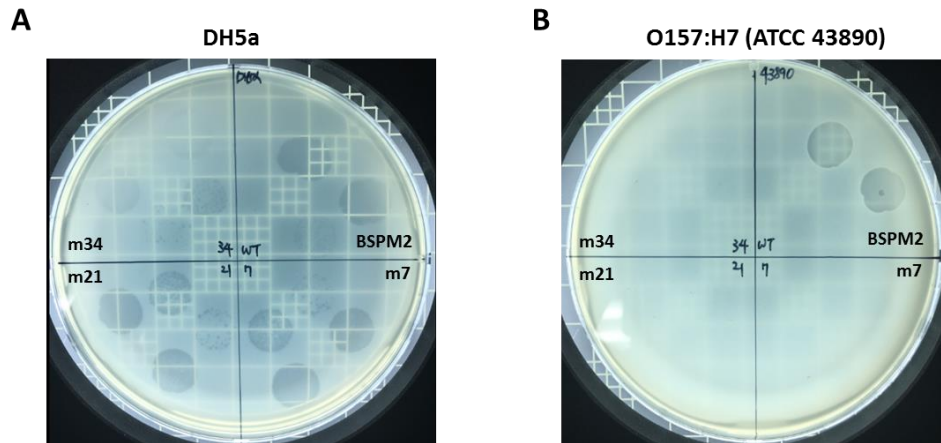
Fig. 2.15. Host range confirmation. Spotting assay results of phage BSPM2 and its derivative mutants on (A) *E. coli* DH5α and (B) *E. coli* O157:H7 ATCC 43890 strains, respectively. Formation of single plaques was determined by spotting the serially diluted phages.
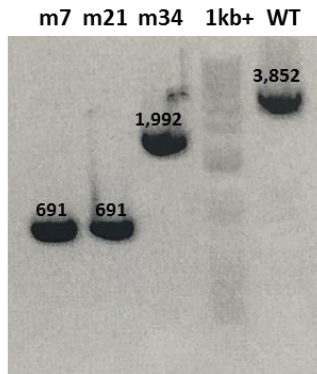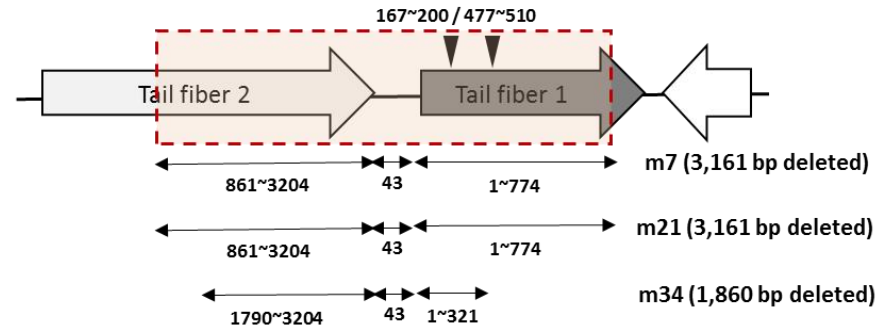
Fig. 2.16. Confirmation of mutated region in BSPM2 phage tail fiber genes. Deleted regions of the phage BSPM2 derivative mutants were confirmed by PCR using a BSPM2 tail fiber-specific primer set (A) and the nucleotide sequences were confirmed by sequencing (B). The orientation of tail fiber genes was reversed for better visualization. A red box showed the truncated regions in tail fiber genes and the arrows indicated the specific nucleotide length in truncated regions. Note that both phage m7 and m21 contained the same mutated regions.

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Chapter III.

Enhanced inhibition of *Salmonella* Typhimurium by a phage cocktail targeting different host receptors

III-1. Introduction

Salmonella is a leading bacteria cause of foodborne infection. It has been estimated that *Salmonella* is responsible for more than 90 million cases of gastroenteritis every year and 155,000 deaths worldwide (1). Most *Salmonella* infections are self-limiting in healthy people but may cause serious patient outcomes, even death, in young and older individuals and immunocompromised patients (2). Fresh produce is considered as the major food implicated in foodborne outbreaks mainly ascribing to the increased consumption of fresh vegetables for their convenience and nutritional aspects. According to a report from Centers for Disease Control and Prevention (CDC) indicated that 46% of foodborne illnesses in the US are caused by fresh produce (3). Among diverse food-borne pathogens, *Salmonella* is one of the most concerning pathogen associated with fresh produce because of its strong persistence and survival in the environment (4, 5). Recently, multistate *Salmonella* outbreak occurred in the US by contaminated cucumber in 2015, resulting in 204 hospitalizations and six deaths (6). Therefore, it is urgent to control *Salmonella* in fresh produce for food safety and public health. Unlike meat products that are cooked at high temperatures, thermal treatment is limited for fresh produce; thus, disinfectants [e.g., chlorine, chlorine dioxide (ClO_2), ozone (O_3), and peracetic acid (PAA)] are widely used for fresh produce production by the industry (7, 8). However, the generation of hazardous by-products, such as trihalomethanes (THMs), haloacetic acids (HAAs), chlorite, chlorate and bromate, are concerns to consumers, and the residual activity of those chemicals needs to be monitored during application (9). With the increasing food safety concerns about fresh produce and limitations of current disinfectant treatments, the

development of novel and safe methods is urgently required to control the contamination of fresh produce (10).

Bacteriophages (phages) are self-replicating virus particles with bacterial lysis activities, indicating that they can infect and lyse the specific host bacteria without additional treatment (11, 12). Phages specifically infect bacteria and are known to have no harm to human cells; this supports the safety of phage treatment (13). Moreover, phage does not affect the properties of food products, such as flavor, color and taste (14), making phages an attractive alternative biocontrol agent in foods. In 2006, ListShield (Intralytix, Inc., Baltimore, MD, USA), a cocktail of six phages targeting *Listeria monocytogenes*, was first approved by the US Food and Drug Administration (FDA) for application in foods in 2006 (15). In addition, SalmoFresh (Intralytix, Inc., Baltimore, MD, USA), a product comprised of six bacterial monophage specific to *Salmonella* enterica (monophage cocktail) got GRAS status in 2013. Therefore, phages have been an interesting subject for biocontrol of food-borne pathogens including *Salmonella* (16).

Despite the advantages of phage as a biocontrol agent, the major hurdle is the emergence of phage-insensitive bacteria strains (17). Since phage initiates infection by adhering to and penetrating into the cell, cell surface receptors are important for phage infection (18); thus, bacteria develop resistance to phage infection usually by modifying surface receptors (19). Up to date, various surface structures have been identified as phage receptors for *Salmonella* infection, including OmpC, FhuA, BtuB, lipopolisaccharides (LPS), core-oligosaccharide (core-OS), and

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flagella (20). Some resistance mechanisms of receptor modifications have been reported in *Salmonella*. For example, *S*. Typhimurium can develop transient resistance against BtuB-targeting phages by O-antigen glucosylation (21). In addition, another study showed that *Salmonella* has developed the O-antigen deficient mutant against the O-antigen-targeting phage (20). Since O-antigen deficient mutant against the O-antigen-targeting phage become sensitive to core-OS-targeting phage infection, co-treatment of a core OS-targeting phage (rough type specific) with an O-antigen-targeting phage suggested to be a good strategy to control *S*. Typhimurium (20). Like this, phage cocktails have been tested to overcome the resistance problem (22). Even though many reports are available for the development of phage cocktails, detailed information about mechanisms for phage infection and genomic features of phages in cocktails have not yet been sufficiently investigated (23-26). Most phage cocktails have thus far been prepared by combining phages that are selected by host ranges, lytic efficiency, and morphological features without mechanistic explanation about phage infection. Recently, a phage cocktail comprised of three virulent phages, which target LPS O-antigen, outer membrane porin OmpU, and an unknown receptor, has been suggested as a promising strategy to prevent *Vibrio* infection in animal models (27). In this study, I hypothesized that the simultaneous treatment of phages targeting different receptors will generate a burden to resistance development in *S*. Typhimurium. Three different phages targeting different receptors of *S*. Typhimurium were isolated from various sources to construct a phage cocktail targeting multiple receptors. Complete

genome sequencing and comparative genomic analysis were performed to understand their host interaction mechanisms and to ensure the safety at the genome level. I observed that the antimicrobial activity was significantly enhanced and reduction of BIMs frequency was achieved by using three phages targeting different host receptors. Furthermore, the antimicrobial effect of the phage cocktail was also tested on iceberg lettuce and cucumber spiked with *S.* Typhimurium. This study provides a receptor-based strategy to develop a novel biocontrol agent against *S.* Typhimurium in fresh produce.

III-2. Materials and Methods

III-2-1. Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. All of these strains were aerobically grown in Luria-Bertani (LB) broth medium (Difco, Detroit, MI, USA) at 37°C or at 30°C in some cases. The LB agar plate was prepared with LB broth supplemented with 1.5% Bacto agar (Difco) and soft top agar containing LB broth was prepared with 0.4% agar for phage plaque confirmation. For phage isolation, prophage-cured *S.* Typhimurium LT2 strain (designated as LT2C) and its derivative mutants were used as host in order to exclude the prophage induction.

Table 3.1. The bacterial strains used in chapter III

Strains [Description]	Refererences[a]	Strains	Refererences[a]
Samonella enterica serovar Typhimurium		*Salmonella* **Typhimurium LT2C mutants**	
SL1344	NTCT	*ΔrfaC*	(20)
UK1	(28)	*ΔbtuB*	(21)
LT2	(29)	*ΔrfbP*	(21)
LT2C [prophage-cured LT2 strain]	(30)	*ΔflgK*	Park (2014)
ATCC14028	ATCC	*ΔbtuBrfbP*	(21)
ATCC19586	ATCC	*ΔflgKrfbP*	(20)
ATCC43147	ATCC	*ΔflgKbtuB*	This study
ATCC13076	ATCC	*ΔbtuBrfbPflgK*	Park (2014)
DT104			
Samonella enterica serovar Typhi Ty 2-b	IVI		
*Samonella enterica*serovar Parathyphi			
A IB 211	IVI	*Escherichia coli* **O157:H7**	
B IB 231	IVI	ATCC35150	ATCC
C IB 2973	IVI	ATCC43890	ATCC
serovar Dublin IB 2973	IVI	ATCC43894	ATCC
Escherichia coli		ATCC43895	ATCC
MG1655	Wild type	O157:NM 3204-92	IVI
DH5a	Invitrogen	O157:NM H-0482	IVI

Table 3.1. The bacterial strains and plasmids used in chapter III (Continued)

Strains [Description]	References[a]	Strains	References[a]
Gram-negative bacteria		**Gram-positive bacteria**	
Shigella flexineri 2a strain 2457T	IVI	*Listeria monocytogenes* ATCC 19114	ATCC
Shigella boydii IB 2474	IVI	*Staphylococcus aureus* ATCC 29213	ATCC
Vibrio fischeri ES-114 ATCC 700601	IVI	*Staphylococcus epidermis* ATCC 35983	ATCC
Pseudomonas aeruginosa ATCC 27853	ATCC	*Bacillus subtilis* ATCC 23857	ATCC
Cronobacter Sakazakii ATCC29544	ATCC	*Bacillus cereus* ATCC 14579	ATCC

Table 3.1. The bacterial strains and plasmids used in chapter III (continued)

Plasmid [Description]	References[a]	Plasmid [Description]	Refererences[a]
Plasmid		**Plasmids for complementation**	
pKD46	(31)	pbtuB	(32)
[P$_{BAD}$-*gam-beta-exo oriR101 repA101^{ts}*; Amp^R]		[pACYC184-btuB; Cm^R]	
pKD13	(31)	prfbP	Kim (2010)
[R6Kγ *ori*, Amp^R-FRT, and Km^R-FRT]		[pUHE21-rfbP; Amp^R]	
pCP20	(31)	pflgK	This study
[pSC101 (Ts) *ori*, Amp^R, Cm^R, *cI857*, and λP$_R$*flp*]		[pUHE21-flgK; Amp^R]	
pACYC184	(33)		
[*repp15A* Cm^R Tet^R]			
pUHE21-2 lacI^q	(34)		
[*reppMB1 lacI^q* Amp^R]			

[a]NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; IVI, International Vaccine Institute.

III-2-2. Bacteriophage isolation, purification and propagation.

S. Typhimurium-targeting bacteriophages were isolated from environmental samples collected from farm soil, pond water, and swine intestine. All samples were used for bacteriophage isolation as described previously with slight modifications (35). Briefly, 25 g of each sample was mixed with 225 mL of sterile Butterfield's phosphate-buffered dilution water (0.25 M KH_2PO_4 adjusted to pH 7.2 with NaOH) in sterile bags and then homogenized for 90 s using a BagMixer 400 blender (Interscience Laboratory Inc., St. Nom, France). Twenty-five milliliter of each homogenized sample was diluted with the same volume of 2X fresh LB broth and incubated at 37°C for overnight with shaking (220 rpm). After incubation, the samples were centrifuged at 10,000 x g for 10 min and filtrated using 0.22 μm pore size filters (Millipore, Billerica, MA) to remove residual bacteria. Ten milliliters of each supernatant was added to 40 mL LB broth containing the overnight culture of the host strains (1%, final concentration) and then incubated. The culture was centrifuged at $10,000 \times g$ for 10 min and the supernatant was filtrated. For the confirmation of the presence of bacteriophages, each supernatant was serially diluted by 10-fold and spotted on molten 0.4% LB soft agar containing 1 % *Salmonella* strain (final concentration). After dried for 15 min, agar plates were incubated at 37 °C for overnight and plaque formation was observed.

For phage propagation, the lysate of a single phage was added at a multiplicity of infection (MOI) of 1 to the culture of desired host *Salmonella* strain (2 h, $OD_{600} = 0.5$) and the mixture was incubated at 37°C for 3 h. Phage lysates were

obtained by centrifugation and filtration as described above. The phage propagation

steps were serially performed with three different culture volumes (3, 50, and 200

mL culture) to obtain enough volume of phage lysate. To prepare a high-titer phage

stock, the phage particles were precipitated using polyethylene glycol (PEG) 6000

(Junsei) in 1M sodium chloride (final concentration) at 4°C for 12h. After

centrifugation (10,000 × g, 20 min, 4°C), precipitated phage particles were

suspended in 1 mL of SM buffer and separated by different CsCl density gradient

(step density = 1.3, 1.45, 1.5 and 1.7 g/mL) ultracentrifugation (himac CP 100β,

Hitachi, Japan) at 78,500 × g, 4°C for 2 h. Finally, only the phage band fraction was

collected and dialyzed two times (1 h each) in 1 Liter of standard dialysis buffer (5

M NaCl, 1 M MgCl$_2$ and 1 M Tris·HCl at pH 8.0) using Spectra/Por dialysis

membrane (molecular weight cutoff, 12,000 to 14,000; Spectrum Laboratories, Inc.).

Concentrated phage stocks were stored in a glass vial at 4°C after phage titer

measurement.

III-2-3. Receptor analysis and complementation

The LT2C derived mutant strains used for phage receptor analysis were

listed in Table S2. Briefly, an overnight culture of various LT2C mutants was mixed

with 0.4% molten LB soft agar to make the bacterial lawn as described above. Each

phage diluents were spotted onto the bacterial lawns and incubated. After incubation,

the formation of the single plaque was examined to determine each phage receptor.

To confirm the phage receptor, complement strains for *rfbP, btuB,* and *flgK* genes

were further subjected to spotting assay. Primers used for mutant construction and gene complementation were listed in Table 3.

Table 3.2. Primers used in chapter III

Primer	Nucleotide sequences [5`→ 3`][a]	Restriction sites
btuB-RED-F-LT2	ATG ATT AAA AAA GCT ACG CTG CTG ACG GCG TTC TCC GTC ATG TAG GCT GGA GCT GCT TCG	-
btuB-RED-R-LT2	TAA TGG CGT ATC GGT AAT CGC ATT ACG CGC ATC AAC GTA A AT TCC GGG GAT CCG TCG ACC	-
btuB-Di-F	CAT CAT CGC GTA CTA TCG AT	-
btuB-Di-R	GAT GTG AGG TGA CCG GAT AT	-
pUHE21-flgK-BamHI-F	GAG TAT TGA A<u>GG ATC C</u>AA AGG AAC CAT CAT	BamHI
pUHE21-flgK-HindIII-R	GGG TAC TGA T<u>AA GCT T</u>GT CAT CCT TCT CCT	HindIII
pUHE21-di-F1	AGA TTC AAT TGT GAG CGG ATA AC	-
pUHE21-di-R1	GGT CAT TAC TGG ATC TAT CAA CA	-

[a]Restriction sites are underlined

III-2-4. Transmission electron microscopy (TEM) analysis

Morphological analysis of the phages was performed with transmission electron microscopy (TEM). Each phage stock diluent (approximately 10^9 to 10^{10} PFU/mL) was placed on a carbon-coated copper grid and negatively stained with aqueous 2% uranyl-acetate (pH 4.0) for 30 s. Phage sample was observed by an energy-filtering transmission electron microscope (LIBRA 120, Carl Zeiss) at an operating voltage of 80 kV at the National Academy of Agricultural Science (Suwon, South Korea). Morphology of phages was identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses (36).

III-2-5. Extraction of bacteriophage genomic DNA

Bacteriophage genomic DNA was purified from concentrated phage high titer stock (about 10^9 to 10^{10}) as previously described (37). Prior to extract the phage DNA, phage lysate was treated with DNase and RNaseA at 37°C for 30 min in order to remove non-phage DNA or RNA contaminants. After then, 20 mM of EDTA (pH 8.0), 50 μg/mL of proteinase K and 0.5% of sodium dodecyl sulfate (SDS) were added and the phage lysate was incubated for 15 min at 65°C. Finally, standard phenol-chloroform DNA purification with ethanol precipitation was carried out to get purified phage genomic DNA.

III-2-6. Genome sequencing and bioinformatics analysis

The extracted phage genomic DNAs were subjected to be sequenced by a Genome Sequencer 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) were performed using the combination of FgenesB (Softberry, Inc., Mount Kisco NY), Glimmer v3.02 (38) and GeneMarkS (39) software. The predicted ORFs were annotated based on the results of the InterProScan (40) and BLASTP (41) analysis. For the comparative analysis of the receptor-related genes, genomes of phages with known receptor were used (Table 2).

III-2-7. Bacteriophage host range determination

Bacterial strains used for phage host range analysis were listed in Table 3.1 Each strain was incubated overnight at 37°C with shaking and then a 100 μL of each bacterial culture was added to 5 mL of the 0.4% molten LB soft agar and mixed. Then the mixture was overlaid on the 1.5% LB agar plate and dried for 20 min at room temperature. Subsequently, serially 10-fold diluted phage lysates were spotted onto the prepared bacterial lawns and incubated at 37°C for 12 h. After incubation, formation of a single plaque was observed to determine the sensitivity of each bacterium. The efficiency of phage infectivity (E.O.P) was determined against each strains in comparison to the E.O.P of the host strain LT2C.

III-2-8. Bacterial challenge assay

Fifty milliliters of LB broth was sub-inoculated with appropriate *S*. Typhimurium strains (1%, final concentration) and this culture was incubated at 37°C with shaking (220 rpm) until it reached the early exponential phase (OD_{600} = 0.3~0.4). At that time, the culture was infected with each phage or cocktail of three phages at an MOI of 1 (approximately 10^8 PFU). Only SM buffer added into the bacterial culture was used as a negative control. After phage infection, bacterial samples were collected every 1 h and OD_{600} was measured. Bacterial samples were collected at indicated time points and plated on LB agar medium to count viable cell numbers. Samples were diluted by 10-folds if necessary. All experiments were performed in triplicate.

III-2-9. Motility assay

The motility of the obtained BIMs after the three phage cocktail treatment was examined. Briefly, one microliter of the overnight culture of resistant strains was inoculated in the 0.3% LB soft agar medium. The plate was dried at room temperature for 20 min and incubated at 37 °C for 5 h. *S*. Typhimurium LT2C was used as a positive control.

III-2-10. The frequency of bacteriophage-insensitive mutants

The frequency of bacteriophage-insensitive mutants (BIMs) emergence was determined as previously described (42). Briefly, an appropriate volume of an overnight culture of *S*. Typhimurium LT2C (10^8 CFU) was mixed with each single

phage, with two phages cocktail or with a cocktail of three phages at an MOI of 10. After incubating for 10 min at 37°C, the mixtures were serially diluted in sterile Butterfield's phosphate-buffered dilution water and plated on LB agar and incubated at 37°C for 12 h. BIMs frequency was calculated by dividing the number of surviving cells by the initial cell numbers. All experiments were performed in triplicated. The obtained BIMs against each single phage were sub-cultured and subjected to susceptibility test with other two phages.

III-2-11. Food application

Fresh iceberg lettuce and cucumber were purchased from the local market and stored at 4°C before use. For the bacterial strain preparation, the host strain was incubated in LB broth at 37 °C with shaking (220 rpm) until the OD_{600} value was reached to 0.3 (~ 1×10^8 CFU/mL). One-milliliter of cell culture was centrifuged at 13000 x g for 2 min to remove LB broth and then resuspended in 1 mL of sterile Butterfield's phosphate-buffered dilution water. The prepared cell was serially (10-fold) diluted and used for the artificial contamination of food samples as previously described (43). Briefly, inner leaves of iceberg lettuce (*Lactuca sativa*) were cut and sliced into the size 2×5 cm (approximately 10 cm^2) after removing the outer leaves. Cucumber (*Cucumis sativus*) was sliced using slicer into the average diameter of 3.6 cm (approximately 10 cm^2) after the surface was washed with 70% ethanol and dried. Both sides of sliced food samples were UV-treated for at least 1 h in the bio-safety cabinet and then 100 μL of prepared *Salmonella* inoculum (~ 1×10^6 CFU/ml) was

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spotted onto each food sample. After 1 h of drying at room temperature in a bio-safety cabinet, each sample was treated by phage cocktail at different MOI values (10^3 to 10^4) and incubated at room temperature (25°C). The same volume of SM buffer was treated for the non-phage treated control group. At the indicated time point, each treated samples were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 90 mL of buffered peptone water (BPW) and homogenized for 2 min with a BagMixer 400 Laboratory Blender (Bagmixer, Interscience). After homogenization, serially diluted or 1 mL of undiluted sample was spread-plated using the traditional overlay method (pouring the Xylose Lysine Desoxycholate agar on top of resuscitated cells on LB agar 3-4 h after incubation) as previously described (44). Time points when low cell levels were expected, 5 mL of undiluted samples were equally distributed into five plates of each medium and plate counting as described previously (45). The absence of other bacterial contamination in non-inoculated samples was confirmed by the same method with the experimental group. All of the tests were conducted in duplicate.

III-3. Results and discussion

III-3-1. Isolation of *S*. Typhimurium-infecting phages with different host receptors.

As a prerequisite to constructing a phage cocktail, *S*. Typhimurium-infecting phages targeting different receptors were isolated from various environmental samples. The selected three phages BSPM4, BSP101, and BSP22A were isolated from the swine intestine, soil and pond water, respectively. According to the receptor screening results, the receptors of phages BSPM4, BSP101, and BSP22A were expected to be flagella, O-antigen, and BtuB, respectively (Table 3.3). Subsequent complementation experiments of three genes restored the sensitivity to each phage (Fig. 3.1), confirming that flagella, O-antigen, and BtuB are the host receptor of phage BSPM4, BSP101, and BSP22A, respectively.

Table 3.3. Receptor screening of newly isolated phages

Strains	BSPM4	BSP101	BSP22A
LT2C	C	C	C
ΔbtuB	C	C	-
ΔrfbP	C	-	C
ΔflgK	-	C	C
ΔbtuBrfbP	C	-	-
ΔrfbPflgK	-	-	C
ΔbtuBflgK	-	C	-
ΔbtuBrfbPflgK	-	-	-
Receptors expected	**Flagella**	**O-antigen**	**BtuB**

C, clear plaque; -, no infection
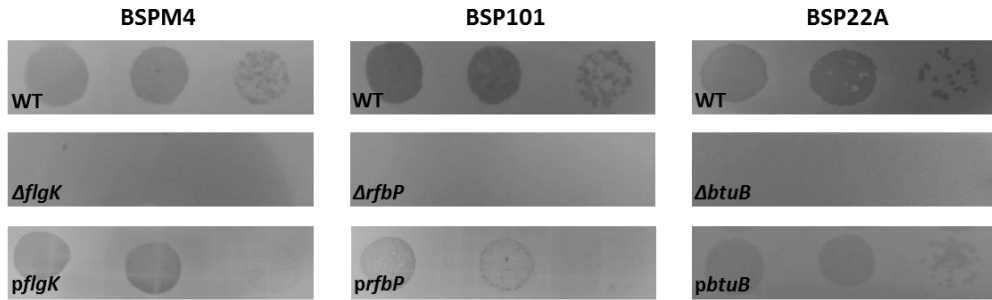
Figure 3.1. Identification of S. Typhimurium host receptor. Receptor gene complementation of the *flgK* gene for BSPM4, *rfbP* gene for BSP101, and *btuB* gene for BSP22A, respectively. Ten-fold serially diluted phage samples were dotted in each chamber.

III-3-2. Morphological analysis

The TEM analysis of phages revealed that BSPM4 and BSP22A belonged to *Siphoviridae* family (Fig. 3.2A and 3.2B). BSPM4 consisted of a long non-contracted tail of 229 ± 5 nm length (N=3) with coiled tail fiber, and its head size was 71 ± 2 nm (N=3). BSP22A has a non-contracted tail (179 ± 5 nm, N=3) without distinct tail fiber structure and an icosahedral capsid (95 ± 2 nm, N=3). In general, the size of phage capsid corresponds to its genome size (46). Therefore, it could be predicted that phage BSP22A will contain a larger genome size than phage BSPM4. Meanwhile, BSP101 had a contracted tail (123 ± 5 nm, n=3) with short tail spikes and a biggest head (97 ± 4 nm, N=3), which is a typical morphology of *Myoviridae* family phage (Fig. 3.2C) (47). TEM analysis indicated that the three phages were morphologically different from each other, and the morphological differences suggest distinct features during infection to the host (Fig. 3.2).
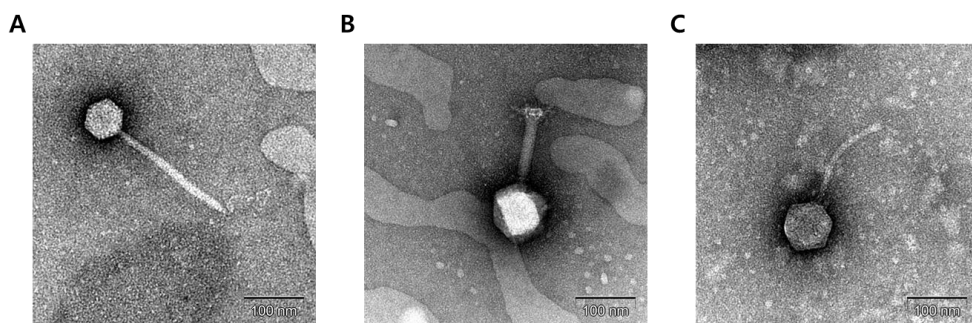
Figure 3.2. TEM image of phages isolated. (A) BSPM4, (B) BSP101, and (C) BSP22A, respectively.

III-3-3. Analysis of genome sequences of the BSPM4, BSP101, and BSP22A phages

Virulent phages are generally considered to be suitable for the development of phage-based biocontrol agent because they are unable to integrate into the host genome and unlikely to mediate horizontal gene transfer (48). In order to examine the genomic characteristics, whole genome sequencing was performed for the three phages; the Genbank accession numbers for phage BSPM4, BSP101, and BSP22 are KY620117, KY787213, and KY787212, respectively. Bioinformatics analysis revealed that phage BSPM4, BSP101, and BSP22A consisted of 78, 219 and 174 open reading frames (ORFs), respectively. In general, phages with clear plaques are regarded as virulent phages in many studies (25, 49, 50). Since all three phages formed clear plaques against the host strain (Table 3.3), they were expected to be virulent phages. Consistently, genes related to phage lysogen decision (integrases, excisionase or repressors) were not identified in all three phage genomes (Fig. 3.3), confirming that they are virulent phages (12). Moreover, genes related to virulence, toxin or drug resistance were not discovered in the genomes of the three phages (Fig. 3.3) (51).

The genome sizes of BSPM4, BSP101, and BSP22A were 59,097 bp, 157,665 bp and 110,741 bp, respectively, and their G+C% contents were 56.5%, 44.5%, and 40.01%, respectively (Fig. 3.3). Although both BSPM4 and BSP22A belong to the *Siphoviridae* family with a long flexible tail (Fig. 3.2A and B), the genome size of BSP22A is almost two fold bigger than that of BSPM4 (Fig. 3.3).

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The results of whole genome sequence analysis indicated BSPM4 and BSP22A are categorized into chi-like phage and T5-like phage, respectively. In addition, BSP101 has a large genome size (ca. 158 Kbp) (Fig. 3.3), which is consistent with most of the *Myovirus* phages with a large genome size usually >125 Kbp ,(52) supporting the TEM analysis result (Fig. 2B).

Genes associated with host recognition were identified in each phage genome, including a tail fiber protein for BSPM4, a tail spike protein for BSP101 and a receptor-binding protein for BSP22A, respectively (Table 3.4). Comparative genomic analysis revealed that those proteins showed high homology to the corresponding proteins in previously-reported phages (Table 3.4), but no homology were observed among the receptor recognition genes of the three phages. A tail fiber protein (ORF_31) of BSPM4 have 98-99% amino acid identity with hypothetical or tail fiber proteins of flagella-targeting phages, such as iEPS5, Chi, and SPN19, confirming that a tail fiber protein of the phage BSPM4 is important for recognizing the host flagella (Table 3.4). Among four tail-related genes in the BSP101 phage, an ORF_81 showed a high amino acid identity of 99% with the tail spike gene of O-antigen-targeting SFP10 that possesses a tail spike gene (ORF_162) responsible for O-antigen recognition (35). This supports that BSP101 recognizes O-antigen for infection. Surprisingly, the tail spike protein of BSP101 also showed a high (80%) amino acid homology with that of a *Siphoviridae* phage 9NA (Table 3.4). Since the tail spike proteins of *Myoviridae* phages (BSP101 and SFP10), a *Siphoviridae* phage (9NA) and a *Podoviridae* phage (P22) showed relatively high homologies ranging

from 36% to 99%, these tail spike proteins may be widely distributed among O-antigen-targeting phages (53) regardless of the phage morphologies. Moreover, all the four tail spike proteins commonly contained a phage P22 tail domain (pfam09251), supporting their functional involvement in O-antigen recognition (Table 3.4). In addition, phage BSP22A had a similar receptor-binding protein with amino acid homologies from 76 to 83% to other BtuB-targeting phages, supporting the receptor of BSP22A is BtuB as other related phages (Table 3.4). Therefore, comparative genome analysis revealed that the genes important for receptor recognition are highly conserved in the phages that utilize the same receptors.

Interestingly, genes related to superinfection immunity were identified in the genome of BSP101 (ORF_146, superinfection exclusion protein) and BSP22A (ORF_170, receptor blocking protein) while BSPM4 have no genes for superinfection immunity (Fig. 3.3). Usually, superinfection immunity occurred against the same or a closed related phage groups (19, 54). TEM analysis and whole genome sequencing results revealed that three phages belong to the different phage groups (Chi-like, T5-like, and Vi01-like family, respectively) and no nucleotide homologies were observed among the three phage genomes (Fig. 3.2 and Fig. 3.3). Therefore, the results suggested that the three phages are free from the superinfection exclusion by other phages. Taken together, genome analysis suggested that three different phages will be good candidates for the development of a phage cocktail.

A

Salmonella Typhimurium
Bacteriophage BSPM4
(59,097-bp)

primase
putative DNA polymerase
putative terminase small subunit
putative terminase large subunit
putative head-to-tail joining protein W
putative lambda family portal protein B
putative prohead protease ClpP
putative decorator protein D
major capsid protein E
putative assembly protein 2
putative assembly protein 1
tail fiber protein
putative lysis protein A (LysA)
Putative lysis protein B (LysB)
putative N-6-adenine-methyltransferase
putative recombination-associated protein RdgC

B

Salmonella Typhimurium
Bacteriophage BSP101
(157,665-bp)

Phage DNA ligase
DNA primase-helicase subunit
UvsX RecA-like recombination protein
dUTP diphosphatase
Thymidylate synthase
DNA end protector protein
Baseplate tail tube initiator
T4-like single-strand DNA-bindign protein
T4-like late promoter transcription accessory protein
Baseplate hub subunit
Baseplate hub subunit and tail associated lysozyme
Baseplate wedge protein
Glutaredoxin
NrdA/ NrdB ribonucleotide-diphosphate reductase
PhoH-like protein
Peptidoglycan bindign protein
DNA primase subunit
Recombination endonuclease subunit
Sigma factor for late transcription
Ribonuclease H
ATP-dependent helicase
DNA-bindign protein
Superinfection exclusion protein
RegA translational repressor protein
Clamp loader subunit
Clamp loader subunit ATPase
Sliding clamp DNA polymerase accessory protein
Helicase
DNA repair/recombination protein UvsY
Tail completion and sheath stabilizer protein
Prohead core protein
Prohead protease
Prohead core scaffold protein
Major capsid protein
Tail completion and sheath stabilizer protein
neck protein2
neck protein1
Tail sheath stabilizer
Terminase large/small subunit
Tail sheath protein
Tail tube protein
Portal protein
Head completion protein
Baseplate tail tube cap
Baseplate wedge subunit
Loader of T4-like helicase
Serine/ threonine phosphatase
DexA exonuclease
DNA topoisomease II medium subunit
DNA topoisomease II large subunit
Tail fiber protein
RIIB
RIIA
DNA polymerase
Baseplate wedge subunit
Tail spike protein
Tail fiber protein
Tail spike protein

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Figure 3.3. Genome map of phages. (A) BSPM4, (B) BSP101, and (C) BSP22A, respectively. The inner circle with the red line indicates the GC content. The outer circle indicates the predicted ORFs by strand. The functional categories and annotation of the ORFs are indicated by specific colors as follow. DNA packaging (purple), replication (orange), phage structure (green), host lysis (red), DNA repair/recombination (blue), nucleotide metabolism (yellow), host interaction (sky blue) and additional function (pink). Black-colored ORFs indicate hypothetical proteins. The scale units are base pairs.

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Table 3.4. Comparative analysis of genes related to the host receptor

ORF	Predicted function	Length[a]	% identity[b]	BLASTP matches	Receptor[c]	Morphology	Reference
BSPM4 (*Siphoviridae*)							
BSPM4_ORF31	Tail fiber protein	246	98% over 246 aa	Hypothetical protein (ORF_043) from *Salmonella* phage iEPS	Flagella	*Siphoviridae*	(55)
			98% over 246 aa	Tail fiber protein from *Salmonella* phage Chi	Flagella	*Siphoviridae*	(56)
			99% over 221 aa	hypothetical protein (ORF_043) from *Salmonella* phage SPN19	Flagella	*Siphoviridae*	(57)
BSP101 (*Myoviridae*)							
BSP101_ORF81	Tail spike protein	698	99% over 698 aa	Tail spike protein (ORF_0162) from *Salmonella* phage SFP10	O-antigen	*Myoviridae*	(35)
			80% over 619 aa	Tail spike protein (ORF_055) from *Salmonella* phage 9NA	O-antigen	*Siphoviridae*	(58)
			51% over 709 aa	Tail spike protein (ORF_708) from *Salmonella* phage Det7	O-antigen	*Myoviridae*	(53)
			36% over 551 aa	Tail spike protein (Gene 9) from *Salmonella* phage P22	O-antigen	*Podoviridae*	(59)
BSP22A (*Siphoviridae*)							
BSP22A_0169	Receptor-binding protein	593	83% over 595 aa	Receptor-binding protein (ORF_0143) from *Salmonella* phage SPC35	BtuB	*Siphoviridae*	(21)
			76% over 595 aa	Receptor-binding protein from *Escherichia* phage BF23	BtuB	*Siphoviridae*	(60)

[a]Number of amino acids; [b]Amino acid (aa) sequence identity; [c]Identified phage receptors.

III-3-4. Host range analysis

The host range was analyzed in BSPM4, BSP101, and BSP22A against *S.* Typhimurium strains, other Gram-negative and Gram-positive strains, and the results revealed that the three phages commonly infected all tested *S.* Typhimurium strains and formed clear plaques (Table 3.5). Additionally, the phage BSP101 infected *E. coli* O157:H7 strains and BSP22A infected other *Salmonella* serovars, including *S.* Typhi, *S.* Paratyphi, *S.* Dublin and *Shigella flexineri* as well (Table 3.5). These results indicate that the phage cocktail using BSPM4, BSP101, and BSP22A may inhibit pathogenic bacteria other than *Salmonella* such as *E. coli* O157:H7 and *S. flexineri*, which are important foodborne and waterborne pathogens. Most recently-reported phage cocktails targeting *Salmonella* show broad host ranges than single phages, but their infection range is only limited to within *Salmonella* subspecies (25, 61, 62).

Table 3.5. Host range analysis of three phages

Bacterial strain	BSPM4	BSP101	BSP22A
Samonella enterica			
serovar Typhimurium			
SL1344	++	+	+
UK1	+++	+	+++
LT2	+	+	+
LT2C	+	+	+
ATCC14028	+	+	+
ATCC19586	++	+	+
ATCC43147	+++	+	++
ATCC13076	+	+	+
DT104	+	+	+
serovar Typhi Ty 2-b	-	-	+
serovar Parathyphi			
A IB 211	-	+	+
B IB 231	-	+	+
serovar Dublin IB 2973	-	+	
Escherichia coli			
MG1655	-	-	+
Escherichia coli **O157:H7**			
ATCC35150	-	+	-
ATCC43890	-	+	-
ATCC43894	-	++	-
ATCC43895	-	++	-
O157:NM 3204-92	-	++	-
O157:NM H-0482	-	++	-
Gram-negative bacteria			
Shigella flexineri 2a strain 2457T	-	+	-
Shigella boydii IB 2474	-	-	-
Vibrio fischeri ES-114 ATCC 700601	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-
Cronobacter Sakazakii ATCC29544	-	-	-
Gram-positive bacteria			
Listeria monocytogenes ATCC 19114	-	-	-
Staphylococcus aureus ATCC 29213	-	-	-
Staphylococcus epidermis ATCC 35983	-	-	-
Bacillus subtilis ATCC 23857	-	-	-
Bacillus cereus ATCC 14579	-	-	-

*, +++, EOP of 2 to 1.5; ++, EOP of 1.5 to 1; +, EOP of 1 to 0.5; −, not susceptible to phages.

†, ATCC, American Type Culture Collection.

III-3-5. Bacterial challenge assay

The efficiency of the phage cocktail was evaluated using the bacterial challenge method *in vitro*. Whereas BSPM4 and BSP101 significantly inhibited the growth of *Salmonella* for a few hours after infection, BSP22A slightly reduced bacterial growth for several hours after infection (Fig. 3.4A). Combinations of two phages (i.e., BSPM4 & BSP101, BSPM4 & BSP22A, and BSP101 & BSP22A) inhibited *Salmonella* as comparably as the single treatment of BSPM4 and BSP101 (Fig. 3.4A). However, simultaneous treatment of the three phages efficiently lysed and inhibited *Salmonella* growth for 6 h (Fig. 3.4A) and significantly reduced the number of viable cells compared to the single and double-phage treatments after 12 h (Fig. 3.4B). These results were similar to a previous study in *Vibrio cholera*-targeting phages, where a phage cocktail treatment achieved lower cell density than single phage treatment groups after 12 h (27). Therefore, simultaneous treatment of three phages targeting different receptors showed a significant enhanced inhibitory effect on the host cell growth. The low cell number in the group treated with the three-phage cocktail may be because of the difficulties for *Salmonella* to develop resistance against the three phages targeting different receptors (63). Moreover, the loss of motility was observed in the bacteriophage-insensitive mutants (BIMs) that emerged after treatment with the three-phage cocktail (Fig. 3.4C). It would be a resistance mechanism to escape from the infection of flagella-targeting phage BSPM4, since it has been reported that most bacteria resistant to flagella-targeting phages tend to lose motility (64). Since motility is an important virulence factor in

Salmonella (65), bacteria resistant to the phage cocktail treatment would have a compromised virulence (66). These results suggest that simultaneous treatment of three phages with different receptors would be a promising strategy to control *S.* Typhimurium by reducing both bacterial number and virulence.

A

B

C

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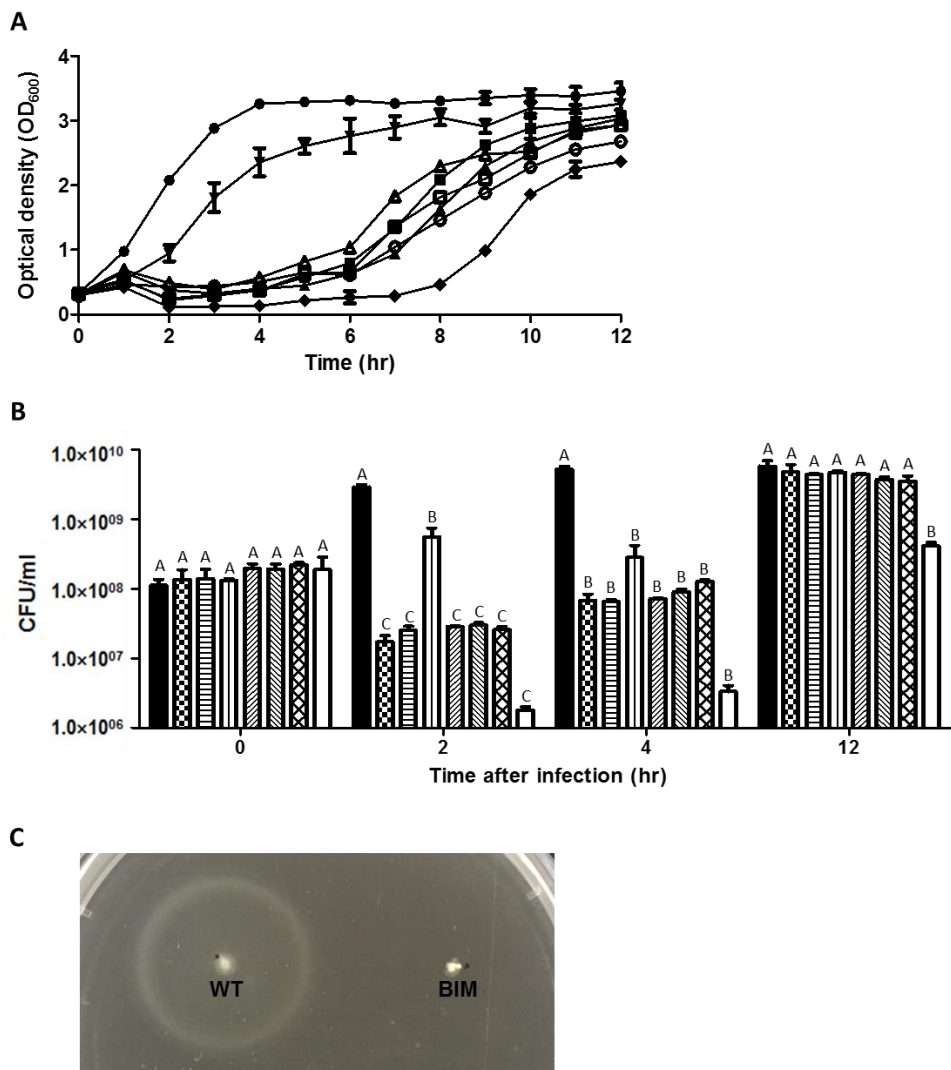
Figure 3.4 Bacterial challenge test of phage BSPM4, BSP101, BSP22A and cocktail of three phages with *S*. Typhimurium LT2C. (A) The graphs show OD values of samples after phage infection. Closed circles, non-phage-infected sample; closed squares, BSPM4-infected sample; closed triangles, BSP101-infected sample; reverse triangles, BSP22A-infected sample; open circles, BSPM4 and BSP101-infected sample; open squares, BSPM4 and BSP22A-infected sample; open triangles, BSP101 and BSP22A-infected sample and closed diamonds, three phage cocktail-infected sample. (B) The numbers of viable cells were determined at indicated time points; non-phage-infected sample (■), BSPM4-infected sample (▣), BSP101-infected sample (▤), BSP22A-infected sample (▥), BSPM4 and BSP101-infected sample (▨), BSPM4 and BSP22A-infected sample (▧), BSP101 and BSP22A-infected sample (▨), and three phage cocktail-infected sample (□), respectively. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. One-way analysis of variance (ANOVA) with Duncan's multiple range tests was used at each time point, respectively. The capital letters indicate significant differences (p value<0.05) between the test groups. (C) Motility of WT LT2C strain and BIMs obtained after the three phage cocktail treatment.

III-3-6. Frequencies of BIM development and phage susceptibility in BIMs

Since the three-phage cocktail extended growth inhibition in *Salmonella* and delayed the emergence of resistant strains, the effect of the three-phage cocktail treatment on the emergence of bacteriophage-insensitive mutants (BIMs) was measured (42). Interestingly, the frequencies of BIM occurrence were significantly decreased in two double-phage cocktails (i.e., BSPM4 & BSP101 and BSP101 & BSP22A) and the three-phage cocktail (Table 3.6). Commonly, the cocktails containing BSP101, which targets O-antigens, significantly inhibited resistance development of the host (Table 3.6). To date, cross-resistance between flagella-targeting phages and O-antigen targeting phages have not been discovered, and our result showed that simultaneous application of phages targeting of flagella and O-antigen efficiently reduced the development of resistant strains (Table 3.6). In addition, co-treatment of phages targeting BtuB and O-antigens by BSP22A and BSP101 phages also effectively reduced the emergence of resistant strains. It has been reported that variations in O-antigen biosynthesis or modifications are a mechanism conferring resistance to the infection of phages targeting O-antigen and BtuB (57, 67). Indeed, continuous O-antigen glucosylation of the host strain by the plasmid expressing the *gtrABC1* genes inhibited the infection of BSP22A (BtuB phage). However, the *gtrABC1* genes expressing strain remained susceptible to BSP101 (O-antigen phage) infection (data not shown), supporting the effectiveness of two different receptor-targeting phage treatment. Moreover, BIMs strains derived

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from phages BSP101 and BSP22A treatment, respectively, were susceptible to the subsequent infection of each other phage (BSP22A and BSP101, respectively), supporting the enhanced effect of the co-treatment of two phages in reducing the bacterial resistance development (Fig. 4). As mentioned above, another possibility is that *Salmonella* would develop the O-antigen deficient mutant other than the transient resistant mutant (20). However, this type of resistance is disadvantageous to the host, since O-antigen deficient mutant can be infected by BtuB-targeting phages including BSP22A (Table S1). Therefore, it can be speculated that development of bacterial resistance against both phages may affect to bacterial growth. Although the exact resistance mechanism remains unknown, our data showed that co-treatment of BSP101 and BSP22A showed enhanced reduction in the occurrence of BIMs of *S*. Typhimurium compared to each phage treatment.

In contrast, double-treatment of BSPM4 and BSP22A showed 1.7-fold increased effect in reducing the resistance development compared to BSP22A single treatment (Table 3.6). Therefore, it can be speculated that only additive effect of two phage mixture in reducing the bacterial resistance development was observed. This may because the resistance mechanisms by the host may independently developed against flagella-targeting phage and BtuB-targeting phage infections as observed previously (57).

Interestingly, the three phage cocktail treatment of this study showed a reduction in the development of BIMs from 3.20 fold to 49.5 fold, compared to the double-phage cocktail treatment (BSPM4 & BSP101) and the single phage (BSP22A)

treatment, respectively (Table 3.6). In contrast, significant differences in BIMs frequency between phage cocktails treatments and single phage treatments have not been observed in previous studies in which bacteriophage receptors were not identified (68, 69). These results indicated that the treatment of the three phages using different receptors in a cocktail may significantly reduce *Salmonella* development of resistance to phage infection.

Meanwhile, the BIMs that were obtained after each phage treatment showed susceptibility to the second infection by the other two phages (Fig. 3.5). These results suggested that phages can independently infect the host using different receptors regardless of the development of the bacterial resistance (70). Therefore, the results of the BIM-development testing clearly showed that simultaneous treatment of phages in a phage cocktail targeting different receptors was effective in reducing the development of bacterial resistance to phage infection. The findings suggested that the three-phage cocktail could be an effective biocontrol agent to control the occurrence of resistance to phage infection. In addition, studies of three different receptor phages treatment provides new insights of the enhanced effects of three phages when treated in combinations.

Table 3. Determinations of the frequencies of BIMs

Phage treatment	BIMs frequency (mean $\pm$ SD[a])	Fold-change
BSPM4	$1.63 \times 10^{-3} \pm 2.63 \times 10^{-4}$	8.25
BSP101	$1.11 \times 10^{-3} \pm 2.08 \times 10^{-4}$	5.64
BSP22A	$9.75 \times 10^{-3} \pm 6.73 \times 10^{-4}$	49.5
BSPM4+BSP101	$6.28 \times 10^{-4} \pm 3.95 \times 10^{-5}$	3.20
BSPM4+BSP22A	$5.92 \times 10^{-3} \pm 6.12 \times 10^{-4}$	30.0
BSP101+BSP22A	$7.40 \times 10^{-4} \pm 7.71 \times 10^{-5}$	3.80
BSPM4+BSP101+BSP22A	$1.97 \times 10^{-4} \pm 9.34 \times 10^{-5}$	1.00

[a]SD, standard deviation. Results were obtained from triplicate experiments.
[b]Fold-change values were represented in comparison to the three phage cocktail treatment result
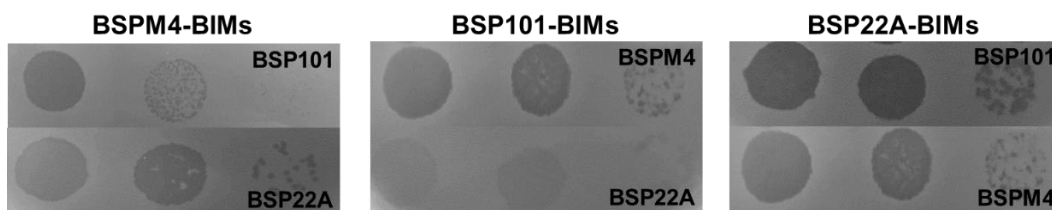
Figure 3.5. **Susceptibility of obtained BIMs to other receptor-targeting phages**.

Infection ability of three phages was confirmed against BIMs obtained after other

two phage treatments. All experiments were conducted in triplicate.

III-3-7. Efficacy validation of the three-phage cocktail in fresh produce spiked with *Salmonella*.

Along with the increasing demands for minimally processed fresh produces, *Salmonella* infection through fresh produces also have been increased annually (71, 72). Therefore, contamination of *Salmonella* in ready-to-eat vegetables is required to be controlled urgently (4). This motivated me to evaluate the efficiency of the phage cocktail treatment in the control of *Salmonella* on fresh produce, including iceberg lettuce and cucumber as model systems. Pieces of fresh produce was inoculated with *S*. Typhimurium LT2C. After 1 h, the three-phage cocktail was treated onto the contaminated fresh produce at an MOI of 10^3 or 10^4 for 12 h at room temperature. SM buffer without phages were pipetted onto the *Salmonella*-contaminated fresh produce as a control. As the results, when the phage cocktail was treated to the fresh produce samples spiked with *S*. Typhimurium LT2C at an MOI of 10^3, the number of bacterial strain was significantly reduced by 1.1 to 1.9 log CFU/cm^2 in iceberg lettuce (Fig. 3.6A) and reduced about 0.7 to 1.2 log CFU/cm^2 in cucumber (Fig. 3.6B) for 4 h at 25°C. When an MOI of 10^4 phages were treated, significantly reduction in the number of bacterial strain was achieved by 2.8 to 3.9 log CFU/cm^2 in iceberg lettuce (Fig. 3.6A) and about 2.5 to 2.8 log CFU/cm^2 in cucumber (Fig. 3.6B) in 4 h at 25°C. Previously, treatment of a cocktail of two phages targeting *S*. Enteritidis in Chinese cabbage showed 3.0 log CFU reduction in 5 h at 25°C (25). Experiment by another group demonstrated that a phage cocktail containing four lytic phages reduced *S*. Enteritidis populations by 2.5 log CFU on

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fresh-cut honeydew melons at room temperature (20°C) (73). Also, treatment of a six phage cocktail (SalmoFresh) to the *Salmonella* Newport contaminated cucumbers decreased bacterial populations to 1.83 log in a day during storage at 4°C (74). A cocktail of three phages used in this study achieved 3.9 and 2.8 log reduction of *S*. Typhimurium number in lettuce and cucumber in 4 h at 25°C, respectively, suggesting that this phage cocktail had strong killing efficiency against S. Typhimurium contaminated in fresh produces. In addition, the bacterial killing effect sustained for more than 12 h in both food samples resulting 5.5 to 5.8 log CFU reduction by treating the MOI of 10^4 phages. Therefore, the results indicate that the antimicrobial effect of the three phage cocktail treatment was maintained even in food samples for 12 h (Fig. 3.6). Therefore, these results suggest that three phages cocktail targeting different receptors would be a useful strategy for the development of novel natural biocontrol agents that can be used to improve the safety of fresh produces by reducing *S*. Typhimurium contamination.
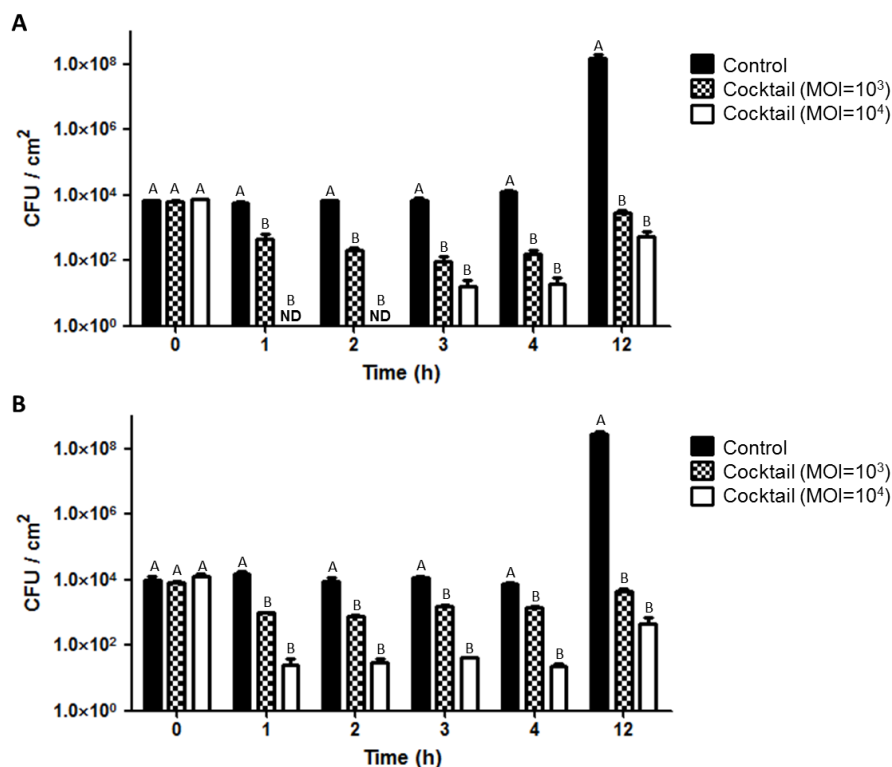
Figure 3.6. Food application of phage cocktail. Efficiency of a three phage cocktail was teste against *S.* Typhimurium in (A) iceberg lettuce and (B) cucumber, respectively. The numbers of viable cells were determined at indicated time points; non-phage-infected sample (■), cocktail-treated samples with an MOI of 10^3 (▣), cocktail-treated samples with an MOI of 10^4 (□), respectively. Detection limit of the experiment is 1.0 log CFU. The mean values from two independent measurements are plotted. One-way analysis of variance (ANOVA) followed by Duncan's multiple range tests was used at

each time point, respectively. The capital letters indicate the significant differences ($p < 0.05$) between the test groups. ND, not detected.

III-4. Conclusion

S. Typhimurium is one of the problematic food-borne pathogen causing frequent human outbreak all over the world. Therefore, this study was carried out to construct a novel phage cocktail using agent against *Salmonella*. For this purpose, three novel phages targeting the different receptors (i.e., flagella, O-antigen and BtuB) of *S*. Typhimurium were isolated and characterized. In addition, genomic study of three phages revealed that all three phages are strictly virulent without no toxin or virulence-related genes in their genomes, suggesting they are safe to be applied on foods (13). *In vitro* challenge assay and BIMs frequency analysis revealed the enhanced effect of three phages cocktail to inhibit the host growth and the occurrence of resistant strains, indicating the preparation of cocktail with phages utilizing different host targets is a powerful strategy to control *S*. Typhimurium. The strategy for phage cocktail preparation based on the receptor utilization in this study may be applied to the development of phage cocktails against other pathogens. Furthermore, the effective inhibition of *S*. Typhimurium by the phage cocktail in food systems showed that the phage cocktail has great potential of being developed as a biocontrol agent against *S*. Typhimurium in fresh produces.

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Chapter IV.

Characterization of a Novel Bacteriophage Lysis Protein and Its Possible Host Lysis Mechanism

IV-1. Introduction

Bacteriophages have adopted diverse cell lysis mechanisms to release their progeny after multiplication inside the host bacteria. Bacterial lysis by phages can be accomplished by at least two different ways. One is peptidoglycan degradation by a holin-endolysin system found in most double-stranded DNA phages and the other one is inhibition of peptidoglycan synthesis seen in single-stranded RNA or DNA phages (1-3).

In most double stranded DNA-containing phages, two proteins called endolysin (peptidoglycan hydrolase) and holin are involved in lytic function. Endolysins encoded by phages have a peptidoglycan hydrolase function. During the late stage of the phage lytic cycle, endolysins are expressed and accumulated in the cytoplasm. In general, a holin protein forms holes in the cytoplasmic inner membrane at a determined time, allowing the endolysin to access to the peptidoglycan substrates. Therefore, rapid cell lysis occurs by osmotic pressure caused by peptidoglycan degradation (4). In contrast, some endolysins have holin-independent export mechanism. Those endolysins contain signal sequences or N-terminal hydrophobic regions working as a signal-arrest-release (SAR) domain which allows them to access to the periplasmic region without help of holin proteins. In these systems, endolysins are translocated by *Sec* translocase and anchored to the cytoplasmic membrane until holins dissipate the membrane proton motive force (5-9).

A putative lysis protein named M4LysA was identified from a double-

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stranded DNA-containing bacteriophage BSPM4. M4LysA protein showed no sequence homology with previously identified catalytic domains from Gram-negative bacteria-targeting phages. When M4LysA was expressed alone in *E. coli* system it caused holin-independent cell lysis which resembled the holin-independent endolysins such as P1 endolysin and Ms6 endolysin (5, 8). Interestingly, M4LysA sequence is highly conserved in flagella-targeting phages, suggesting those phages may share the similar cell lysis system which may be distinct from previously reported endolysins. However, experimental evidences about the M4LysA function in the host lysis are still remained unknown. Therefore, in this study, the function of M4LysA was newly investigated in order to understand the cell lysis mechanism of the BSPM4 phage.

Although conserved catalytic domains were not predicted in the M4LysA sequence, turbidity reduction assay showed that a single 'virion protein (PHA02564)' domain in N-terminal region showed petidoglycan lytic activity. Moreover, the specific enzymatic activity of virion protein domain was confirmed to be an endopeptidase. In addition, M4LysA contained an unusual 'transmembrane domain (TMD)' at its C-terminal region and it was revealed to be important for M4LysA function. Moreover, inhibition of the *Sec*-translocase, a major protein secretion system, did not completely inhibit the cell lysis by M4LysA expression, suggesting that secretion of M4LysA may be different to other *Sec*-dependent endolysins (5, 9).

Meanwhile, deletion of the C-terminal TMD caused reduced lysis activity, suggesting the importance of the TMD in M4LysA protein function. Surprisingly,

co-expression of the M4LysA⊿TMD and C-terminal TMD showed the host cell lysis while separate expression of each region did not cause cell lysis. The results indicated that C-terminal TMD in M4LysA may function as a membrane permeabilizer which may enabled the lytic domain (M4LysA⊿TMD) reach to its peptidoglycan substrate, consequently resulted in cell lysis.

IV-2. Materials and methods

IV-2-1. Bacterial strains, media and growth conditions

The bacterial strains used in this study are listed in Table 4.1. Bacterial strains were grown in Luria-Bertani (LB) medium (Difco, Detroit, MI, USA) at 37°C with aeration. Competent *E. coli* DH5α and BL21 (DE3) strains were used for cloning and protein expression, respectively. Appropriate antibiotics (50 μg/ml of ampicillin or 50 μg/ml of kanamycin; final concentration) were used if necessary. When indicated, isopropyl-β-thiogalactopyranoside (IPTG), sodium azide (NaN$_3$) and sodium thiocyanate (NaSCN) were added at 0.1 mM, 1-10 mM, and 10-30 mM (final concentrations), respectively.

Table 4.1 Bacterial strains and plasmids in chapter IV

Strains	Description	Reference
Escherichia coli		
BL21 (DE3)	F− *ompT hsdSB* (r_B- m_B-) *gal dcm* (DE3)	(10)
Plasmids		
pET28a(+)	Expression vector with a hexahistidine tag, Kan^r	Novagen
pETDuet-1	Co-expression vector with a hexahistidine tag, Amp^r	Novagen
pM4LysA	pET28a-*lysA*	This study
pM4LysB	pETDuet-1-*lysB*	This study
pM4LysA_B	pETDuet-1-*lysA.lysB*	This study
pM4LysA*ΔTMD*	pETDuet-1-*lysA ΔTMD*$_{1-206}$	This study
pM4TMD	pETDuet-1-*TMD*$_{158-237}$	This study
pM4LysA*ΔTMD*_TMD	pETDuet-1- *lysA ΔTMD*$_{1-206}$.*TMD*$_{158-237}$	This study
pM4LysA$_{226}$	pET28a-*lysA*$_{1-226}$	This study
pM4LysA$_{225}$	pET28a-*lysA*$_{1-225}$	This study
pM4LysA$_{224}$	pET28a-*lysA*$_{1-224}$	This study
pP22hol	pETDuet-1-p22*holin*	This study
pP22lys	pETDuet-1-p22*lysin*	This study
pM4LysA*ΔTMD*_P22hol	pETDuet-1-*lysA ΔTMD*$_{1-206}$.p22*holin*	This study
pM4TMD_P22lys	pETDuet-1-*TMD*$_{158-237}$.p22*lysin*	This study

IV-2-2. Cloning and expression of the lysis proteins

Genes encoding two putative lysis proteins, named M4LysA and M4LysB, were predicted from the *S*. Typhimurium-targeting phage BSPM4 according to the complete genome sequence analysis results. The gene encoding a putative lysis protein LysA (BSPM4_ORF_38) was amplified by polymerase chain reaction (PCR) with the primer sets BSPM4lysA-F-NdeI and BSPM4lysA-R-XhoI using the phage BSPM4 DNA as a template. The PCR product was digested with desired restriction enzymes and cloned into the pETDuet-1 vector (Novagen, Madison, WI, USA) and sequences were confirmed. The recombinant plasmids were than transformed into *E. coli* BL21 (DE3) strain for expression test. Another putative lysis protein M4LysB (BSPM4_ORF_37)-coding gene was PCR amplified with the primer sets BSPM4lysB-F-NcoI and BSPM4lysB-R-HindIII and cloned into pETDuet-1 vector (Novagen, Madison, WI, USA) in the same way. For co-expression of M4LysA and M4LysB, both genes were cloned into the pETDuet-1 vector using primers above, respectively. Primers used for PCR amplification were listed in Table 4.2. To express the cloned M4LysA and M4LysB protein, 0.1 mM of isopropyl-β-thiogalactopyranoside (IPTG) was added 3 h after incubation and lysis kinetic of each strain was observed every 5 min as previously described (5). Cell lysis activity of M4LysA protein was further confirmed with addition of IPTG (0.0 to 1.0 mM). Non-recombinant plasmid containing *E. coli* BL21 (DE3) strain was used as a negative control.

Table 4.2 Primers used in chapter IV

Primer	Nucleotide sequences [5`→ 3`][a]	Restriction sites
BSPM4lysA-F-NdeI	ATG AGG AAA TAA <u>CAT ATG</u> GCT AAA CAG AAG	NdeI
BSPM4lysA-R-XhoI	GAA AAC GAG CGC <u>CTC GAG</u> GAC GCC CGT CTT	XhoI
BSPM4lysA-43-F-NdeI	AGC CGC TTC GCG CAG TTC AAA <u>CAT ATG</u> GCA TCC GGC ATC CGC GCC ATT	NdeI
BSPM4lysA2-R-XhoI	GGC CGC GCC GAA <u>CTC GAG</u> GCG TAC TCA ATC ACC	XhoI
BSPM4lysA5_3-R-XhoI	CTT CCG GTA <u>CTC GAG</u> TCA TCC CAT GTA CAG	XhoI
BSPM4lysA5_4-R-XhoI	CCG ATG CTT <u>CTC GAG</u> CGC TCA CCA TCC CAT	XhoI
BSPM4lysA6-R-XhoI	CCC GGC CCG ATG <u>CTC GAG</u> GTA CTA GAC CCA TCC	XhoI
BSPM4lysB-F-NcoI	CCC GCT AAT TTT TTG TGA GGA <u>CCA TGG</u> GC A TGA GCG AAA TGG AAC G	NcoI
BSPM4lysB-R-HindIII	GTT ATT GCG AAT CCC GCG <u>AAG CTT</u> CTG TTT AGC ATC GG	HindIII
pETDuet-M4TMD-NcoI-F	CGA GGA AGG TCT GC<u>C CAT GG</u>G GGG CAT CGT TAA G	NcoI
pETDuet-M4TMD-HindIII-R	AAA ACG AGC GCC GC<u>A AGC TT</u>G CCC GTC TTG ATC C	HindIII
pETDuet-P22holin-NcoI-F	ATC CTC ACG TTC GTG A<u>CC ATG G</u>AC ATG AAA AAG ATG CCA	NcoI
pETDuet-P22holin-HindIII-R	TCA CCT TCT TCA CGT TTT <u>AAG CTT</u> GTG ATT CCG TTA CTG	HindIII
pETDuet-P22lysin-NdeI-F	CTG CTA AAA AAG CCG GAG <u>CAT ATG</u> ATG CAA ATC AGC AGT	NdeI
pETDuet-P22lysin-XhoI-R	CAC GAA AGA CAG ACG ATG <u>CTC GAG</u> ATA ATG ACA GAC GCA	XhoI
Duet-UP1-F	GAT GCG TCC GGC GTA GAG G	-
Duet-DOWN1-R	CGA TTA TGC GGC CGT GTA CAA T	-
Duet-UP2-F	ATT GTA CAC GGC CGC ATA ATC G	-
T7-promoter-F	TAATACGACTCACTATAGGG	Universal
T7-terminator-R	GCT AGT TAT TGC TCA GCG GTG	Universal

IV-2-3. *In silico* analysis

Conserved domains of M4LysA and M4LysB proteins were analyzed using the BLASTP (11), Interproscan (12), and the NCBI Conserved Domain Database (CDD) (13) tools. The transmembrane topology was predicted by Phobius web server (14). Amino acid sequence alignments of the proteins were conducted with the ClustalX2 algorithm and edited using GeneDoc tool (15, 16). To determine the phylogenetic position of the M4LysA protein, phylogenetic trees were constructed based on the alignment of the amino acid sequences from 42 Gram-negative bacteria-infecting phage endolysins which are available in the NCBI nucleotide databases. Phylogenetic trees were created using the ClustalX2 program (15) and constructed with MEGA7 by the neighbor-joining method with *P*-distance values (17).

IV-2-4. Overexpression and purification of M4LysA⊿TMD protein

A soluble form of M4LysA protein lacking the C-terminal TMD was constructed, expressed, and designated as M4LysA⊿TMD. The M4LysA⊿TMD coding gene was cloned into pET28a vector which had N-terminal hexahistidine (His6)-tag sequences as described above. The recombinant plasmid containing *E. coli* BL21 (DE3) strain was incubated for 2 h until OD_{600} reached 0.5 and then supplemented with 0.5 mM IPTG (final concentration) to express the M4LysA⊿TMD protein. After incubation for 3 h, cells suspended in a lysis buffer (20 mM Tris-Cl, 200 mM NaCl, pH 8.0) were sonicated (Branson Ultrasonics, Danbury, CT) to break the bacterial cell wall. Sonicated cells were centrifuged at

15,000 x g for 20 min to obtain the supernatant containing soluble proteins. His-tagged M4LysA⊿TMD protein was purified using a Ni-nitrilotriacetic acid (NTA) Superflow column (Qiagen GmbH, Germany) according to the manufacturer's instructions. The purified protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stored in the storage buffer (20 mM Tris-Cl, 200 mM NaCl, 30% glycerol, pH 8.0) after buffer changing using a PD Miditrap G-25 (GE healthcare, Amersham, Bucks, UK) (18).

IV-2-5. Activity and the host range of M4LysA⊿TMD

The lytic activity of purified M4LysA⊿TMD protein was determined using the purified *E. coli* peptidoglycan as substrates. Crude peptidoglycan of *E. coli* BL21(DE3) was extracted as described previously with slight modifications (19). In brief, 400 mL of exponentially grown *E. coli* cell (OD_{578} = 0.6) was harvested and rapid cooling in ice. Cell was then centrifuged at 12,000 x g for 15 min and suspended in 3 mL of cold distilled water. Cells suspensions were slowly dropped into 8% boiling SDS with vigorous stirring to lyse the cells (in 10 min) and boiled for 30 min. After cooling at room temperature for overnight with slight stirring, the mixture was centrifuged at 130.000 x g at 25°C for 1 h. The crude cell wall was washed using cold distilled water 4 times and glycan was degraded using α-amylase (100 μg in 1 mL of 10 mM Tris-HCl buffer, pH 7.5) for 2 h at 37°C. Sample (1 mL) was mixed with 8% SDS (1 mL) and boiled for 15 min and washed for 3 times. The muropeptide was suspended in D.W. and stored in 4°C until use. Peptidoglycan lysis

activity of M4LysA⊿TMD were examined by measuring the OD_{600} reduction of the peptidoglycan solution. For the host range analysis, purified M4LysA⊿TMD was spotted on the lawn of each autoclaved cell and incubated at 37°C for 12 h and the turbidity of the plates were determined.

IV-2-6. Enzymatic activity confirmation

Enzymatic activity of purified M4Lys⊿TMD protein was confirmed using colorimetric assays. Endopeptidase activity was measured by quantification of free amino groups liberated from the peptidoglycan after the M4Lys⊿TMD treatment. Crude peptidoglycan of *E. coli* BL21(DE3) and pre-existing amino groups were blocked by acetylation (20). Then, free amino groups generated by cleavage of the cell wall by M4Lys⊿TMD were assayed by the TNBS method (21). Glycosidase assay and amidase assay was performed as described previously (20, 22).

IV-2-7. Functional analysis of M4LysA

Truncation of the C-terminal TMD of M4LysA protein was performed using various primer sets. Primers BSPM4lysA-F-NdeI and BSPM4lysA2-R-XhoI were used to generate C-terminal TMD truncated construct M4LysA-2. Another two primer sets were used to generate partially truncated TMD; BSPM4lysA-F-NdeI and BSPM4lysA5_3-R-XhoI for M4LysA$_{1-224}$ and BSPM4lysA-F-NdeI and BSPM4lysA6-R-XhoI for M4LysA$_{1-226}$, respectively. The functional analysis of N-terminal domain was examined by N-terminal truncation using primer sets of

BSPM4lysA_43-F-NdeI and BSPM4lysA-R-XhoI. All PCR amplified constructs were cloned into pET28a vector and used for activity test with IPTG induction. For the expression of M4LysA⊿TMD and C-terminal TMD region, each gene was PCR amplified and cloned into the pETDuet-1 co-expression vector and expressed using IPTG as described above. For the complementation test with other lysis system, lysin and holin genes from phage P22 were PCR amplified and cloned into the pETDuet-1 vector, respectively. Primers used for PCR amplification were listed in Table 4.2.

IV-3. Results and discussion

IV-3-1. Identification of putative lysis protein in BSPM4 phage

A phage BSPM4 was previously isolated and its genome was fully sequenced (NCBI accession number: KY620117). A lysis cassette of BSPM4 phage was comprised of two putative lysis proteins, M4LysA and M4LysB (Fig. 4.1A). Therefore, each gene was cloned into the vector and expressed in *E. coli* BL21 (DE3) strains respectively, in order to examine their role in host lysis. Interestingly, cell lysis was observed only when M4LysA was expressed, however, induction of M4LysB alone did not cause cell lysis (Fig. 4.1B). In addition, co-expression of M4LysB with M4LysA showed slight delay in lysis but, it did not inhibit cell lysis. The results indicated that the M4LysA may be related to host cell lysis.
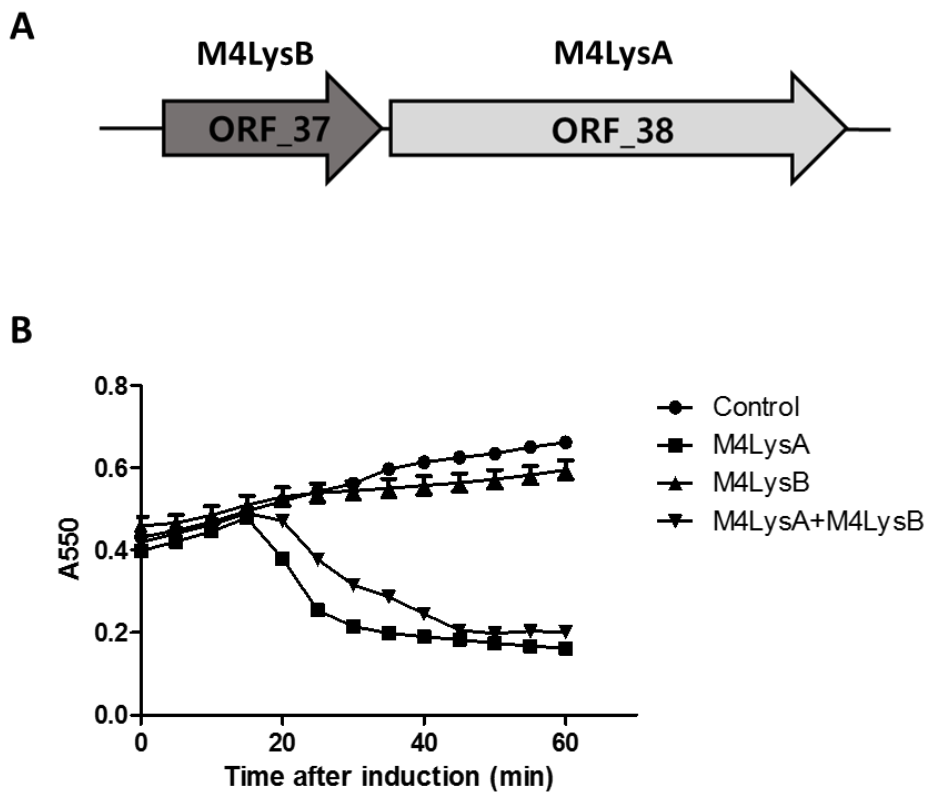
Figure 4.1. *In-silico* **prediction and lysis activity of putative lysis proteins of phage BSPM4.** (A) Schematic representation of the phage BSPM4 lysis cassette. (B) Growth kinetics of the strains expressing M4LysA and M4LysB proteins were monitored. Closed circles, empty vector; closed triangles, expression of M4LysB; closed squares, expression of M4LysA; reverse closed triangles, co-expression of M4LysA and M4LysB. Lysis activity of the putative lysis proteins were confirmed by 0.1 mM IPTG induction and the turbidities of the cells were observed.

IV-3-2. M4LysA sequence is conserved in Chi-like phages

Since M4lysA have a novel cell lysis mechanism distinct to other previously reported endolysins, comparative genomic analysis was performed in order to understand the genomic features of M4LysA. Phylogenetic analysis with 37 available endolysin sequences revealed that M4LysA was classified as a new type of lysis protein group with some phages including SPN19, iEPS5, FSLSP088, and Chi based on the domain composition (Fig. 4.2A). Subsequent BlastP homology search and amino acid sequence alignments revealed that M4LysA sequence was highly conserved among the Chi-like phages with amino acid identity from 97 to 99% (Fig. 4.2B). Since phage BSPM4 also utilizes flagella as a receptor like other phages above (23, 24), the result suggested that flagella-targeting phages may share the similar host lysis mechanism. Though M4LysA protein sequence is highly conserved in flagella-targeting phages, it is still unknown why this unique lysis system is utilized by those phages.

A

Group I
- BSPM4
- SPN19
- iEPS5
- FSLSP088
- Chi

Group II
- S394
- Stitch
- Eps7
- T5
- vB CsaP Ss1
- vB CfrM CfP1

Group III
- K11
- KP32
- T7
- T3

Group IV
- Fels-1
- SPN1S
- PVP-SE1
- vB AbaP CEB1
- Abp1
- ABP-01
- ABP-04

Group V
- phage 10
- Vil
- 0507-KN2-1
- ST201phi2-1

Group VI
- T4
- phi68
- P1
- ERA103
- SPN3UB
- phiKMV
- Fels-2
- vB EcoM-ep3
- P22
- Mu
- 22
- SPN9CC

0.10

B

Figure 4.2. Amino acid sequence alignments of M4LysA with other endolysins. (A) Phylogenetic analysis of the endolysins from Gram-negative bacteria-infecting phages. Each group was indicated according to the functional domains, Group I, V superfamily; Group II, D-alanyl-D-alanine-carboxypeptidase; Group III, *N*-acetyl muramoyl L-alanine amidase; Group IV, Glycoside hydrolase family 19; Group V, *N*-acetylmuramidase; Group VI, Glycoside hydrolase family 24, respectively. (B) Amino acid sequence alignments of the protein M4LysA with other putative lysis protein of relevant phages including SPN19, iEPS5, FSLSP088, and Chi. Conserved and identical residues are shaded in gray and black.

IV-3-3. Domain analysis of M4LysA and its lytic activity

Up to date, at least five different catalytic domains including *N*-acetylmuramidases (lysozymes), *N*-acetyl-*β*-D-glucosaminidases (glycosidases), *N*-acetylmuramoyl L-alanine amidases, L-alanoyl-D-glutamate endopeptidases, and interpeptide bridge-specific endopeptidases have been studied (25). Since M4LysA protein had strong lytic activity when induced inside the cells, domain analysis of this protein was performed. The conserved domain searched by NCBI Conserved Domain Database and interproscan database analysis revealed that it contained a provisional virion protein domain (V superfamily, PHA02564) with a distinct C-terminal TMD (Fig. 4.3A). Among 278 endolysins identified from Gram-negative bacteria-targeting phages, the majority of endolysins (93.9%) have globular structure with one catalytic domain. Although such domains were not predicted in M4LysA sequence, expression of the full-length of M4LysA protein caused rapid cell lysis (Fig. 4.1B). Therefore, it was expected that the N-terminal virion protein domain may function as a catalytic domain. Actually, when 43 amino acids from virion protein domain of the N-terminus of M4LysA were deleted, cell lysis activities did not show any more (data not shown), suggesting that the virion protein domain may be important for the enzymatic activity. Therefore, in order to examine the enzymatic activity of M4LysA, purification of M4LysA protein was performed. As the full-length protein was insoluble with the presence of the C-terminal TMD (data not shown), protein purification was carried out using the protein without the C-terminal TMD region (M4LysAΔTMD$_{1\text{-}206}$). The soluble form of M4LysAΔTMD$_{1\text{-}206}$ was

successfully purified by affinity chromatography and SDS-PAGE analysis revealed a single band approximately 24.5 kDa size (Fig. 4.3B), which was consistent with the predicted molecular mass of M4LysA⊿TMD$_{1-206}$ protein. As expected, when M4LysA⊿TMD$_{1-206}$ was treated to the purified aqueous peptidoglycan, it showed rapid reduction in optical density, indicating M4LysA⊿TMD$_{1-206}$ has peptidoglycan degradation activity (Fig. 4.3C). Therefore, these results showed that M4LysA caused the host cell lysis by degrading the peptidoglycan layer.

Generally, expression of the endolysins in *E. coli* system did not trigger cell lysis without a holin since they accumulate in the cytosol (26, 27). However, M4LysA caused cell lysis more quickly as IPTG concentrations increased, indicating that the cell lysis occurred M4LysA protein concentration-dependent manner (Fig. 4.3D). Therefore, these results suggested that the accumulation of M4LysA may trigger gradual release of the protein to the periplasm and degrades the peptidoglycan layer, resulting in the host cell lysis.

In addition, endopeptidase activity of purified M4LysA⊿TMD was confirmed by adding the trinitrobenzene sulfonic acid (TNBS) to detect the free amino groups from peptidoglycan after hydrolysis by M4LysA⊿TMD. A yellow trinitrophenyl derivative was measured at 340 nm after incubation with M4LysA⊿TMD and lysozyme, respectively (2 μg, for 1 h). Indeed, only M4LysA⊿TMD treated group generated strong yellow color indicating that M4LysA⊿TMD had an endopeptidase activity (Fig. 4.4A and B). Meanwhile, M4LysA⊿TMD did not show any *N*-acetylmuramoyl-L-alanine amidase (Fig. 4.4B)

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or glycosidase activity (Fig. 4.4C).

When the purified M4LysA$\varDelta$TMD$_{1\text{-}206}$ protein was treated to various bacterial cells, it showed a broad lytic spectrum among Gram-negative bacteria tested while the parental phage BSPM4 only could infect *S*. Typhimurium strains (Table 4.3). Usually, it has been reported that the antimicrobial spectrum of the endolysin is broader than that of the parental phage (25, 28-30). Therefore, M4LysA protein may be a novel phage-derived lysis protein which has broad host range in Gram-negative bacteria.

A

16   157   207   228 237

Virion protein (V superfamily)   TM helix

B

(kDa) M   F.T   W   P

95
70
55
43
34
26
17
6

M : Molecular weight markers
F.T : flow through
W : washing
P : purified M4LysAΔTMD

C

- Control
- 10 µg/mL
- 40 µg/ml

OD_{600}

Time (min)

D

0   10   50   100   200   500   1000   IPTG (µM)
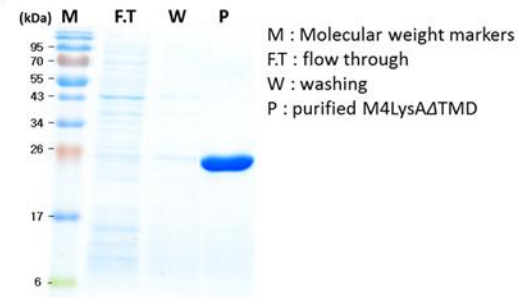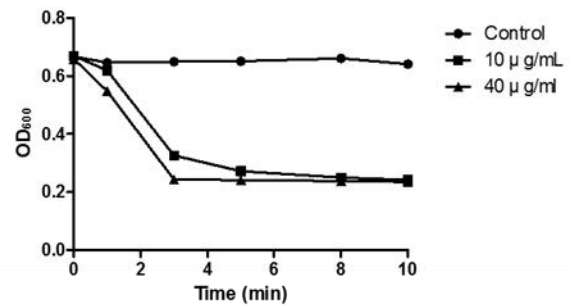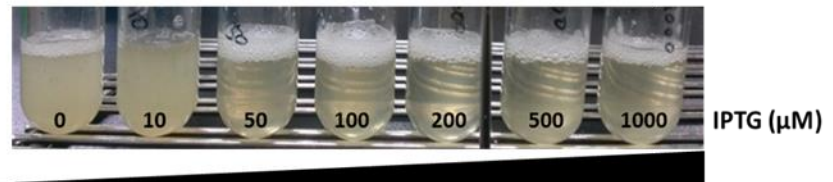
Figure 4.3. Domain prediction of M4LysA and its activity. (A) Domain analysis of M4LysA protein. The number represented the amino acid positions. (B) Purification of the M4LysA⊿TMD using affinity chromatography. (C) Turbidity reduction assay of purified M4LysA⊿TMD using the *E. coli* peptidoglycan. Closed circles, buffer treatment; closed squares, 10 μg/ml of M4LysA⊿TMD treatment; closed triangles, 40 μg/ml of M4LysA⊿TMD treatment, respectively. (D) Cell lysis activity of M4LysA was observed with addition of various concentrations of IPTG.

A

OD$_{340}$

B

PGN   PGN + M4Lys   M4Lys   PGN + Lyso

C

OD$_{560}$

D

OD$_{690}$

PGN
PGN + M4LysAΔTMD
M4LysAΔTMD
PGN + Lyso
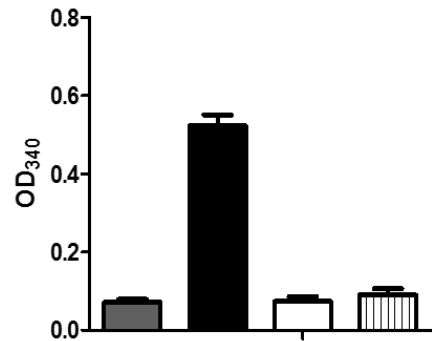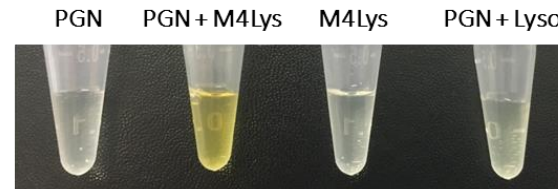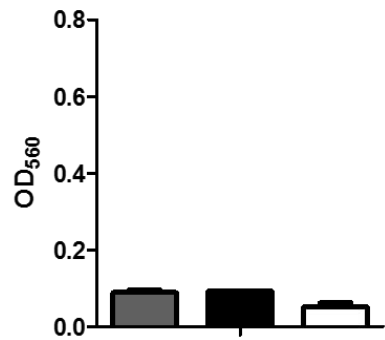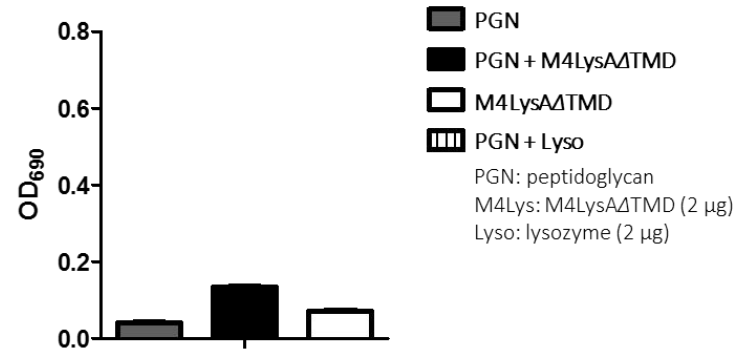
PGN: peptidoglycan
M4Lys: M4LysAΔTMD (2 μg)
Lyso: lysozyme (2 μg)

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Figure 4.4. Enzymatic activity of M4LysA⊿TMD$_{1-206}$. (A) Endopeptidase activity of M4LysA⊿TMD$_{1-206}$ was measured at 340 nm after endolysin treatment. (B) An orange TNBS complex was change to yellow in M4LysA⊿TMD$_{1-206}$ treated group. Non-treated group and lysozyme treated group were used as controls. (C and D) *N*-acetylmuramoyl-L-alanine amidase or glycosidase activity activity of M4LysA⊿TMD was measured at 560 nm and at 690, respectively, after treatment.

Table 4.3 Host range analysis of M4LysA⊿TMD

Bacterial strain	BSPM4 (phage)	M4LysA⊿TMD (lysis protein)
Salmonella enterica		
serovar Typhimurium		
SL1344	CC	++
UK1	CCC	++
LT2	C	+
LT2C	C	+
ATCC14028	C	++
ATCC19586	C	+
ATCC43147	CCC	+
ATCC13076	C	++
DT104	C	+
serovar Parathyphi		
A IB 211	-	+
B IB 231	-	++
serovar Dublin IB 2973	-	++
Escherichia coli		
BL21	-	+++
Escherichia coli **O157:H7**		
ATCC35150	-	+++
ATCC43890	-	+
ATCC43894	-	+++
ATCC43895	-	++
O157:NM 3204-92	-	++
O157:NM H-0482	-	+
Gram-negative bacteria		
Vibrio fischeri ES-114 ATCC 700601	-	-
Pseudomonas aeruginosa ATCC 27853	-	+++
Cronobacter Sakazakii ATCC29544	-	+
Gram-positive bacteria		
Staphylococcus aureus ATCC 29213	-	-
Staphylococcus epidermis ATCC 35983	-	-
Bacillus subtilis ATCC 23857	-	-
Bacillus cereus ATCC 14579	-	-

†, ATCC, American Type Culture Collection.

*, CCC, EOP of 2 to 1.5; CC, EOP of 1.5 to 1; C, EOP of 1 to 0.5; −, not susceptible to phages.

#, +++, OD reduction of 0.6 to 0.4; ++, OD reduction of 0.4 to 0.3; +, OD reduction of 0.3 to 0.2; -, no OD reduction.

IV-3-4. M4LysA is a *Sec* translocase-independent membrane protein

In bacteria, most membrane proteins are translocated into the membrane by the well-conserved *Sec* translocase using N-terminal signal sequences (31). Likewise, some endolysins contained N-terminal signal peptide sequences or N-terminal signal-arrest-release (SAR) domains for their secretion to the periplasm (5, 7, 9). Usually, the signal sequences and SAR domains allow them to be secreted by *Sec*-system, consequently localized to the periplasm. Since, M4LysA neither contain a distinct N-terminal signal sequence nor a SAR domain, I verified whether the *Sec* system is involved in the M4LysA secretion or not. Sodium azide (NaN_3) is a well-known inhibitor of *Sec* translocase which is necessary for the protein translocation through the membrane (9). The host cell lysis kinetics of M4LysA were observed with addition of various concentrations of sodium azide. Interestingly, addition of sodium azide that compromises the *Sec* function could not inhibit but slightly retard the host cell lysis by M4LysA expression (Fig. 4.5A). Meanwhile, inhibition of another major membrane protein secretion pathway, a Twin-arginine translocation (*Tat*) pathway by treating sodium thiocyanate (NaSCN) (32) could not inhibit the cell lysis by M4LysA induction, indicating *Tat* pathway is not associated with the M4LysA secretion (data not shown). As described above, M4LysA contained a C-terminal TMD rather than the N-terminal secretion sequences or SAR domains. This unusual structure of M4LysA was very similar to tail-anchored membrane proteins (TAMPs) which are recently identified in the bacteria genomes having one or two TMDs near the N-terminus (33). TAMPs are classified as a class of proteins that

characterized by absence of N-terminal signal sequence and *Sec*-independent membrane targeting (34). TAMPs play important roles in eukaryotic organism while this class of membrane proteins were recently identified in bacteria (33). As TAMPs are lacking N-terminal signal sequences, their translocations are occurred in *Sec*-independent way and the C-terminally located transmembrane domain sequences are sufficient for TAMPs localization to the membrane (33). Therefore, it was speculated that the *Sec* pathway-independent mechanism of M4LysA may derived from the C-terminal TMD, like TAMPs. Indeed, when 11 amino acids from the C-terminus TMD (M4LysA$_{1-226}$) was deleted, it showed decreased lytic activity and when 13 amino acids from C-terminus TMD (M4LysA$_{1-224}$) was deleted, cell lysis did not occur anymore (Fig. 4.5B), supporting the importance of TMD for M4LysA translocation. Taken together, localization of M4LysA may be caused by the C-terminal TMD, actually not by the *Sec* or *Tat* pathways, which is different from the previously reported holin-independent endolysins (5, 7, 9).

A
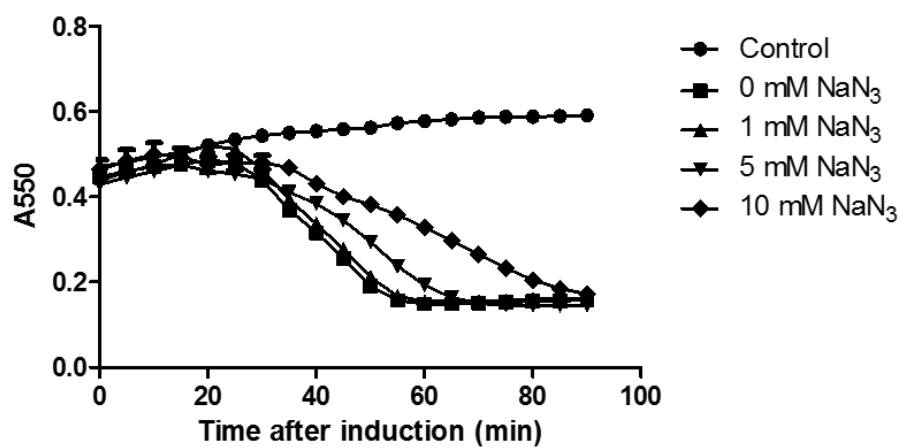
B
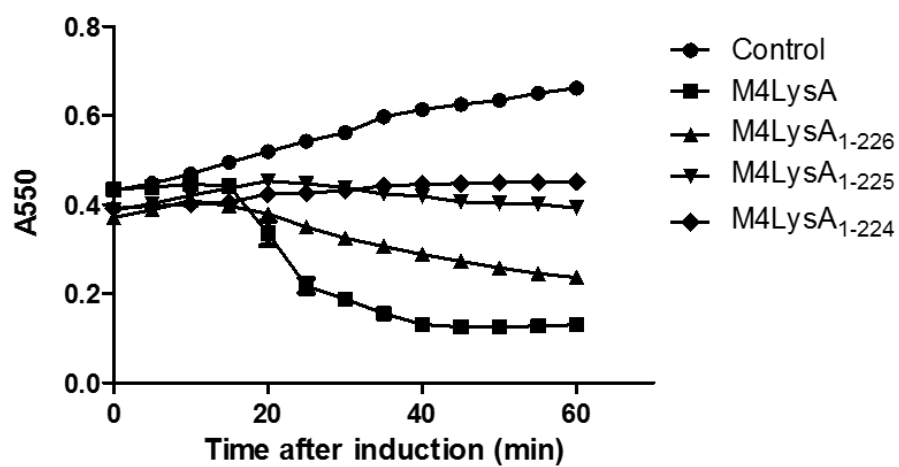
Figure 4.5. The cell lysis kinetics in the presence of translocase inhibitor and functional analysis of M4LysA TMD. In all experiments, M4LysA were expressed by adding 0.1 mM of IPTG. (A) Inhibition of *Sec*-pathway by adding NaN_3. Closed squares, no NaN_3; closed triangles, 1 mM NaN_3; reverse triangles, 5 mM NaN_3; diamonds, 10 mM NaN_3 and only 10 mM NaN_3 treatment sample without IPTG induction (closed circles) was used as a negative control. (B) Lysis activity of two TMD mutants were compared with the full length M4LysA activity. Closed circles, empty vector; closed squares, full length M4LysA; closed triangles, 11 amino acids deletion in TMD of M4LysA; reverse triangles, 12 amino acids deletion in TMD of M4LysA; closed diamonds, 13 amino acids deletion in TMD of M4LysA.

IV-3-5. C-terminal TMD are required for M4LysA activity

As showed above, the N-terminal virion protein domain had peptidoglycan lytic activity (Fig. 4.3D), suggesting N-terminal domain is an enzymatic activity domain. Interestingly, expression of the C-terminal TMD deleted M4lysA protein in the *E. coli* cells could not cause the host cell lysis (Fig. 4.5B). Therefore, I hypothesized that the C-terminal TMD may play an important role for the translocation of the N-terminal virion protein domain to the periplasm. Indeed, when the active domain of M4lysA (M4LysA$\mathit{\Delta}$TMD$_{1-206}$) was expressed in the cell, it did not trigger the host cell lysis (Fig. 4.6A), suggesting this protein may remain in the cytoplasm. Surprisingly, simultaneous expression of M4LysA$\mathit{\Delta}$TMD$_{1-206}$ and the C-terminal region of M4lysA (TMD$_{158-237}$) showed rapid cell lysis after 20 min of induction (Fig. 4.6A). In addition, expression of the C-terminal domain (TMD$_{158-237}$) alone inhibited the bacterial growth, but no cell lysis was observed (Fig. 4.6A). These results suggested that the overexpression of C-terminal domain (TMD$_{158-237}$) was toxic to bacterial cells which is similar to overexpression of the membrane proteins or holin proteins (35, 36). Taken together, the results suggested that release of the catalytic domain to the periplasm may be mediated by the C-terminal TMD of M4LysA, thus causing the host cell lysis.

According to the previous study, lysis system of phage P22 can be functionally exchangeable with other lysis systems such as phage lambda and T4 those are composed of holin and endolysin (37), indicating that they may share the similar lysis mechanism. However, complementation test by the plasmids expressing

P22 endolysin with TMD$_{158-237}$ and P22 holin with M4LysA$\varDelta$TMD$_{1-206}$, respectively, did not exhibited the cell lysis (Fig. 4.6B). Therefore, the results suggested that BSPM4 phage has a unique cell lysis system distinct to that of other phages.
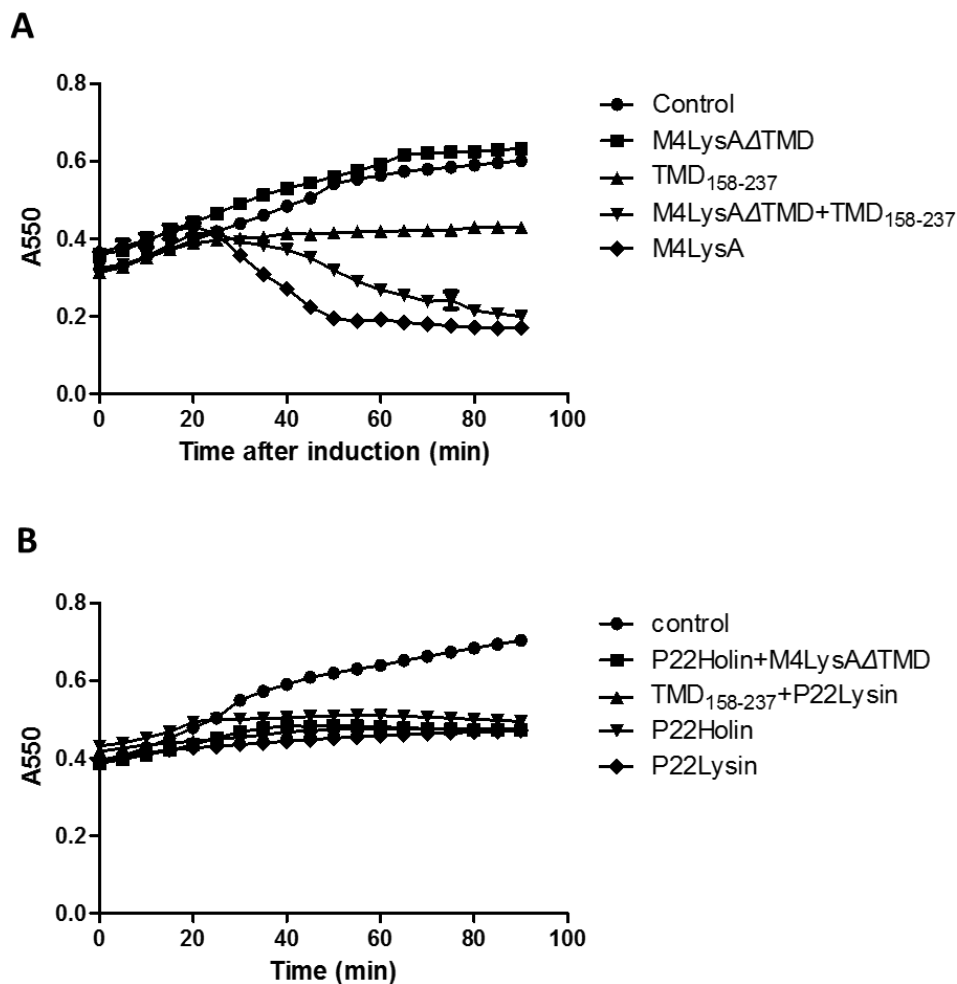
Figure 4.6. Functional analysis of the TMD of M4LysA. (A) Virion protein domain and C-terminal TMD region were expressed separately or co-expressed in the *E. coli* BL21 (DE3) cells. Closed circles, empty vector; closed square, expression of M4LysA$\varDelta$TMD$_{1\text{-}206}$; closed triangles, expression of TMD$_{158\text{-}237}$; reverse triangles, co-expression of M4LysA$\varDelta$TMD$_{1\text{-}206}$ and TMD$_{158\text{-}237}$; closed diamonds, expression of M4LysA. (B) Complement test of M4LysA domains with phage P22 lysin and

holin. Closed circles, empty vector; closed square, co-expression of M4LysAΔTMD$_{1\text{-}206}$ and P22 holin; closed triangles, co-expression of TMD$_{158\text{-}237}$ and P22 lysin; closed reverse triangles, expression of P22 holin; closed diamonds, expression of p22 Lysin. All proteins were induced by addition of 0.1 mM IPTG.

IV-4. Conclusion

Most double-stranded DNA containing-phages targeting Gram-negative bacteria have developed a cell lysis system comprised of holin and endolysin. Endolysins usually contain conserved catalytic domains such as phage lysozyme, amidase, and peptidase. Timing-regulated expression of holins makes holes in cytoplasmic membrane for releasing the endolysin to the periplasm at indicated time. Therefore, holins play critical roles in the endolysin secretion. In contrast, some endolysins contained secretion sequences or SAR domains at the N-terminus revealed to be secreted by *Sec* pathway. In these cases, holin play a subsidiary role in disrupting the membrane potential.

M4LysA, a lysis protein identified from a double-stranded DNA-containing phage, did not have sequence homologous to previously identified endolysin or holin of Gram-negative bacteria-targeting phages. However, turbidity reduction assay and enzymatic activity assay using the purified peptidoglycan revealed that M4LysA contained a virion protein domain which has endopeptidase activity. Interestingly, M4LysA contained an unusual C-terminal membrane domain which are superficially similar to the tail-anchored membrane protein (TAMPs). M4LysA could be translocated independent of *Sec* system and the C-terminal TMD was sufficient for membrane targeting like TAMPs. This may because M4LysA does not have signal sequences such as signal peptides or SAR. Surprisingly, TMD of M4LysA plays an important role in the translocation of the enzymatic activity domain to the periplasm. Therefore, the results showed that the virion protein domain and a TMD are

correspond to the endolysin and holin of other phages, respectively. However, when each domain of M4LysA was co-expressed with the holin and endolysin of phage P22, respectively, they were not complemented functionally, indicating that they may not share the lysis system. The results suggested that M4LysA contained dual functional modules including a catalytic domain and a holin-like domain. The unusual chimeric structure of M4LysA may be originated from the recombination of a lysis module and a TMD module. The results of this study show that the cell lysis mechanism by M4LysA is novel and may be widely distributed among flagella-targeting phages. Though the reason why these two domains are combined in a protein still remains unknown, the specific interaction of two domains in M4LysA is currently under the study.

IV-5. References

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Chapter V.

Liposome-mediated delivery of phage endolysins

to penetrate Gram-negative bacteria cell wall

V-1. Introduction

Since its development in the 20th century, antibiotics have been widely used to fight infections because of its rapid effectiveness to pathogenic bacteria (1). However, a widespread and inappropriate overuse of antibiotics have led to the problems with emergence of resistant bacteria (2). Moreover, the development of new types of antibiotics is getting harder while the emergence of resistant strains including *Salmonella* is rising worldwide (3, 4). Therefore, it is urgent to develop an alternative strategy to control the antibiotic-resistant bacteria (5).

Bacteriophages are viruses with an ability to infect bacteria specifically and, in most cases, kill bacterial cells (6). To escape from inside of bacteria after infection, phages express a peptidoglycan degrading enzyme called "endolysin" (7). Endolysin is a natural material known to be harmless to human cells due to its target specificity (8). In addition, it has been known to have strong bactericidal activities including the resistant bacteria (9, 10). Therefore, endolysins have been widely studied and attracted much attention in controlling the Gram-positive bacteria such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Clostridium perfrigens* (11).

On the other hand, in the case of Gram-negative bacteria, the presence of outer membrane inhibits the endolysin accession to the target peptidoglycan substrates and makes it insensitive to external endolysin treatment (12). Since, the peptidoglycan layer in Gram-negative species is much thinner than that of Gram-positive bacteria, it is suggested that small amount of endolysin would be sufficient to control Gram-negative bacteria. Therefore, many researchers have shown interest

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to various physical, chemical, and biological approaches for endolysin to pass through the outer membrane barrier in Gram-negative bacteria (13).

Liposomes, lipid nano vesicles, are spherical structures composed by one or more phospholipid bilayers with aqueous core (14). Due to the presence of aqueous phases in the structure of lipid vesicles, they have been widely utilized in the entrapment, delivery, and release of water-soluble drugs and antimicrobial peptides (15-17). In addition, liposomes have been widely applied in food industry to deliver proteins, enzymes, vitamins, and antioxidants (18, 19). Previous studies revealed that liposome stabilized the encapsulated materials against a range of environmental conditions, including extreme pH and temperature (14, 20).

In this study, a phage-derived peptidoglycan hydrolase (endolysin) was purified and characterized. Moreover, this highly soluble endolysin protein was subjected to encapsulate into the lipid nano vesicles and its antimicrobial activities were investigated. Although endolysin is a water soluble enzyme, it is difficult to apply because the endolysin is usually susceptible to various external environmental conditions such as heat, pH, and buffer conditions (21, 22). Therefore, encapsulation and delivery of endolysin by lipid nano vesicle could be a promising strategy to control Gram-negative bacteria with two major advantages that it could not only penetrate the cell wall of Gram-negative bacteria but also protect endolysin from the diverse environmental physical or chemical stresses.

V-2. Materials and Methods

V-2-1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 5.1. All strains were aerobically grown in Luria-Bertani (LB) broth or LB broth with 1.5% bacto agar medium (Difco, Detroit, MI, USA) at 37°C. Antibiotic was added for the maintenance of the endolysin expression vector: kanamycin, 50 µg/ml (final concentration).

Table 5.1. The bacterial strains and plasmids used in chapter V

Strains	Refererences
Samonella enterica serovar Typhimurium	
LT2	(23)
Escherichia coli	
DH5α	Invitrogen
BL21 (DE3)	37
Plasmid	
pET28a(+)	Novagen

V-2-2. Cloning, expression, and purification of BSP16Lys endolysin

The *N*-acetylmuramonyl-L-alanine amidase gene was identified previously from a phage BSP16 (BSP16_ORF33) by functional domain analysis using the NCBI Conserved Domain Database (CDD) (24) and InterProScan database (25). The gene was PCR amplified with the primers BSP16lys-F-NdeI (CA AGA AGG AGA AGA A<u>CA T ATG</u> CCA AAG GTA CAA) and BSP16lys-R-XhoI (GCG GTA AAT CAT <u>CTC GAG</u> CAT TCC CAT CTC ATG). The PCR product was cloned into pET28a(+) vector (Novagen, Madison, WI) to make an N-terminal hexahistidine (His$_6$)-tagged protein. The resultant recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) cell after sequence confirmation. The cells harboring the recombinant plasmid was then supplemented with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG; final concentration) at OD$_{600}$ 0.5 to express the BSP16Lys endolysin and incubated at 37°C. After incubate for 3 h, harvested cells were suspended in a lysis buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) and sonicated (Branson Ultrasonics, Danbury, CT). The cells were centrifuged at 15,000 x g for 20 min to obtain the supernatants containing soluble proteins filtrated using 0.22-μm pore size filter (Millipore, Germany). His-tagged BSP16Lys protein was purified using a Ni-nitrilotriacetic acid (NTA) Superflow column (Qiagen Gnbh, Germany) according to the manufacturer's instructions and confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (26). The purified protein was stored at -80°C before use after the buffer changing (storage buffer; 50

mM Tris-HCl, 100 mM NaCl, 30% glycerol, pH 8.0) using a PD Miditrap G-25 (GE healthcare, Amersham, Bucks, UK).

V-2-3. Lysis activities of BSP16Lys endolysin

An exponentially grown cultures of *Salmonella* Typhimurium LT2 cells were treated with ethylene diamine tetra acetic acid (EDTA) with 100 mM concentration for 10 min to permeabilize the outer membrane (27, 28). Then, cells were washed with Tris-HCl buffer twice and suspended in the same buffer (29). Different amounts of BSP16Lys endolysin (1 μg, 3 μg, and 5 μg) were added to the 1 ml of EDTA-treated *S.* Typhimurium LT2 cell suspension. The antimicrobial activity of BSP16Lys was determined by measuring the turbidity reduction of the cells (optical density at 600 nm) (30). Storage buffer-treated cells were used as a negative control. Antimicrobial activities of BSP16Lys endolysin were also examined against non-EDTA treated *S.* Typhimurium LT2 and a *S. aureus* ATCC 13301 cells.

V-2-4. Effects of pH, temperature, and NaCl concentrations on the enzymatic activities of BSP16Lys endolysin

Relative antibacterial activities of BSP16Lys endolysin were determined under various conditions as described previously with some modifications (30). To test the enzyme activities under various pH condition, 3 μg of BSP16Lys endolysin were added to the *S.* Typhimurium LT2 cell suspension in various buffers and

turbidity reductions were observed. The buffers with diverse pH are as follows; 0.1% trifluoroacetic acid (pH 2.0), 50 mM sodium acetate (pH 4.3), 50 mM 2-(N-morpholino) ethanesulfonic acid (pH 6.0), 50 mM bis-tris (pH 6.5), 50 mM bis-tris (pH 7.4), 50 mM Tris-HCl (pH 8.0), 50 mM Tris-HCl (pH 8.8), 50 mM glycine (pH 9.5), and 50 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (pH 10.0). To know stability of BSP16Lys endolysin under various temperature conditions, the enzyme was incubated at different temperatures from 25 to 75°C for 30 min. After incubation, turbidity reduction assay was performed against the EDTA treated *S.* Typhimurium LT2 cells. The effects of sodium chloride (NaCl) on BSP16Lys endolysin activity were examined with different NaCl concentrations (0 to 500 mM) at 25°C. The relative activities of BSP16Lys endolysin were calculated in proportion to the highest OD_{600} reduction value obtained in the tested groups.

V-2-5. Liposome preparation

Lipid mixtures of dipalmitoylphosphatidylcholine (DPPC), cholesterol, and hexadecylamine (HDA) were used for the preparation of cationic liposomes. Molar ratio of 8:2:1 was used for BSP16Lys and molar ratio of 8:2:2 was used for chicken egg white lysozyme (SigmaAldrich, St. Louis). Liposomes were prepared by thin-film hydration method written previously with some modifications (31). Briefly, lipids and surfactants were dissolved in chloroform, at indicated ratio, to make total lipid concentration to 2 mM. The solvent was evaporated using nitrogen gas to make a thin lipid film. Then the lipid film was hydrated at 42°C (above the transient

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temperature of DPPC) with Tris-HCl buffer (pH 8.0) or buffer containing purified BSP16Lys endolysin or lysozyme, respectively. The resulting heterogeneous multi lamellar vesicles (MLVs) were then downsized by sonication to make small unilamellar vesicles (SUVs) of uniform size. Sucrose of 10 mM was employed to improve the encapsulation efficiency and to stabilize proteins from the heat treatment (32, 33).

V-2-6. Dynamic light scattering (DLS)

Size distributions and polydiversity index (PDI) of the lipid vesicles were determined using a Zetasizer nano ZS (Malvern Instruments LTd., UK). Each sample (1 ml) was applied into disposable cuvette and measured in triplicate under the following measurement conditions; refractive index (1.330), viscosity (0.8916 cp), equilibration time (1 min), temperature (25℃), and measurement angle (173° backscattering). To measure the zeta potentials of the vesicles, each sample was loaded into a disposable folded capillary cell (DTS1070, Malvern Instruments Ltd., UK) and bubbles were removed before the measurement. All measurements were conducted at 25℃ in triplicate.

V-2-7. Transmission electron microscopy (TEM)

Formation of lipid vesicles were confirmed by TEM analysis. Each liposome sample was placed on a Formvar-coated carbon grid (200 mesh) and negatively stained with aqueous 2% uranyl-acetate (pH 4.0) for 1 min. After washing

with distilled water two times, the grid was completely dried. Then liposomes were visualized by TEM (LIBRA 120, Carl Zeiss) at 120 kV accelerating voltage.

V-2-8. Entrapment efficiency

The encapsulation efficiency of BSP16Lys endolysin and lysozyme into the vesicle was determined by BCA (bicinchoninic acid) protein assay kit (Pierce) according to the manufacturer's instructions. In brief, vesicles were centrifuged at 4000 x g more than three times using the Amicon Ultra-4 of 30 NMWL pore size cellulose filter (Millipore, Germany) with addition of buffer to remove residual proteins. After then, vesicles which contain proteins were mixed with 0.5% Triton-X 100 (final concentration), 2% SDS (final concentration), and vortexed vigorously to break the lipid vesicles for protein release. The amounts of entrapped proteins were measured by BCA method and calculated using BSA as a standard. The encapsulation efficiency (%) was calculated as follow formula;

$$\text{Entrapment efficiency (E.E., \%)} = \frac{\text{Amount of encapsulated protein}}{\text{Amount of total protein}} \times 100$$

where the amount of total protein is the amount of initial protein used and the amount of encapsulated protein is the amount of protein in the vesicles.

V-2-9. Antimicrobial activity of liposome-encapsulated BSP16Lys endolysin and lysozyme

The antimicrobial activity of liposome-encapsulated BSP16Lys and lysozyme was determined against *S.* Typhimurium LT2 strain. Briefly, each protein -containing vesicles were mixed with an exponentially grown *S.* Typhimurium LT2 strain (~10^3 CFU/mL) containing 20 mM of $CaCl_2$ and incubated for 1 h at 25°C. The mixture was then plated on the LB agar plate and incubated to enumerate remain bacteria cells. The amount of the treated protein-containing vesicles were indicated as the concentrations of lipids (0.5 mM or 2 mM). Treatments including proteins only, buffer containing liposomes, and co-treatment of buffer containing liposomes with each protein were used as controls.

V-3. Results and discussion

V-3-1. Purification and antibacterial activities of BSP16Lys endolysin

The genome of phage BSP16 contained a peptidoglycan hydrolase which was predicted as an *N*-acetylmuramonyl-L-alanine amidase (BSP16_ORF33). To verify the bacterial cell lysis activity of the endolysin, a recombinant plasmid containing the endolysin gene was constructed and overexpressed in the *E. coli* system with IPTG induction. The BSP16Lys endolysin was purified using Ni-NTA affinity chromatography and confirmed by SDS-PAGE. Approximately, the size of 19 kDa of BSP16Lys endolysin was successfully obtained, indicating this protein is highly soluble (Fig. 5.1A). Concentration of purified BSP16Lys endolysin was about 500 μg/mL. Since water soluble proteins can be loaded in lipid vesicles, BSP16Lys endolysin can be easily entrapped into the lipid vesicles (34). To test the lytic activities of purified BSP16Lys endolysin, EDTA treated *S.* Typhimurium LT2 cells were used as a substrate. When BSP16Lys endolysin was treated at various concentrations, at least 3 μg/mL of endolysin showed rapid lytic activity within 7 min (Fig. 5.1B), suggesting the antimicrobial potential of BSP16Lys endolysin. In contrast, BSP16Lys endolysin did not show any lytic activities against the EDTA non-treated Gram-negative cells, indicating the outer membrane prohibited access of BSP16Lys endolysin to the peptidoglycan substrates (Fig. 5.2A). In addition, treatment of BSP16Lys endolysin could not occur cell lysis against a Gram-positive *S. aureus* cell (Fig. 5.2B). Therefore, BSP16Lys endolysin is highly specific to Gram-negative bacteria, since the type of peptidoglycan structures are different in

Salmonella and *S. aureus* cells (35).
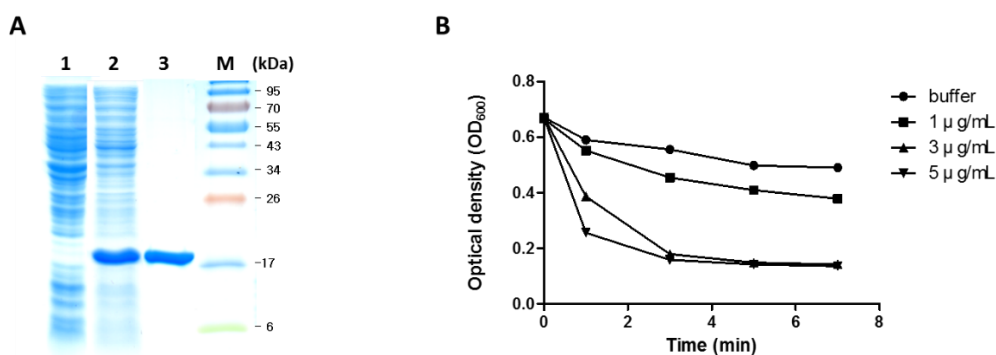
Fig. 5.1. Purification of BSP16Lys endolysin and its antibacterial activities. (A) *E. coli* BL21(DE3) cell containing the empty backbone plasmid (lane 1) or recombinant plasmid (lane 2) were induced with 0.5 mM IPTG, and the total cell lysates were separated using a 15% acrylamide gel. The recombinant BSP16Lys endolysin (~ 19 kDa) was purified using affinity chromatography (lane 3). M, protein molecular weight markers with size. (B) The lytic activities of purified BSP16Lys endolysin was examined using EDTA-treated *S.* Typhimurium LT2 cells. Concentrations from 0 to 5 μg/mL of endolysin were treated to the substrate and OD_{600} was measured every minute.

200
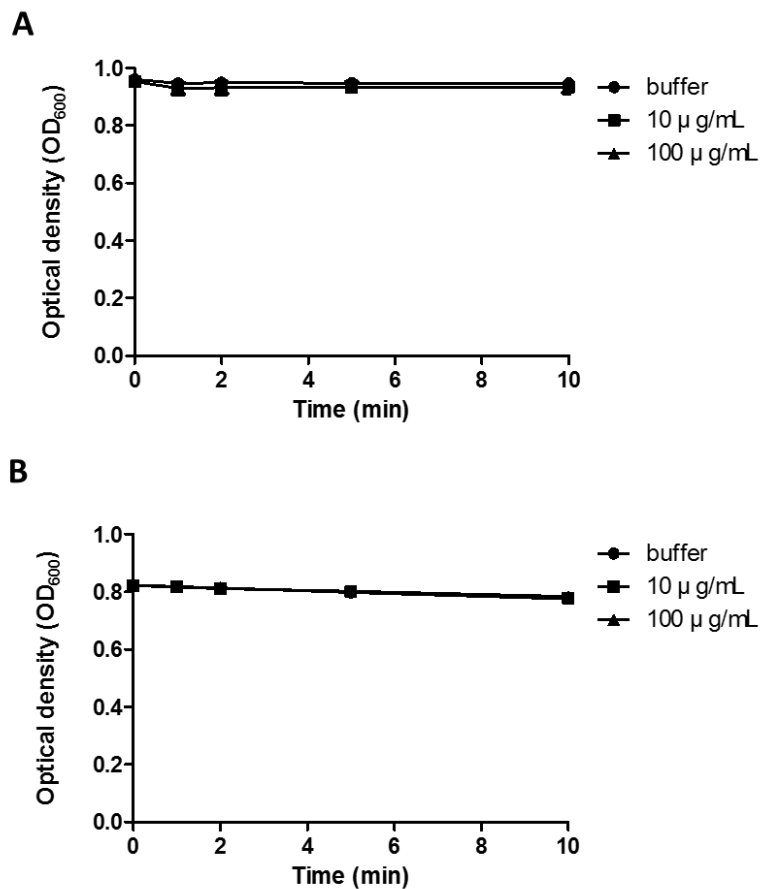
Fig. 5.2. Antimicrobial activities of BSP16Lys endolysin. (A) A large amount of purified BSP16Lys endolysin (0 to 100 μg/mL) was treated to EDTA non-treated *S. Typhimurium* LT2C cells and (B) concentrations from 0 to 100 μg/mL of endolysin were treated to exponentially grown *S. aureus* cells and OD_{600} was measured.

V-3-2. Optimal pH, temperature and NaCl concentrations for enzyme activity

The effects of pH, temperature, and NaCl concentration were determined to set the optimum condition for the encapsulation of BSP16Lys endolysin into the lipid vesicle. As a result, BSP16Lys endolysin showed similar activities at neutral and weak basic pH values (pH 7.4 to 9.5) and showed the maximum activity at pH 8.8 (Fig. 5.3A). In addition, as the temperature increased from 25 to 75°C, the activities of BSP16Lys endolysin drastically decreased over 60°C (Fig. 5.3B). The highest enzyme activity was shown in the presence of 100 mM NaCl condition and the activity got reduced as higher concentrations of NaCl were added (Fig. 5.3C). Based on the characteristics of BSP16Lys endolysin, the buffer condition for the protein encapsulation into the cationic liposome were set as Tris-HCl containing 100 mM NaCl. It is notable that pH 8.0 was selected for the storage buffer rather than pH 8.8 or 9.5 to obtain plus-charged endolysin. Since the predicted isoelectric point (pI) value of BSP16Lys endolysin was 8.62, this protein will have plus charge under the pH value. In consequence, the plus-charged endolysin will hardly aggregate with the positively charged liposomes (36). In addition, high pH value could result in extensive aggregation of liposomes (37).
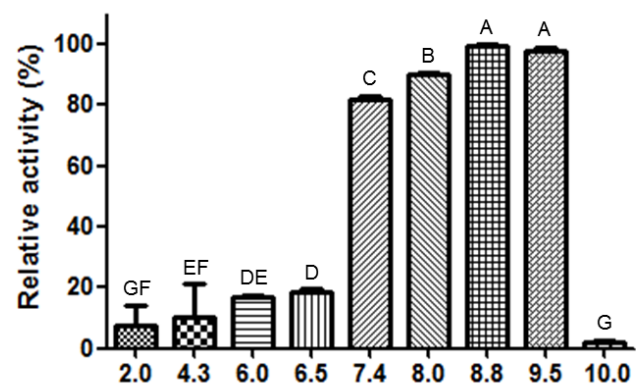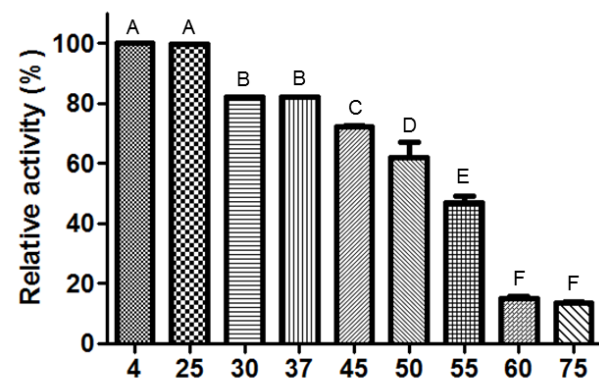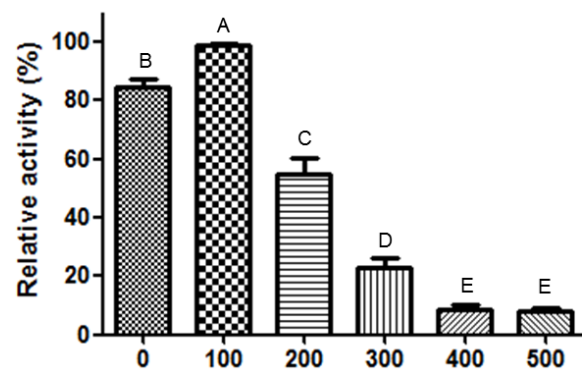
Fig. 5.3. The optimum conditions for the enzymatic activity of BSPLys endolysin. (A) The relative lysis activity of BSP16LysA endolysin was determined in buffers with different pH values for 10 min at room temperature, (B) at different temperatures for 30 min, and (C) with various NaCl concentrations buffer, respectively. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. One-way analysis of variance (ANOVA) with Duncan's multiple range tests was used for the comparison in each condition, respectively. The capital letters above the error bars indicate significant differences ($P < 0.05$) between the test groups.

V-3-3. Physicochemical properties of liposomes

To develop a liposome as an antimicrobial delivery system, it is important to understand the main characteristics of the liposome, such as vesicle size, zeta-potential, and encapsulation efficiency (38). In order to encapsulate the BSP16Lys endolysin into the liposome, a lipid mixture of DPPC, cholesterol, and HDA (8:2:1 molar ratio) was set for the preparation of cationic liposome as described above. Each buffer and buffer-containing BSP16Lys endolysin was separately used to hydrate lipid thin film in order to make liposomes. DLS analysis indicated that the mean sizes of the empty liposome and BSP16Lys endolysin-encapsulated liposome are 184.03 ± 4.53 nm and 303.50 ± 4.27 nm, respectively (Fig. 5.4A and B). The polydispersity index (PDI) value of both vesicle was lower than 0.5, indicating both liposomes were relatively uniform and homogeneous (Table 5.2) (39). The size of endolysin encapsulated liposome was about 1.8 times bigger than the empty liposome, indicating a large amount of soluble protein may have entrapped into the lipid vesicles. As expected, the zeta potential of both liposomes containing HDA was positive (40) with averages of 46.1 ± 6.78 and 34.7 ± 3.01, respectively (Table 5.2). In general, liposomes with higher electrical charges (negative or positive) are expected to be more stable than neutral liposomes because of increment in repulsive interactions between liposomes, consequently reduce the frequency of liposome aggregation (16). Therefore, both empty liposome and BSP16Lys endolysin encapsulated liposome are anticipated to be stable. Meanwhile, the net surface charge of the endolysin-harboring vesicle was lower than that of empty vesicle. The

electrostatic potential may be decreased because of the interaction between positively charged endolysin and a zwitterionic lipid DPPC as previously reported (41). The endolysin entrapment efficiency was 35.27% (Table 5.2) in average of two separate thin-film hydration preparations, which is slightly lower than those of nisin-containing vesicles (47 to 63%) (38). The addition of cholesterol in lipid compositions may result in encapsulation efficiency decrement of endolysin containing liposome (42). However, the endolysin load in the DPPC liposome would be an adequate value for application (43) and the presence of cholesterol will enhance the rigidity of liposome thus inhibiting leakage of the protein in liposome compositions (36, 44). Indeed, previous study showed that nisin encapsulation efficiency was increased with the addition of cholesterol when compared to liposome which was consisted only with phosphatidylcholine (PC) (45). Meanwhile, lysozyme from chicken egg white was also encapsulated into DPPC liposome. In order to encapsulate the lysozyme into the liposome, a lipid mixture of DPPC, cholesterol, and HDA (8:2:2 molar ratio) was used for cationic liposome preparation as described above. DLS analysis indicated that the mean sizes of the empty liposome and lysozyme-encapsulated liposome are 180.50 ± 3.39 nm and 182.60 ± 1.18 nm, respectively (Fig. 5.4C and D). In contrast to the endolysin containing vesicle, the size of lysozyme encapsulated liposome was similar to the empty liposome, indicating only small amount of soluble protein may have entrapped into the lipid vesicles. As expected lysozyme entrapment efficiency was 22.81%, which was lower than that of BSP16Lys (Table 5.2). The polydispersity index (PDI) value of both

vesicles were about 0.274 and 0.300, respectively, indicating both liposomes were homogeneous (Table 5.2). The zeta potential of both liposomes containing HDA was positive with averages of 58.8 ± 4.59 and 57.3 ± 3.32, respectively (Table 5.2).

Morphological properties of liposomes were observed by TEM analysis. The results showed that all prepared liposomes were homogeneously formed into spherical nano-particles (Fig 5.5).

C

D
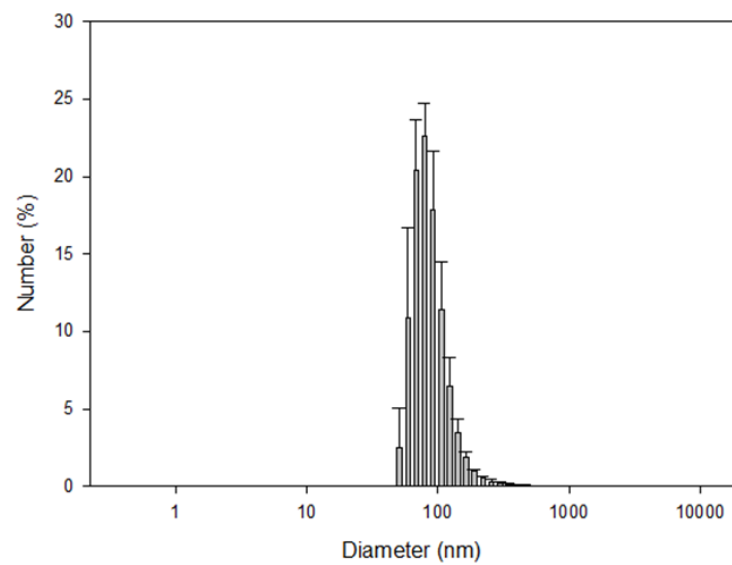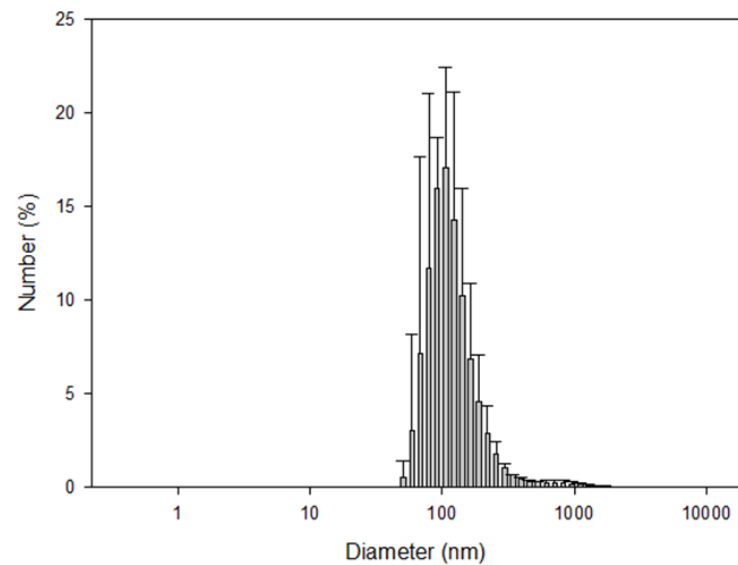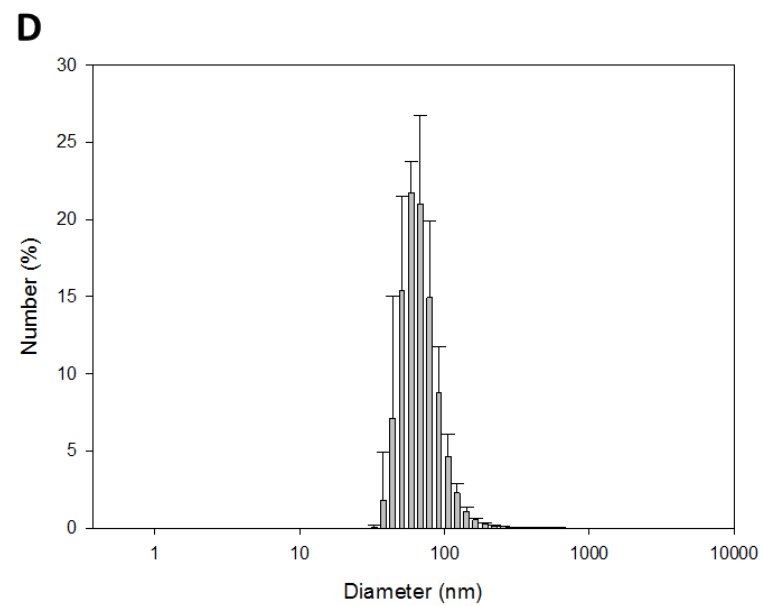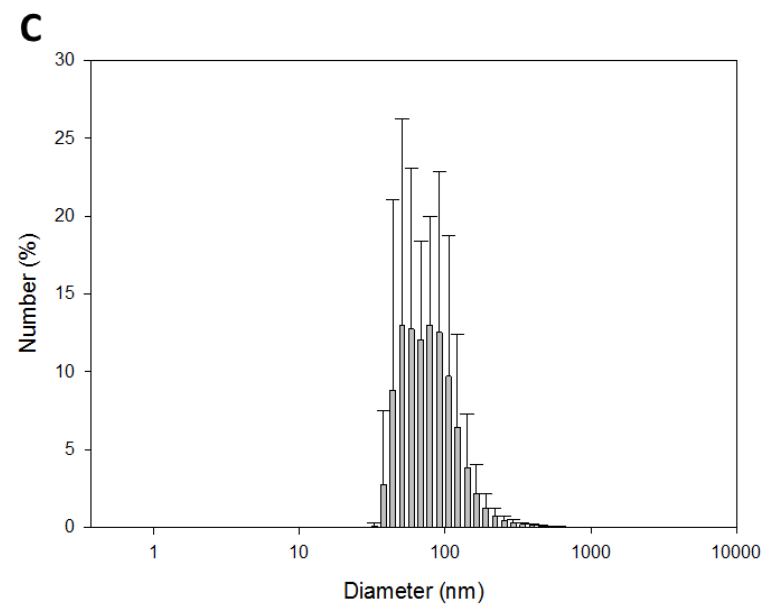
Fig. 5.4. DLS number distribution of DPPC liposomes. The number of lipid vesicles was determined using a Zetasizer nano instrument at 25°C. (A and C) size distribution of empty liposomes containing buffer and (B and D) size distribution of liposomes containing BSP16Lys endolysin and lysozyme, respectively.

Table 5.2. Physicochemical properties liposomes

Liposome cargo	Size (nm)[a]	PDI[a]	Zeta potential (mV)[a]	Entrapment efficiency (%)
Buffer	184.03 ± 4.53	0.333 ± 0.050	46.1 ± 6.78	N.D.
BSP16Lys	303.50 ± 4.27	0.445 ± 0.023	34.7 ± 3.01	35.27
Buffer (HDA 2X)	180.50 ± 3.39	0.274 ± 0.008	58.8 ± 4.59	N.D.
Lysozyme (HDA 2X)	182.60 ± 1.18	0.300 ± 0.025	57.3 ± 3.32	22.81

[a]mean ± standard deviation of three replicates; N.D., not determined.
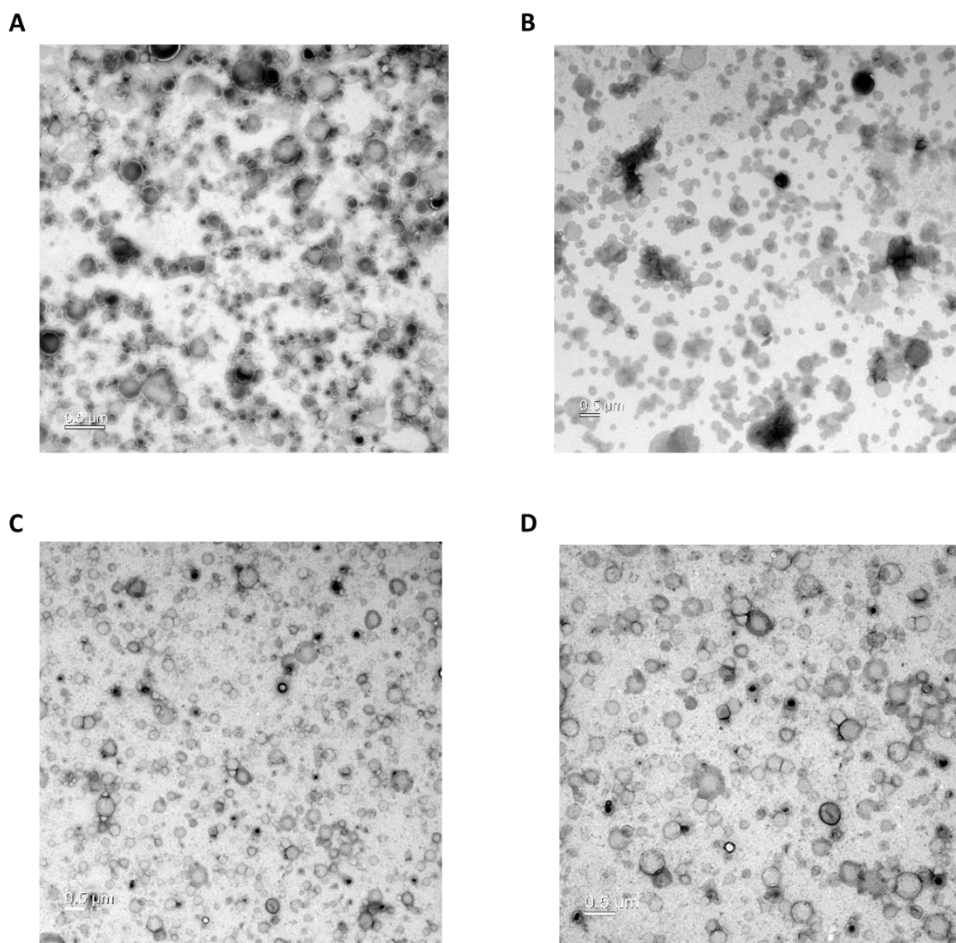
Fig. 5.5. Morphological analysis of liposomes. (A) Buffer-containing liposome composed of DPPC, cholesterol, and HDA (8:2:1 molar ratio). (B) BSP16Lys loaded liposome composed of DPPC, cholesterol, and HDA (8:2:1 molar ratio). (C) Buffer-containing liposome consisted of DPPC, cholesterol, and HDA of 8:2:2 molar ratios. (D) Lysozyme containing liposome consisted of DPPC, cholesterol, and HDA (8:2:2 molar ratio).

V-3-4. Antimicrobial activity of liposomes

Previous studies have demonstrated that the lipid vesicles interact with bacteria by membrane fusion (37, 45, 46). Therefore, I hypothesized that hydrolases-containing liposomes would fuse with the outer membrane of Gram-negative bacteria. In order to examine the antimicrobial efficiency of protein-containing liposome, exponentially grown *S*. Typhimurium LT2 cells were subjected to be mixed with the liposome without EDTA treatment. Surprisingly, BSP16Lys-containing liposome treatment groups showed strong antimicrobial activity after 1 h incubation (Fig. 5.6A). When the liposomes amount corresponding to a 0.5 mM lipid concentration (final concentration) was treated, about 1.1 log cell number reduction was achieved. Moreover, the treatment of liposome containing 2.0 mM lipid (final concentration) successfully eradicate *S*. Typhimurium resulting in about 3.2 log reduction (Fig. 5.6A). Meanwhile, treatment of lysozyme-containing liposome also showed antimicrobial activity after 1 h incubation (Fig. 5.6B). When the liposome amount corresponding to a 0.5 mM lipid (final concentration) was treated, cells were reduced about 0.3 log. In addition, 1.7 log reduction of *S*. Typhimurium was shown by the liposome treatment with higher lipid concentration (final concentration; 2.0 mM lipid) (Fig. 5.6B). Through the results, better antimicrobial activity was observed when BSP16Lys-containing liposome was treated. This may be occurred because BSP16LysA showed higher encapsulation efficiency than lysozyme as determined above (Table. 5.2).

The results suggested that lipid vesicle may deliver hydrolases across the outer membrane of Gram-negative bacteria. No decrease in cell number were observed in control groups (Fig. 5.6A and B). These results showed the potential of peptidoglycan hydrolase delivery into the Gram-negative cells without treating membrane permeabilizers which are generally harmful to human cells. Up to date, delivery of a phage-derived peptidoglycan hydrolase by lipid vesicles is the first study and further analysis such as membrane fusion assay and liposome stability are necessary to develop this system as a novel antimicrobial agent.
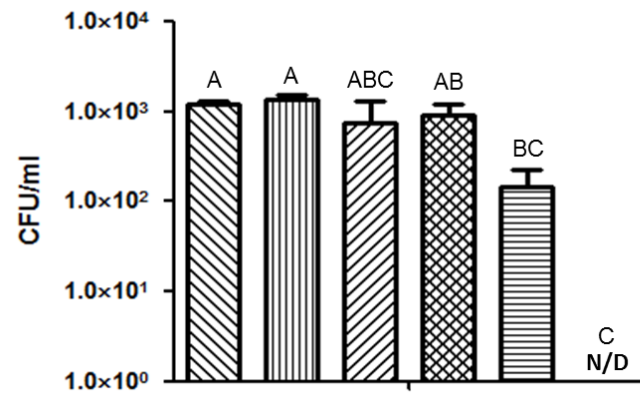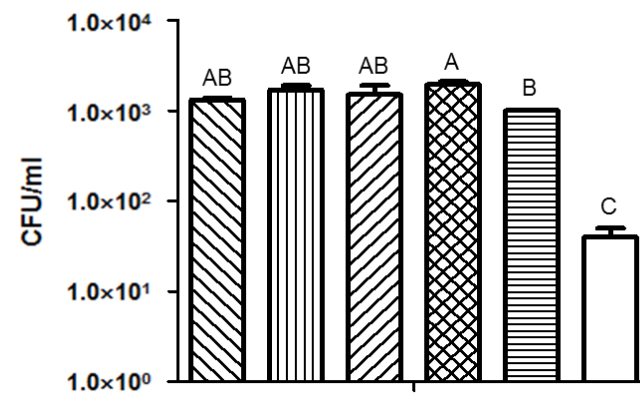
Fig. 5.6. Antimicrobial activities of endolysin and lysozyme containing liposomes. (A) The numbers of viable cells were determined after 1 h treatment with 100 µg/mL of BSP16Lys (▦), buffer-containing liposome (2 mM of lipid, ▨), BSP16Lys (100 µg/m) with buffer-containing liposome (2 mM of lipid, ⊠), BSP16Lys loaded liposome (0.5 mM of lipid, ▤), and BSP16Lys loaded liposome (2 mM of lipid, ☐). (B) The numbers of viable cells were determined after 1 h treatment with 100 µg/mL of lysozyme (▦), buffer-containing liposome (2 mM of lipid, ▨), lysozyme (100 µg/m) with buffer-containing liposome (2 mM of lipid, ⊠), lysozyme-containing liposome (0.5 mM of lipid, ▤), and lysozyme-containing liposome (2 mM of lipid, ☐). Only Tris-HCl buffer (pH 8.0)-treated *S.* Typhimurium (⧅) was used as a control in both experiments. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. One-way analysis of variance (ANOVA) with Duncan's multiple range tests was used for the comparison in each condition, respectively. The capital letters above the error bars indicate significant differences ($P < 0.05$) between the test groups.

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Appendix 1:

A Study of *Cronobacter Sakazakii*-targeting phage CR5

A Novel Bacteriophage Targeting *Cronobacter sakazakii* is a Potential Biocontrol Agent in Foods

(Published in Applied and Environmental Microbiology, 2016)

VI-1. Abstract

Cronobacter sakazakii is an important pathogen with high mortality in infants. Due to its occasional antibiotic resistance, a bacteriophage approach could be an alternative effective method for the control of this pathogen. To develop a novel biocontrol agent using bacteriophages, the *C. sakazakii*-infecting phage CR5 was newly isolated and characterized. Interestingly, this phage exhibited efficient and relatively durable host lysis activity. In addition, a specific gene knockout study and subsequent complementation experiment revealed that this phage infected the host strain using the bacterial flagella. The complete genome sequence analysis of phage CR5 showed that its genome contains 223,989-bp DNA, including 231 predicted open reading frames (ORFs), with a GC content of 50.06%. The annotated ORFs were classified into six functional groups (structure, packaging, host lysis, DNA manipulation, transcription, and additional functions); no gene was found to be related to virulence, toxin, or lysogen formation, but more than 80% of the predicted ORFs are unknown. In addition, a phage proteomic analysis using SDS-PAGE gel electrophoresis and MALDI-TOF/MS revealed that seven phage structural proteins are indeed present, supporting ORF prediction. To verify the potential of

this phage as a biocontrol agent against *C. sakazakii*, it was added to infant formula milk contaminated with *C. sakazakii* clinical isolate or food isolate, revealing the complete growth inhibition of the isolates by the addition of the phage CR5 when multiplicity of infection (MOI) was 10^5.

VI-2. Introduction

Enterobacter sakazakii was first defined in 1980 (1) and was reclassified into a new genus, *Cronobacter*, in 2007 based on its f-AFLP (fluorescent amplified fragment length polymorphism) fingerprints, ribopatterns, and 16S rRNA sequencing (2). *C. sakazakii* is a generally well-known pathogen in infant milk formula powders that causes bacteremia, meningitis, and necrotizing enterocolitis in neonates with high fatality rates (3, 4). Due to this high risk to infants, *C. sakazakii* pathogenesis has attracted broad public attention. This pathogen was recently reported to infect elderly adults in Taiwan, suggesting that there may be various routes of *C. sakazakii* infection (5). When *C. sakazakii* was defined, antibiotic susceptibility tests revealed that this species had occasional antibiotic resistance (1, 6), suggesting that the antibiotic resistance of *C. sakazakii* has resulted in limited antibiotic therapies for the control of this pathogen. Therefore, novel alternative biocontrol agents should be developed.

Bacteriophages are bacterial viruses that infect and lyse specific host bacteria for their replication and propagation (7). Due to their host specificity and lysis activity, phages have been considered alternative biocontrol agents for the control of pathogenic bacteria. Although this approach has been used as a therapeutic approach in the Soviet Union and Eastern Europe for several decades, the application of bacteriophages for the control of pathogens has only been suggested and evaluated in Western countries over the last decade (8). Because of the high risk of *C. sakazakii* and the emergence of antibiotic-resistant strains, a bacteriophage agent could be very

useful for the control of *C. sakazakii*. Previous US Food and Drug Administration (FDA) approval of bacteriophages for food applications, such as ListShield (Intralytix, Baltimore, MD, USA) and Listex P-100 (Micros Food Safety, Wageningen, The Netherlands), supports this.

To date (Sep. 2015), 18 complete genome sequences of bacteriophages specific for *Cronobacter sakazakii* have been reported (9-19). These phage genome analyses have revealed that they have no virulence-related genes encoding virulence factors and toxins. A few attempts using *C. sakazakii* phages have been made in food applications for the control of *C. sakazakii*. Selected *C. sakazakii* phages have been tested in reconstituted infant formula, and the results show that they can efficiently prevent the bacterial growth of *C. sakazakii* in the formula (20). In addition, 67 newly isolated *C. sakazakii* phages were tested, and some of them reduced up to 4 log (CFU/ml) of *C. sakazakii* in pure broth culture, highlighting their potential as biocontrol agents against *C. sakazakii* in foods (21). Furthermore, phage therapy using *C. sakazakii* phages showed high efficiency for alleviation of *Cronobacter*-induced urinary tract infection in mice (22).

In this study, the *C. sakazakii*-infecting bacteriophage CR5 was isolated and purified, and its general features were experimentally characterized to evaluate the possibility as a novel efficient biocontrol agent. In addition, its host receptor was confirmed using specific gene knockout of the host strain to elucidate its host infection mechanisms. Furthermore, its genome was completely sequenced, and its core structural proteins were analyzed to further understand its characteristics at the

genomic and proteomic levels. In addition, the application of the phage CR5 to infant formula milk was assessed, suggesting its potential as a novel biocontrol agent against *C. sakazakii* in foods.

VI-3. Materials and methods

VI-3-1. Bacterial strains and growth conditions.

The bacterial strains used in this study are listed in Table 6.1. *C. sakazakii* ATCC 29544 was used for the isolation and propagation of bacteriophage CR5. All of the bacteria were grown at 37°C (*Enterobacter cloacae* and *Enterobacter agglomerans* grown at 30°C) for 12 h in Tryptic soy broth (TSB) medium (Difco, Detroit, MI, USA).

Table 6.1. Host range of *C. sakazakii* phage CR5

Bacterial strain	Plaque formation*	Source or reference†
Cronobacter sakazakii type strains		
C. sakazakii ATCC 29544	+++	ATCC
C. sakazakii ATCC 29544 *ΔflgK*	−	This study
C. sakazakii ATCC 29544 *ΔflgK* pBAD18::*flgK*	+++++	This study
C. sakazakii ATCC 51329	+	ATCC
C. sakazakii BAA-894	I	(44)
C. sakazakii ST4 (ATCC 29004)	I	ATCC
Cronobacter sakazakii isolates		
1-2 (dried fish samples)	+++++	(45)
3-2 (dried fish samples)	I	(45)
4-1 (dried fish samples)	++++	(45)
5-2 (dried fish samples)	++	(45)
7-1 (dried fish samples)	I	(45)
10-2 (dried fish samples)	I	(45)
2-1 (dried powdered vegetable samples)	+++	(45)
16-2 (Sunsik samples)	+++	(45)
20-2 (Sunsik samples)	+++	(45)
27-1 (Sunsik samples)	I	(45)
29-1 (Sunsik samples)	I	(45)
31-3 (Sunsik samples)	++++	(45)
Other *Cronobacter* type strains		
Cronobacter malonaticus DSM 18702	+	DSM
Cronobacter turicensis DSM 18703	+	DSM
Cronobacter dublinensis DSM 18705	I	DSM
Cronobacter dublinensis lactaridi DSM 18707	+	DSM

*, +++++, EOP of 4 to 2; ++++, EOP of 2 to 1; +++, EOP of 1 to 0.5; ++, EOP of 0.5 to 0.2; +, EOP less than 0.2;

I, formation of inhibition zone; −, not susceptible to phage CR5.

†, ATCC, American Type Culture Collection.

Table 6.1. Host range of *C. sakazakii* phage CR5 (continued)

Bacterial strain	Plaque formation*	Source or reference†
Gram-negative bacteria		
Klebsiella pneumoniae KCTC 2242	−	KCTC
Klebsiella oxytoca KCTC 1686	−	KCTC
Klebsiella oxytoca ATCC 43863	−	ATCC
Enterobacter aerogenes ATCC 35028	−	ATCC
Enterobacter cloacae ATCC 7256	I	ATCC
Enterobacter agglomerans ATCC 27987	I	ATCC
Salmonella Typhimurium ATCC 19586	−	ATCC
Salmonella. Enteritidis ATCC 13076	−	ATCC
Escherichia coli DH5α	−	Invitrogen
Vibrio fischeri ATCC 700601	−	ATCC
Pseudomonas. Aeruginosa ATCC 27853	−	ATCC
Gram-positive bacteria		
Listeria monocytogenes ATCC 19114	−	ATCC
Staphylococcus aureus ATCC 29213	−	ATCC
Bacillus cereus ATCC 14579	−	ATCC
Enterococcus faecalis ATCC 29212	−	ATCC

*, +++++, EOP of 4 to 2; ++++, EOP of 2 to 1; +++, EOP of 1 to 0.5; ++, EOP of 0.5 to 0.2; +, EOP less than 0.2;

I, formation of inhibition zone; −, not susceptible to phage CR5.

†, ATCC, American Type Culture Collection.

VI-3-2. Bacteriophage isolation and purification.

Environmental samples from farm soil in Suwon, South Korea were used for the isolation of *C. sakazakii*-targeting bacteriophages. To isolate the bacteriophages, 25 g of each sample was homogenized with 225 ml of sodium chloride-magnesium sulfate (SM) buffer (100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, and 50 mM Tris·HCl, pH 7.5). After homogenization, 25 ml of each homogenized sample was diluted twice with 25 ml of 2X concentrated TSB, and the mixture was incubated with vigorous shaking at 37°C for 12 h. The collected samples were centrifuged at 6,000 × g for 10 min, and the supernatants were filtered using 0.22-µm-diameter filters (Millipore, Billerica, MA, USA). Ten milliliters of each filtrate was mixed with 50 ml of TSB medium containing 10^9 CFU/ml of an overnight culture of *C. sakazakii* ATCC 29544 as the propagation strain. The mixture was then incubated at 37°C for 12 h with vigorous shaking. The culture was centrifuged at 6,000 × g for 10 min, and the supernatant containing the phages was filtered using a 0.22-µm-diameter filter to remove the residual bacterial cells. Then, ten-fold serial dilutions of the supernatant were spotted on molten 0.4% TSB soft agar containing 10^9 CFU/ml of an overnight culture of *Cronobacter sakazakii* ATCC 29544. Individual plaques were picked, and the phages were resuspended with SM buffer. This plaque isolation and phage resuspension steps were repeated at least five times to isolate and purify the individual phages. When the optical density (OD) at 600 nm of the culture of *C. sakazakii* ATCC 29544 reached 1.0, bacteria were infected with the purified phage CR5 at a MOI of 1 and incubated at 37°C for 4 h. For purification of phage CR5 after propagation, the host cell debris was removed by subsequent

centrifugation at 6,000 × g for 10 min, the supernatant was filtered with 0.22-µm-diameter filters, and the phage particles were precipitated by treatment with polyethylene glycol (PEG) 6,000 (Junsei, Tokyo, Japan). As a final step, cesium chloride (CsCl) density gradient ultracentrifugation (HIMAC CP 100β, Hitachi, Tokyo, Japan) with different CsCl steps (step density = 1.3, 1.45, 1.5 and 1.7 g/ml) was performed at 78,500 × g and 4°C for 2 h. The band containing the viral particles was recovered by puncturing the centrifuge tube with a sterilized needle followed by dialysis using standard dialysis buffer (10 mM NaCl, 10 mM $MgSO_4$, and 1 M Tris·HCl at pH 8.0). The purified phages were stored at 4°C for further experiments.

VI-3-3. Transmission electron microscopy.

The morphology of the purified phage CR5 was observed using transmission electron microscope (TEM) analysis. This analysis was conducted following the procedure described by Kim and Ryu (23) with some modifications. Briefly, 10 µl of CR5 phage stock was loaded onto a copper grid and incubated for 1 min and the excessive solution was removed. Then, the same amount of 2% uranyl acetate (pH 4.0) was applied and washed with ultrapure water. The stained grid was subjected to TEM analysis with JEM-2100F (JEOL, Tokyo, Japan) at 200 kV. Identification and classification of CR5 was conducted according to the guidelines of the International Committee on the Taxonomy of Viruses (ICTV) (24).

VI-3-4. Host range analysis.

Five milliliters of molten 0.4% TSB agar containing 100 µl of each test

bacterial culture was overlaid on 1.5% TSB agar plates. Ten microliters of each serially diluted phage CR5 suspension from 10^2 to 10^9 PFU/ml was spotted on the overlaid plates. The host lysis activity of the test bacteria by phage CR5 was determined by the formation of plaques in the spots. The efficiency of plating (EOP) was calculated by comparison of titers between the selected test bacteria and the propagation host strain *C. sakazakii* ATCC 29544.

VI-3-5. Bacterial challenge test.

C. sakazakii ATCC 29544 was inoculated into TSB broth medium and grown at 37°C for 12 h with agitation (220 rpm), and then 1% of this culture was sub-inoculated into 50 ml of fresh TSB broth and incubated at 37°C with agitation. To confirm the bacterial lytic activity of the phage, phage CR5 (MOI = 1.0) was added to an exponentially growing *C. sakazakii* ATCC 29544 culture at $OD_{600 \, nm}$ 1.0, and the culture OD was monitored at 600 nm at 1-h intervals. A culture of *C. sakazakii* ATCC 29544 without the phage was used as a control. All of the tests were conducted in triplicate.

VI-3-6. Mutant construction and complementation for identification of host receptor.

The mutants of *C. sakazakii* ATCC 29544 for identification of host receptors were constructed using the one-step gene inactivation method previously described by Kim et al. (25). For construction of the mutant, five host receptor-associated genes including *rfaC* for O-antigen of LPS, *lamB* or *ompC* for outer

membrane protein, *fhuA* for ferric ion uptake transporter, and *flgK* for flagella were selected and their specific primer sets were designed (Table 6.2). The kanamycin resistance cassette for integration into the selected each gene in the host chromosome was PCR amplified from pKD13 using one of the specific gene-targeting primer sets, which contains identical small DNA sequences upstream of the start codon and downstream of the stop codon of the selected each gene. The PCR product was then electroporated into *C. sakazakii* ATCC 29544 containing pKD46 with an integrase gene for integration of the PCR product into the host chromosome via homologous recombination. After integration of the PCR product, the mutant of each gene was selected using TSB agar plates containing 50 μg/ml kanamycin sulfate (Sigma, St. Louis, MO). The kanamycin resistance gene was removed from the selected mutant using the pCP20 plasmid (26). For complementation of the *flgK* mutant, the host *flgK* gene was PCR amplified using the *flgK*-comple-F forward primer (*Hind*III) (5'-GTG TAC TAA TAA GCT TCG TTC GGT TCC CTG-3') and *flgK*-comple-R reverse primer (*Nhe*I) (5'-TAA GCG CTA GCG ATA ATT ATC GTC AGG ACC-3'). After purification of the PCR product, it was cloned into the pBAD18 expression vector. After the transformation of pBAD18-*flgK* into the *C. sakazakii flgK* mutant and selection with 50 μg/ml ampicillin, arabinose (0.2%, final concentration) was used as an induction reagent for the complementation of the *flgK* gene in the mutant.

Table 6. 2. Primers used for construction of *C. sakazakii* mutants

Gene	Primer Name	Oligonucleotide sequence 5' – 3'[a]
rfaC	rfaC-red-F	AAC GGA TGT TTC CCC GCA AAG CCA GGG ACG CAG TTG TTC AAA AAC GGT AGC GGC GTG TAG GCT GGA GCT GCT TCG
	rfaC-red-R	TGC CCG CGT TCT GGA GAC GCT CAA CGA ACT GCT GCT GAA CGA GGA AGC CTG ACG GAT TCC GGG GAT CCG TCG ACC
lamB	lamB-red-F	GAG ATA GAA TGA TGA TAA CTC TGC GTA AAC TCC CTC TGG CTG TGG CCG TGA TGG CTG TAG GCT GGA GCT GCT TCG
	lamB-red-R	TAC CAC CAG ATT TCC ATC TGG GCA CCG AAG GTC CAC TCA TCA TTG TCG CCA CGG CAT TCC GGG GAT CCG TCG ACC
ompC	ompC-red-F	TCG GAC AAT GGA TTT GCC CGC TAG TTC CCT GAA TTA GTG AGC AGT GGC AAT AAT ATG TAG GCT GGA GCT GCT TCG
	ompC-red-R	GGA GCC CGC AGG CTC CTT TTG CAC ATC AGG TCG GGG ATT AGA ACT GGT AAA CCA GAT TCC GGG GAT CCG TCG ACC
fhuA	fhuA-red-F	TCA AAC AGG TTA TTG ACG TTT AAG GCG ACA GAC GAG CCC GGC AGG CCT AAA CGC GTG TAG GCT GGA GCT GCT TCG
	fhuA-red-R	TAG CAT GGC GCG TTC CAC TCA CAC TCA GAT CAA TAC CAG GAT TTG CAG ACT GGC GAT TCC GGG GAT CCG TCG ACC
flgK	flgK-red-F	CGC ATG TTC TGC TGA TAC ATC ATT TGT GTA CTA ATA CGC ATC GTT CGG TTC CCT GTG TAG GCT GGA GCT GCT TCG
	flgK-red-R	GCG CTG CCG ATA ATT ATC GTC AGG ACC CGC ATA TGA ATG TTC AAA AGG AAC CTC CAT TCC GGG GAT CCG TCG ACC

VI-3-7. Bacteriophage DNA isolation and purification.

Before isolation of the phage genomic DNA, the phage particles were treated with DNase I and RNase A at 37°C for 30 min to remove any bacterial DNA and RNA, respectively. The overall isolation and purification of the phage genomic DNA of CR5 was conducted according to Wilcox et al. (27) and Sambrook et al. (28).

VI-3-8. Bacteriophage genome sequencing and bioinformatics analysis.

The phage genomic DNA was sheared using a HydroShear DNA shearing machine (Digilab, Holliston, MA, USA) and then sequenced using the Genome Sequencer FLX (GS-FLX) instrument (Roche, Mannheim, Germany) at Macrogen Inc. (Seoul, South Korea). After pyrosequencing, the filtered sequence reads were assembled with Newbler v2.3 (Roche). All of the open reading frames (ORFs) were predicted with bacteria genetic code parameter using the Glimmer v3.02 (29), GeneMarkS (30), and FgenesB software programs (Softberry, Inc. Mount Kisco, NY, USA), and their ribosomal binding sites were confirmed by RBSFinder (J. Craig Venter Institute, Rockville, MD, USA). The annotation and functional analysis of the predicted ORFs was conducted using the BLASTP (31) and InterProScan databases (32). Virulence factor analysis was performed using the Virulence Factor Database (http://www.mgc.ac.cn/VFs/). The complete genome sequence and ORF annotations were handled using Artemis v14 (33).

VI-3-9. Proteomic analysis of the phage structural proteins.

To analyze the total phage protein profiles of phage CR5 using SDS-PAGE,

a purified phage stock (10^{11} PFU/ml) was 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). Then, this sample was treated in boiling water for 5 min, and the denatured phage proteins were subsequently separated using a 12% SDS-polyacrylamide gel. After SDS-PAGE, gels containing eight major visible bands were excised, destained with 50% acetonitrile containing 10 mM NH_4HCO_3, and dehydrated with 100% acetonitrile. The dried gels were rehydrated with 25 mM NH_4HCO_3 and digested with 400 mM trypsin overnight at 37°C. The digested peptides were extracted, dried, and redissolved in equilibration buffer (5% acetonitrile and 0.5% acetic acid). ZipTip C_{18} pipette tips (Millipore, Billerica, MA, USA) were used for the desalting and clean-up of the samples. The MADLI matrix was α-cyano-4-hydroxycinnamic acid (CHCA) in a mixture solution of 50% acetonitrile and 0.1% trifluoroacetic acid. Mass spectrometry was performed using the AB SCIEX TOF/TOF™ 5800 system (Framingham, MA, USA) in the positive reflector mode by the Korea Basic Science Institute (KBSI; Seoul, Korea). The scan parameters in the MS mode were as follows: the mass range was 800 to 4,000 Da, and the total number of laser shots was 400. After the MS scan, fragmentation of the selected precursors was performed at 1-kV collision energy with air. The metastable suppressor mode was selected. The output peptide sequences were searched against a database containing all protein sequences of phage CR5. The protein identification was processed with the ProteinPilot 4.0 software using the Paragon algorithm (AB Sciex, Framingham, MA). Then, the statistical cut-off values were used at the peptide confidence of 95%.

VI-3-10. Food application.

Reconstituted infant formula milk was prepared according to the manufacturer's specifications. Approximately 33.6 g of Premium Goat Infant Formula Stage 1 powder (Ildong Foodis, Seoul, Korea) was resuspended in 240 ml of sterilized water, according to the manufacturer's instruction. Host strain (*C. sakazakii* ATCC 29544 as a clinical isolate, a food isolate 31-3, and their mixture; 10^2 CFU/ml, final concentration) and the phage CR5 at different MOI values (10^4 to 10^5) were added to 20 ml of the prepared infant formula milk. Bacterial cultures without the phage were used as controls. The mixture was incubated with shaking (200 rpm) at 37°C for up to 10 h. The number of viable cells was counted at 2-h intervals using the standard serial dilution and viable cell counting method. All of the tests were conducted in triplicate.

VI-3-11. Nucleotide sequence accession number.

The complete genome sequence of *C. sakazakii*-infecting phage CR5 is available in the GenBank database under accession number JX094500.

VI-4. Results

VI-4-1. Isolation of bacteriophage CR5.

Phage CR5 was isolated and purified from a soil sample from cow farm in Suwon, South Korea, having lytic activity against *C. sakazakii* ATCC 29544. The TEM analysis of phage CR5 revealed that it belongs to the *Myoviridae* family (Fig. 6.1). The diameters of the isomeric head and length of tail were approximately 98 nm and 200 nm, respectively.
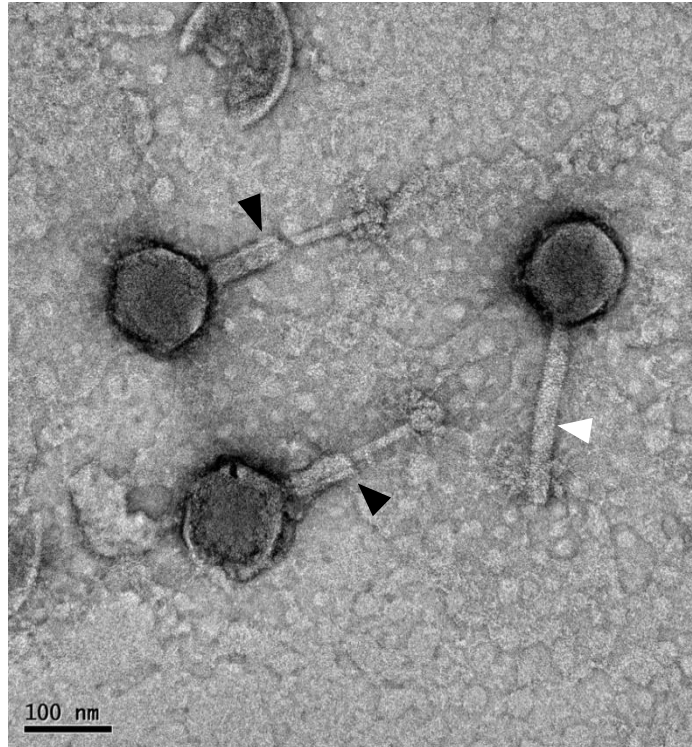
Figure 6.1 TEM image of phage CR5 belonging to the family *Myoviridae*. The black triangles and the white triangle indicate the contracted and non-contracted tails, respectively. The phage were negatively stained with 2% (w/v) uranyl acetate and observed using TEM JEM-2100 (JEOL, Tokyo, Japan) at 200 kV.

VI-4-2. Host range analysis and bacterial challenge test.

The host range analysis of phage CR5 with *C. sakazakii* type strains, *C. sakazakii* isolates from ingredients of infant foods, and other *Cronobacter* species type strains revealed that this phage can infect or inhibit growth of *C. sakazakii* and a few other *Cronobacter* species type strains, indicating its high specificity for infection to *Cronobacter* (Table 6.1). Interestingly, while *Cronobacter* and *Enterobacter* share closely related taxonomic relationship, *E. cloacae* ATCC 7256 and *E. agglomerans* ATCC 27987 showed very weak turbid growth inhibition zones, indicating that phage CR5 does not infect them (Table 6.1). The host lysis activity of phage CR5 was determined using the bacterial challenge method. As soon as phage CR5 (MOI = 1) was added to the culture of *C. sakazakii* ATCC 29544 at $OD_{600 nm}$ = 0.5, the host strain was efficiently lysed, and the host growth inhibition activity was sustained, even after 10 h (Fig. 6.2), indicating that the duration of host growth inhibition activity of the phage CR5 is longer than those of other *C. sakazakii* phages that have been reported (20).
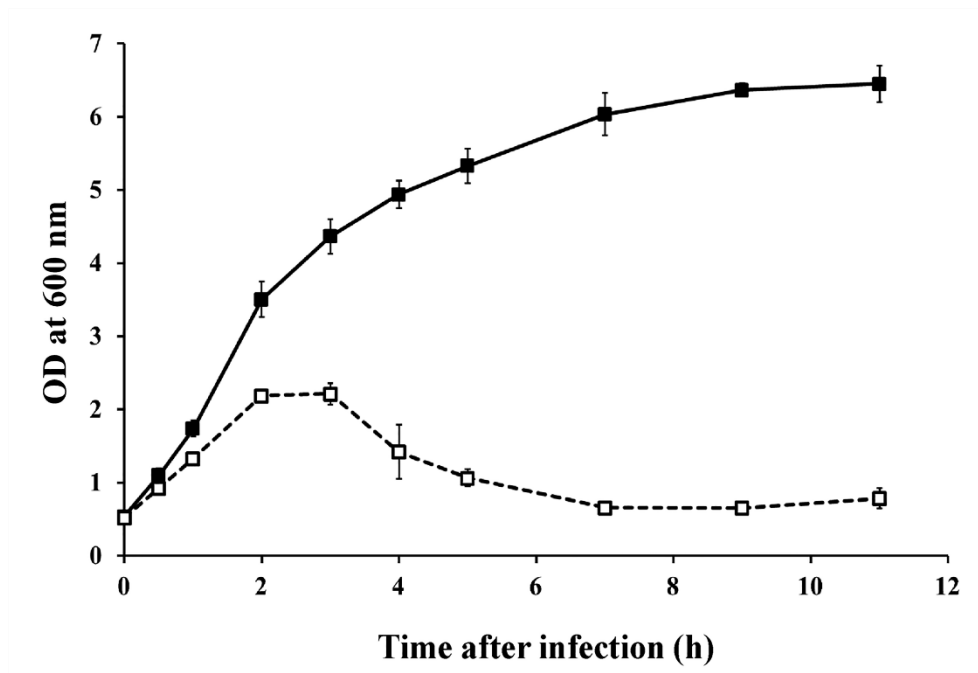
Figure 6.2. Bacterial challenge test of phage CR5 with *C. sakazakii* ATCC 29544 (MOI = 1). The closed square indicates non-phage-treated *C. sakazakii* ATCC 29544, and the open square indicates phage CR5-treated *C. sakazakii* ATCC 29544. Error bars indicate standard deviations in triplicate experiments.

VI-4-3. Identification of the host receptor.

As mentioned previously, phage CR5 infects *C. sakazakii* ATCC 29544 and lyses the host cells (Fig. 6.3A). To determine the host receptor of phage CR5, five well-known host receptor genes, which encode the O-antigen of lipopolysaccharides (LPS), LamB (maltose transporter), OmpC (outer membrane protein), FhuA (ferric ion transporter), and FlgK of flagella (flagellar hook-filament junction protein) were specifically deleted to find the specific *C. sakazakii* ATCC 29544 host receptor of phage CR5 (data not shown), and only the *ΔflgK* mutant showed resistance activity to phage CR5 (Fig. 6.3B), suggesting that the specific host receptor of phage CR5 is the flagellum of *C. sakazakii* ATCC 29544. To confirm this host receptor, a subsequent complementation experiment via the expression of the *flgK* gene was conducted. The *flgK* gene was cloned into the pBAD18 expression vector and expressed in the *ΔflgK* mutant, and the results showed recovery of the sensitivity to phage CR5 and thereby substantiated the finding that the host flagellum is indeed the host receptor of phage CR5 (Fig. 6.3C). Flagella formation or motility has been known to be associated with the virulence of *C. sakazakii* (34) and most resistant bacteria against phages using flagella as a receptor tend to lose their motility (35), suggesting that *C. sakazakii* resistant to the flagella targeting CR5 is expected to be avirulent. Therefore, CR5 could be a good candidate for the control of pathogenic *C. sakazakii*.
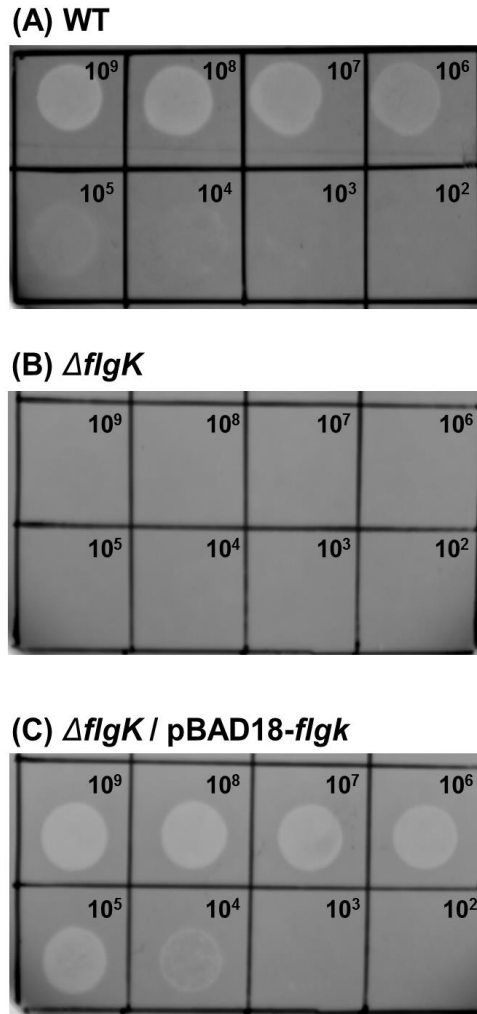
Figure 6.3. Identification of the *C. sakazakii* host receptor by deletion and complementation of the *flgK* gene. (A) Wild-type *C. sakazakii* ATCC 29544. (B) *C. sakazakii* ATCC 29544/*ΔflgK* mutant. (C) *C. sakazakii* ATCC 29544/*ΔflgK* mutant containing pBAD18::*flgK* for complementation. Ten-fold serially diluted phage CR5 samples were dotted from 10^2 to 10^9 PFU/ml in each chamber.

VI-4-4. Genome sequence analysis.

The genome of bacteriophage CR5 contains 223,989-bp DNA with a G+C content of 50.06% and 231 predicted ORFs with no tRNA genes (Fig. 6.4). The average gene length is 912 bp, and the gene coding percentage is 94.1%. The functional ORFs were classified into six groups: structure (major capsid protein, tail fiber protein, tail sheath protein, and many virion structural proteins), packaging (terminase large subunit), host lysis (endolysin), DNA manipulation (nuclease SbcCD D subunit, helicases, endodeoxyribonuclease, and ribonuclease H), transcription (RNA polymerase beta and beta' subunits), and additional functions (N-acetyltransferases, PhoH-like protein, thymidylate synthase, thymidylate kinase, and heat shock protein). However, more than 80% of the predicted ORFs in this genome (185 of the 231 ORFs) remain hypothetical proteins, likely due to insufficient annotation data of *C. sakazakii* bacteriophage genomes in the genome databases.

Because the flagella were identified as the host receptors of phage CR5 in this study, it is likely that the tail fiber protein targets these host flagella for phage infection. This genome has only one gene encoding a tail fiber protein, and it is likely associated with the host specificity of phage CR5. Although the host lysis activity of phage CR5 is strong and consistent, this genome has only one host lysis-related gene encoding endolysin without any genes encoding holin and Rz/Rz1, which is different from other Gram-negative bacteria-infecting phages. The thymidylate synthase and thymidylate kinase of the phage may help the host's folate metabolism as an additional function for the host strain. Interestingly, the phage genome has six phage RNA polymerase beta/beta' subunits, and they did not share protein domains with

each other. Each predicted phage RNA polymerase of CR5 showed high amino acid sequence identities (55-73%) with previously reported RNA polymerase beta/beta' subunits of other two phages, *Salmonella* phage SPN3US and *Erwinia* phage phiEaH2 (36, 37), suggesting that those phages may share the phage gene transcription mechanism (Fig. 6.5). However, the comparative sequence analysis of RNA polymerase beta/beta' subunits between the phage CR5 and the host strain *C. sakazakii* ATCC 29544 revealed that the amino acid sequences of RNA polymerase beta subunits are quite different (data not shown), suggesting that they may have different gene transcription mechanisms. Furthermore, the presence of multi-copies of RNA polymerase beta/beta' subunits in phage CR5 suggests that phage gene transcription may be dominant rather than host gene transcription with a single copy of the host RNA polymerase beta/beta' subunit (36). However, functions of the phage RNA polymerase beta/beta' subunits are still unknown and remain to be elucidated.

terminase large subunit
virion structural protein 21
(A) virion structural protein 20
tail sheath protein
(G) virion structural protein 19
virion structural protein 18
DNA-directed RNA polymerase beta subunit 3
DNA-directed RNA polymerase beta subunit 2
tail fiber protein
virion structural protein 17
ribonuclease H
endodeoxyribonuclease
(D) virion structural protein 16
putative endolysin

possible DNA-directed RNA polymerase beta' subunit
nuclease SbcCD D subunit
DNA-directed RNA polymerase beta subunit 1
DNA-directed RNA polymerase beta' subunit 1
possible helicase
DNA-directed RNA polymerase beta' subunit 2
virion structural protein 1
virion structural protein 2
virion structural protein 3
virion structural protein 4
virion structural protein 5
virion structural protein 6
virion structural protein 7
helicase
major capsid protein (B, C)
virion structural protein 8
virion structural protein 9
virion structural protein 10 (H)
acetyltransferase
PhoH family protein
ribosomal-protein-alanine N-acetyltransferase
thymidylate kinase
GCN5-related N-acetyltransferase 1
GCN5-related N-acetyltransferase 2
thymidylate synthase
heat shock protein ATPase subunit HslU domain-containing protein

Cronobacter sakazakii
Bacteriophage CR5
223,989-bp

(E) virion structural protein 15
virion structural protein 14
virion structural protein 13
virion structural protein 12
virion structural protein 11

(F)

| Structure | Transcription | DNA manipulation | Packaging | Host lysis | Additional function |

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Figure 6.4. Genome map of phage CR5. The inner circle with the red line indicates the GC content. The outer circle indicates the predicted ORFs by strand. The functional categories and annotation of the ORFs are indicated by specific colors according to the legend. Black-colored ORFs indicate hypothetical proteins. A to H letters indicate the major structural proteins. The scale units are base pairs.
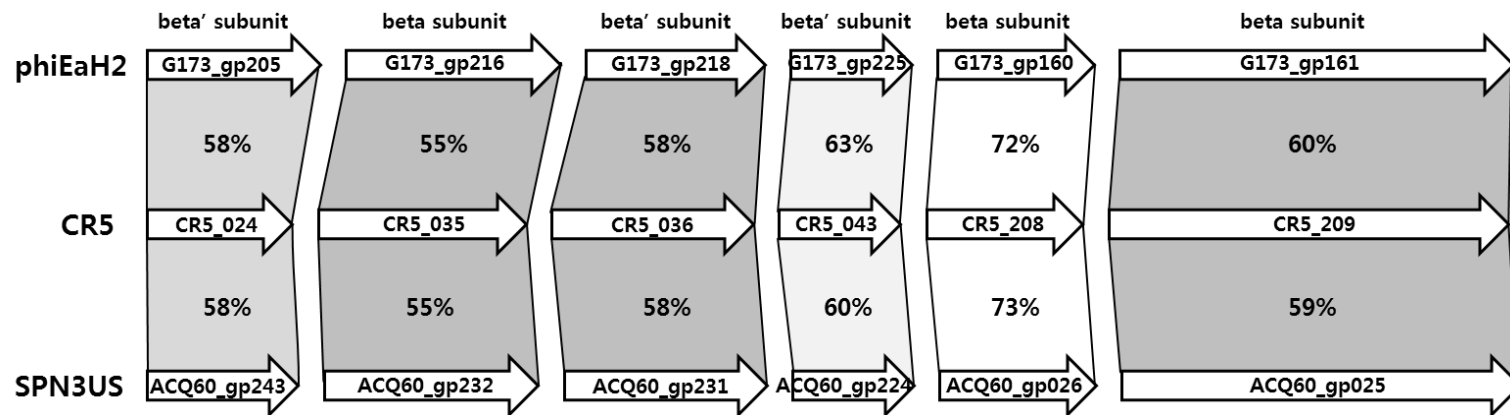
Figure 6.5. Comparative sequence analysis of RNA polymerase beta/beta' subunits in the CR5 phage with those of *Erwinia* phage phiEaH2 and *Salmonella* phage SPN3US. The protein sequence identities are indicated between the arrows. Locus tag of each gene is indicated in the middle of each arrow, respectively.

VI-4-5. Proteomic analysis of the phage structural proteins.

To confirm the expression and identification of the major phage structural proteins, a proteomic analysis using SDS-PAGE and MALDI-TOF/MS was performed. The comparative analysis of these partial peptide sequences with the genome sequence analysis results allowed for the identification of these proteins (Fig. 6.6). Interestingly, eight protein bands were identified as a putative major capsid protein (CR5_071; Band B and C), five putative virion structural proteins (CR5_078; Band H, CR5_158; Band E, CR5_182; Band D, CR5_219; Band G, and CR5_222; Band A), and a hypothetical protein (CR5_137; Band F). Interestingly, one major band (Band C; ~68 kDa) and one minor band (Band B; ~83 kDa) were detected and identified as a putative major capsid protein using protein sequence analysis (Fig. 6.6). The protein sequence analysis predicted the protein size of the putative major capsid protein to be 83.6 kDa (Fig. 6.6B), indicating that the minor band may be the procapsid protein before maturation. Previously, it was reported that the cleavage of the procapsid protein by a proteolysis enzyme is essential for maturation of the major capsid protein (38). Therefore, overall proteomic experiments of phage CR5 may be required to extend our understanding of proteomic characteristics and the maturation process of these structural phage proteins

(A)
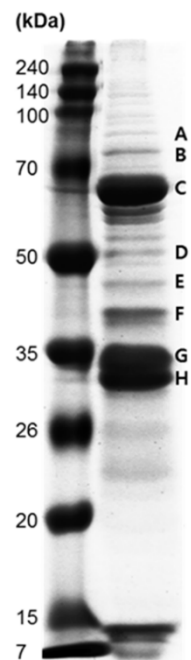
(B)

Band	Locus_tag	Predicted M.W. (kDa)[a]	Predicted pI	Identified peptides	Sequence coverage (%)	Annotation
A	CR5_222	95.3	5.86	1	1.91	Putative virion structural protein 20
B	CR5_071	83.6	5.61	14	26.23	Major capsid protein
C	CR5_071	83.6	5.61	23	35.69	Major capsid protein
D	CR5_182	50.5	5.02	4	12.13	Putative virion structural protein16
E	CR5_158	43.0	5.33	4	13.53	Putative virion structural protein15
F	CR5_137	33.1	4.88	7	24.84	Hypothetical protein
G	CR5_219	32.6	5.48	1	4.45	Putative virion structural protein19
H	CR5_078	32.7	4.96	1	4.44	Putative virion structural protein10

[a], molecular weight

Figure 6.6 Proteomic analysis of the structural proteins of phage CR5 using (A) SDS-PAGE and (B) MALDI-TOF/MS. In the SDS-PAGE, eight major bands were picked and labeled from A to H. Comparing with the genome annotation information of the phage CR5, these major bands were identified with molecular weights and partial peptide sequences using MALDI-TOF/MS. In addition, A to H letters are also added to the CR5 genome map to indicate the locations of major structural proteins (See Fig. 6.4).

VI-4-6. Food application.

To verify the potential of using phage CR5 as a novel biocontrol agent against *C. sakazakii* in food samples, one or two of *C. sakazakii* strains, ATCC 29544 or/and food isolate 31-3, were added to an infant formula milk sample containing phage CR5 at an MOI of 10^4 or 10^5 and the viable cell numbers of *C. sakazakii* in the sample were monitored. After the phage CR5 at an MOI of 10^4 was added to the sample containing the clinical isolate ATCC 29544, the bacterial strain was lysed in 2 h, but recovered. However, after addition at an MOI of 10^5, it was also lysed in 2 h and never recovered up to 10 h, suggesting that the addition of phage CR5 at an MOI of at least 10^5 is required to completely control the *C. sakazakii* ATCC 29544 strain in the sample (Fig. 6.7A). Furthermore, after the phage CR5 at an MOI of 10^4 or 10^5 was added to the sample containing the food isolate 31-3, the bacterial strain was lysed and never recovered, even though the lysis times of CR5 at an MOI of 10^4 and 10^5 were 6 h and 2 h, respectively (Fig. 6.7B).

In addition, a mixed culture of both clinical strain and food isolate was tested with phage CR5. After the phage CR5 at a MOI of 10^4 was added to the mixed culture, growth curve of the host strains is very similar to that of a clinical strain, probably due to the presence of the clinical strain in the mixed culture. The host strains were lysed in 2 h, but regrown (Fig. 6.7C). However, when a MOI 10^5 of phage CR5 was added, the bacterial strains in the mixed culture were lysed in 2 h and never regrown up to 10 h (Fig. 6.7C), which is very similar to both a clinical strain and a food isolate. Therefore, the mixed culture shares characteristics of both strains for the control of *C. sakazakii* using phage CR5. These results suggest that the MOI of CR5 for complete control of both clinical and food isolates is at least 10^5 , which is much

lower than those of previously reported *C. sakazakii* phages (10^7 to 10^9) (20). Therefore, the high host lysis activity of phage CR5 against both clinical and food isolates of *C. sakazakii* in infant formula milk may imply that it could be a possible candidate for the development of a novel biocontrol agent or natural food preservative against *C. sakazakii*.

(A)
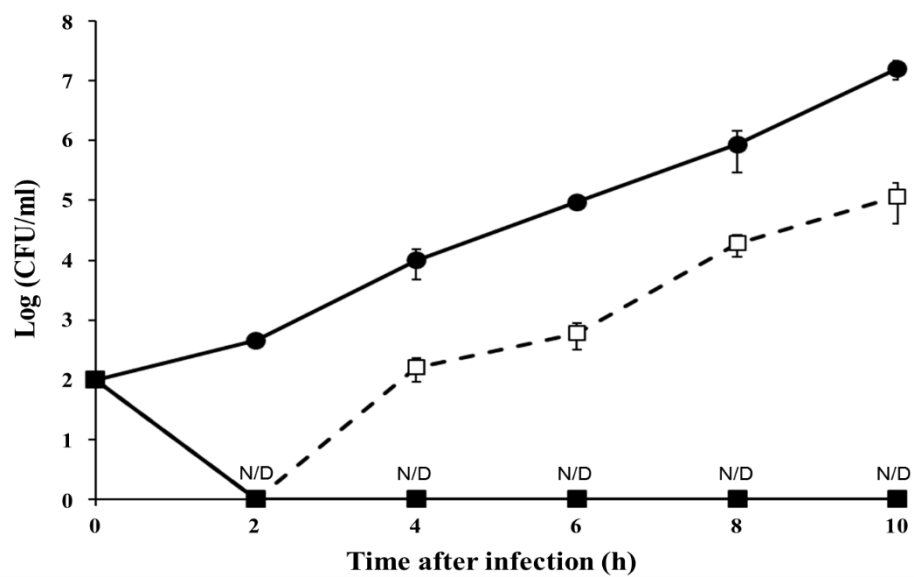
(B)
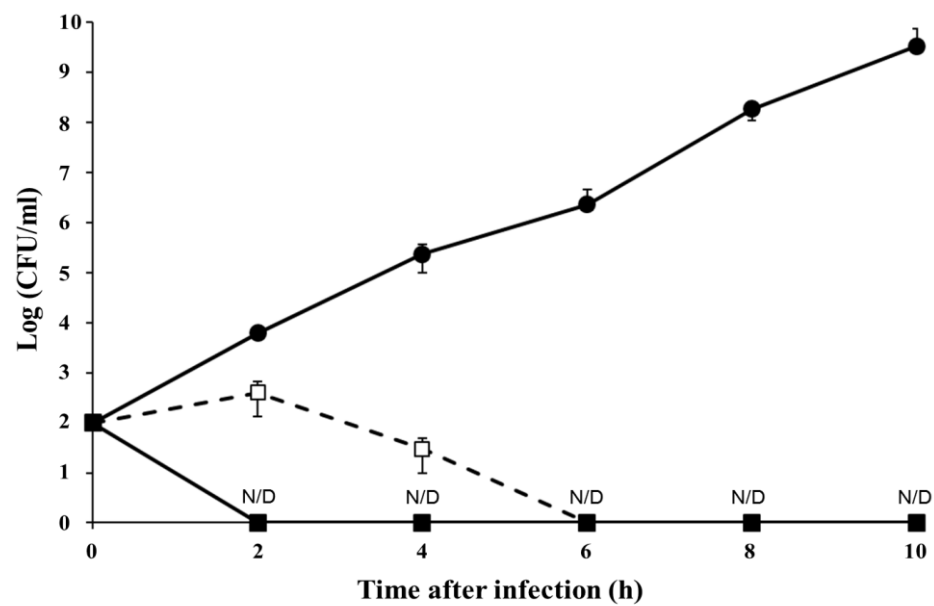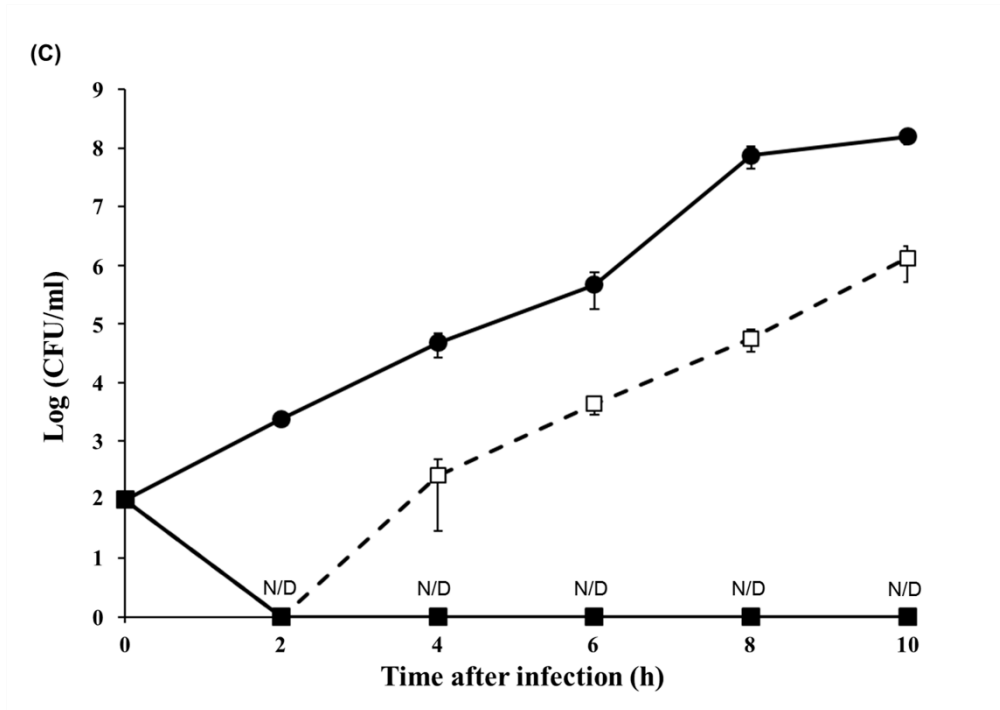
Figure 6.7. Food application of phage CR5 against (A) *C. sakazakii* ATCC 29544, (B) food isolate 31-3, and (C) their mixture in infant formula milk. The closed circles indicate the non-phage-treated samples as negative controls. The open squares with dashed lines indicate the phage CR5-treated samples with an MOI of 10^4, and the closed squares indicate the phage CR5-treated samples with an MOI of 10^5. N/D, not detected.

VI-5. Discussion

C. sakazakii is a fatal food-borne pathogen with high mortality that is generally found in infant milk formula powders (3). Therefore, *C. sakazakii* in foods needs to be urgently controlled. However, a biocontrol agent or a natural food preservative to control this pathogen is not yet available because antibiotic usage is not allowed in foods. The bacteriophage approach has been revisited to control various pathogens because of its bacterial host specificity, bactericidal activity, and human safety (39).

In this study, I isolated and characterized a novel *C. sakazakii*-infecting phage CR5 with the host lysis activity. In addition, the phage CR5 maintained the growth inhibition activity against *C. sakazakii* ATCC 29544 up to over 10 h (Fig. 2). The host growth inhibition activity of the phage CR5 is longer than those of other *C. sakazakii* phages that have been reported (20). The duration of this host growth inhibition activity of phage CR5 in the bacterial challenge assay suggests that phage CR5 may be a candidate as a potential biocontrol agent against *C. sakazakii*. In addition, the host receptor of this *C. sakazakii* phage was identified through specific gene knockout studies and subsequent complementation experiments to understand the host-phage interaction for phage infection. To date, one receptor identification experiment has been conducted and it confirmed that only one *C. sakazakii* phage CR3 infects the host strains using flagella as the host receptors (13).

Interestingly, the genome study of the phage CR5 revealed a few new molecular characteristics. Comparative genome analysis of CR5 with 17 other *C. sakazakii* phages showed extremely low sequence identities, suggesting that they

may not share genomic characteristics (data not shown). The absence of endolysin-supporting proteins (e.g., holin and Rz/Rz1 proteins) in this genome suggests that the single endolysin may lyse the host cells with full function. To make sure there is no holin gene in the CR5 genome, GenBank annotation data of all available 17 *C. sakazakii* genomes were collected and checked if there is any holin gene in the genomes. Only two genomes (ENT47670 and vB_CsaM_GAP161) have holin genes, implying that the presence of holin gene may be not general in *C. sakazakii* phage genomes. DNA sequence alignment analysis of these holin genes with CR5 complete genome sequence showed that there is no DNA sequence match in the genome, indicating that there may be no holin gene in CR5 genome. Subsequent protein domain analysis of these two holin proteins using InterProScan program (32) showed that they have conserved protein domains (PF16083, LydA phage holin, holin superfamily III for holin protein from the phage ENT47670; PF11031, Bacteriophage T holin for holin protein from the phage vB_CsaM_GAP161). Additional InterProScan analysis of all 231 ORFs of CR5 phage genome revealed that there is no gene containing these conserved protein domains, suggesting that there is no holin gene in CR5 genome. However, it is still possible that holin or holin-like gene in *C. sakazakii* phage genomes could be detected when more *C. sakazakii* phage genome annotation results are available in the sequence databases. Interestingly, additional SignalP analysis of endolysin proteins in four *C. sakazakii* phage genomes without holin genes (CR5, ESP2949-1, phiES15, and vB_CskP_GAP227) revealed that they have signal peptides in the N-terminus for secretion without holin, supporting this (data not shown). In addition, phage CR5 genome has six copies of RNA polymerase beta/beta' subunits. The presence of

multi-copies of RNA polymerase beta/beta' subunits in the genome and the lack of similarity of RNA polymerase beta/beta' subunits between the phage and host genomes suggest that the phage has different gene transcription mechanisms and phage gene transcription may be dominant rather than that of the host. To further understand the functions of multi-copies of RNA polymerase beta/beta' subunits in the genome of phage CR5, three different *Pseudomonas* phage genomes, including 201phi2-1, phiKZ, and phiPA3 (40-42), were analyzed and compared. Pfam protein domain analysis of genes encoding RNA polymerase beta' subunits in two phages 201phi2-1 and phiPA3 revealed that a gene encoding the RNA polymerase beta' subunit in each phage genome (gp275 of 201phi2-1 and ORF54 of phiPA3) has a highly conserved protein sequence motif, Rbp1_domain_2 (NA**DFDGD**), associated with Mg^{2+} binding(40, 42), while no Rbp1_domain_2 motif was detected in the phage phiKZ. Three aspartic acid (D) residues in the motif have been suggested to be necessary for holding Mg^{2+} in the active center of the RNA polymerase beta' subunit. These Mg^{2+} ions in the active center were reported to attract negatively charged phosphate of NTPs and to allow it to interact with the 3'-OH end of the RNA transcript for RNA elongation (43). Interestingly, the gene encoding the RNA polymerase beta' subunit (CR5_024 of CR5) also contains this Rbp1_domain_2 motif in the protein sequence, suggesting that this RNA polymerase beta' subunit may be associated with RNA elongation by Mg^{2+} binding in the active center. Therefore, the RNA polymerase beta/beta' subunits of phage CR5 may play similar roles to those of two *Pseudomonas* phages in RNA transcription and elongation.

To evaluate the lysis activity of phage CR5 against *C. sakazakii* by bacterial challenge assay for food application, it was added to infant formula milk containing

C. sakazakii clinical or/and food isolates (ATCC 29544 or food isolate 31-3). Surprisingly, phage CR5 controlled both isolates completely up to 10 h at only MOI of 10^5, which is much lower than those of previously reported *C. sakazakii* phages (20), suggesting that phage CR5 is a good candidate for biocontrol agent against both clinical and food isolates in infant formula milk. However, these food applications using CR5 phage is just a model experiment with specific food conditions to control *C. sakazakii* in infant formula. For real industrial applications, cheap phage purification and concentration method as well as optimized food application method should be developed. In addition, although US FDA approved the phage application to foods as natural food preservatives or as biocontrol agent, customers may not prefer to using phage in some sensitive foods including milk formula. Therefore, food applications to control food-borne pathogens using phage need to be considered carefully. This study provides the characteristic and genomic insights of phage CR5 for further development of a novel phage biocontrol agent against *C. sakazakii* in foods.

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Appendix 2:

A Review of

Bacteriophages and Endolysins applications

Biocontrol and Rapid Detection of Food-borne Pathogens Using Bacteriophages and Endolysins

(Published in Frontiers in Microbiology, 2016)

VII-1. Abstract

Bacteriophages have been suggested as natural food preservatives as well as rapid detection materials for food-borne pathogens in various foods. Since *Listeria* monocytogenes-targeting phage cocktail (ListShield) was approved for applications in foods, numerous phages have been screened and experimentally characterized for phage applications in foods. A single phage and phage cocktail treatments to various foods contaminated with food-borne pathogens including *E. coli* O157:H7, *Salmonella enterica, Campylobacter jejuni, Listeria monocytogenes, Staphylococcus aureus, Cronobacter sakazakii,* and *Vibrio* spp. revealed that they have great potential to control various food-borne pathogens and may be alternative for conventional food preservatives. In addition, phage-derived endolysins with high host specificity and host lysis activities may be preferred to food applications rather than phages. For rapid detection of food-borne pathogens, cell-wall binding domains (CBDs) from endolysins have been suggested due to their high host-specific binding. Fluorescence-tagged CBDs have been successfully evaluated and suggested to be alternative materials of expensive antibodies for various detection applications. Most recently, reporter phage systems have been developed and tested to confirm their usability and accuracy for specific detection. These systems revealed some

advantages like rapid detection of only viable pathogenic cells without interference by food components in a very short reaction time, suggesting that these systems may be suitable for monitoring of pathogens in foods. Consequently, phage is the next-generation biocontrol agent as well as rapid detection tool to confirm and even identify the food-borne pathogens present in various foods.

VII-2. Introduction

Food safety is one of the major concerns due to threatening human health by various food-borne pathogens. Every year in the United States, about 9.4 million cases of foodborne illness with about 56,000 hospitalizations and 1,300 deaths caused by major food-borne pathogens including *Salmonella*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Campylobacter* have been reported (1). Because of food contaminations by pathogens, about 25% of their food productions were lost in food industries every year (2). In general, control of these food-borne pathogens has been done using various natural or chemical food preservatives. Natural preservatives such as organic acids, bacteriocins, chitosan, and lactoferrin have tendency to exhibit weak and limited antimicrobial activities (3). However, consumers generally do not prefer chemical preservatives due to their known side effects (4). Furthermore, while antibiotics have strong and stable antimicrobial activities, they are not allowed for applications in foods.

Bacteriophages are bacterial viruses with host specificity and lysis activities, indicating that they can infect and lyse the specific host bacteria for their replication and propagation (5). Therefore, bacteriophages have been suggested as natural biocontrol agents against food-borne pathogens without any harm to human cells, indicating their safety (6). In general, phages containing double-stranded DNA genomes have specific host cell wall lysis enzymes called endolysin for bacterial host lysis (7). This enzyme has two protein domains, peptidoglycan-hydrolyzing enzymatic activity domain (EAD) for host cell lysis and cell wall binding domain

(CBD) for specific host recognition (8). In general, endolysin is externally added to lyse gram-positive bacteriaand the related animal study showed no side effect, suggesting that it should be safe for human (9-11). Due to these distinct characteristics, endolysin has been considered as a novel type natural food preservative against food-borne pathogens (12).

In addition to the biocontrol of food-borne pathogens in foods using phage or endolysin, their rapid detection is also important in the prevention of food-borne outbreaks (13). For the rapid detection without enrichment step of food-borne pathogens, PCR- and antibody-based rapid detection methods have been developed and broadly used. However, these methods have some limitations including detection limit (antibody) and requirement of long amplification time (PCR) (14-16). To reduce these limitations, phage-derived CBD and genetically engineered reporter phage have been newly proposed and considered for rapid detection of food-borne pathogens in foods (16, 17). These new rapid detection methods can overcome limitations of conventional detection methods and enhance the detection limit and sensitivity in foods (18, 19). In addition, these novel rapid detection methods could be used for monitoring of pathogens in foods. Therefore, these new technologies would provide novel approaches for rapid detection of food-borne pathogens in food environments.

This review is focused on the biocontrol and rapid detection of various food-borne pathogens in foods using phages and their derivatives including endolysin, CBD, and reporter phage. Therefore, general features and various food applications of phages and endolysins for biocontrol of food-borne pathogens would be explained and discussed in this review. In addition, CBD and reporter phage

would be reviewed as a novel type of rapid detection and monitoring of food-borne pathogens with most recent study cases. This review would provide novel insights into applications of phages and their derivatives for efficient biocontrol and rapid detection of various food-borne pathogens in highly complexed food environments.

VII-3. Bacteriophage Biology

VII-3-1. General features and phylogeny

Bacteriophages are the most abundant microorganisms on Earth, and also have the ability to infect bacteria. Basic structure of phages in the order *Caudovirales* consists of two parts: phage head and its tail. The phage head contains a genetic material in a form of DNA or RNA (20). Linked to the phage head, the phage tail generally plays roles in recognition and adsorption of the specific bacterial host receptor (21).After binding to the host bacterium, phage injects its genetic material into the host cytosol via tail structure by diffusion, osmotic pressure, or transport by specific protein (22, 23). The injected genetic material undergoes host genome integration for lysogenic cycle or replication for lytic cycle. During the lytic cycle, structural proteins are produced from encoded genes in the phage genome. After replication of the genetic material and production of structural proteins, progeny phages are assembled with them and released from the host bacterium (5, 24).

Since the first bacteriophage was discovered and characterized by Felix d'Herelle in 1917 (25), tailed bacteriophages in the order *Caudovirales* are the most abundant (about 96% of all phages). This order consists of three major families

including *Siphoviridae, Myoviridae* and *Podoviridae* with different morphological characteristics. Among the reported phages to date, phages in the *Siphoviridae* family are the most abundant (61.6% of all phages) with long flexible noncontractile tails ranged from 79 to 539 nm. The phages in the *Myoviridae* family are the second most abundant (24.5%) and they have larger heads ranged from 53 to 160 nm in comparison to those of other two families. Moreover, contractile tails give *Myoviridae* its unique characteristics. The phages in the *Podoviridae* family (13.9%) have a distinctly short non-contractile tail ranged from 3 to 40 nm (26).

VII-3-2. Phage therapy

The first clinical study using phages was a direct phage injection in six patients with staphylococcal boils in 1921 (27). Since then, phages have been used to cure various diseases caused by bacterial infections for several decades in Eastern Europe. However, antibiotics have been widely used for the same purpose in other parts of the world and this resulted in the emergence of antibiotic-resistant bacteria. Therefore, it has been big issue how to control these antibiotic-resistant bacteria. Because phage has recently attracted the public attention due to its high host specificity and efficient host lysis, phage therapy has been revisited to control these problematic bacteria in Western Europe (20).

To date, numerous clinical phage trials have been reported against various pathogens including *E. coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeroginosa,* and *Salmonella* Typhimurium. As an example, in Poland, 550 patients with gastrointestinal, head, neck, and skin infections caused by these

pathogens were successfully treated and symptoms of 506 patients (92%) were relieved (24). In addition, in Russia, 1,340 patients with conjunctivitis, dermatitis, pharyngitis, and rhinitis were divided into three groups for different treatment regimens: phage treatment (360 patients), antibiotics treatment (404 patients), and combination (576 patients) (24). Interestingly, the phage-treated and the combination groups were clinically improved up to 86% and 83%, respectively. However, the antibiotics-treated group showed minor improvement up to 48%, suggesting that phage therapy may be effective to control these pathogens but combination of phages and antibiotics did not show synergistic effect. In Ireland, ten methicillin-resistant *S. aureus* (MRSA) DPC5246-infected human hands were soaked in a solution containing 10^8 PFU/ml of a single phage K, revealing 2 log reduction, suggesting that MRSA can be controlled using a specific phage (28, 29). Based on the clinical studies, many commercial phage therapy products were developed and produced in Eastern Europe, including "Phagestaph" (JSC Biochimpharm, Tbilisi, Georgia), "*E.coli* bacteriophage" (Microgen, Moscow, Russia), and "Complex pyobacteriophage" (Microgen). In addition, other countries have many phage therapy-related companies producing commercial products: USA (Elanco Food Solutions, Gangagen Inc., Intralytix, Neurophage Pharmaceuticals, New Horizons Diagnostics, OmniLytics Inc., Phage International, Targanta Therapeutics, Viridax), UK (AmpliPhi Biosciences Corporation, Blaze Venture Technologies, BigDNA, Novolytics, Phico), Georgia (Biopharm Ltd., JSC Biochimpharm, Phage Therapy Center), Australia (Special Phage Services Pty, Ltd.), Canada (Biophage Pharma Inc.), Germany (Hexal Genentech), India (Gangagen Biotechnologies PVT Ltd.), Ireland (Phage Works), Israel (Phage Biotech Ltd.), Portugal (Innophage), South

Korea (CJ CheilJedang Corporation) and the Netherlands (EBI Food Safety) (30). Consequently, phage therapy would provide novel insights and approaches to overcome the limitations of antibiotics and biocontrol of various antibiotics-resistant bacteria without any side effect in humans.

VII-3-3. Food applications

In addition to the phage therapy, phages can be used for biocontrol of various food-borne pathogens. The advantage of phage applications in foods is efficient inhibition of food-borne pathogens as well as no harm to human. In addition, antibiotics are not allowed for food applications. Therefore, food application using phages could be a good alternative approach for biocontrol of food-borne pathogens in foods. ListSheild (Intralytix, Inc., Baltimore, MD, USA), a cocktail of six phages targeting *L. monocytogenes*, was first approved by the United States Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA)'s Food Safety and Inspection Service (FSIS) for applications in foods in 2006 and re-approved as GRAS status by FDA in 2014. In addition, EcoShield (Intralytix), a cocktail of three phages (ECP-100) targeting *E. coli* O157:H7, was also approved by FDA and FSIS for food applications in 2011. Listex P100 (Micreos Food Safety, Wageningen, The Netherlands), a single phage targeting *L. monocytogenes*, was approved as GRAS status by FDA in 2006. Recently, SalmoFresh (Intralytix), a cocktail of six *Salmonella*-targeting phage, was also approved as GRAS status by FDA in 2013 (31). Therefore, these phage products are allowed to use in foods as food preservatives to control specific food-borne pathogens. In addition to direct food applications of

phages, they can be used to prevent cross contamination of pathogens in food-contact materials as well as food processing facilities (32). Furthermore, phages can be used to sanitize human hands and utensils. Therefore, phage applications would be useful for extension of food preservation periods and food safety.

VII-4. Biocontrol of food-borne pathogens using phages and endolysins

VII-4-1. Phage applications

As discussed previously, phages can control food-borne pathogens by host recognition, infection, and lysis. In this section, phage applications would be explained and discussed with various research reports of each host pathogenic bacterium. Various phage applications in foods are summarized and listed in Table 7.1.

Table 7.1. Examples of phages used in studies related to pathogen reduction in foods

Host	Phage	Applications	Results	Ref
E.coli **O157:H7**	FAHEc1	thinly sliced beef pieces	2.7 log reduction occurred with 3.2×10^7 PFU/4 cm^2 treatment	(33)
	DT1, DT6 (phage cocktail)	cow meat	2.2 log reductions at 5°C after 24 h	(34)
	DT1, DT6 (phage cocktail)	milk fermentation	3.0 log reduction (total inactivation)	(35)
	ECP100 (3 phages cocktail)	hard surfaces	reduction of 99.99%, 98%, and 94% of viable cell number with 10^{10}, 10^9, and 10^8 PFU/ml treatment , respectively.	(36)
	ECP100 (3 phage cocktail)	tomato slice, spinich, ground beef	(tomato slice) reduction of 99%, 94%, and 96% of viable cell number during storage at 10°C for 24 h, 120 h, and 168 h, respectively (spinich) 100% reduction of viable cell number during storage at 10°C for 24 h and 120 h 99% reduction of viable cell number during storage at 10°C for 168 h (ground beef) 95% reduction of viable cell number during storage at 10°C for 24 h	(36)
	e11/2, e4/1c	ex vivo rumen model	(phage e11/2) reduction below the detection limit within 1 h (Phage e4/1c) reduction of bacterial cell numbers within 2 h	(37)
	e11/2, e4/1c (phage cocktail)	hide samples	2.02 log10 CFU/cm2 reduction after 1 h	(38)
	e11/2, e4/1c, PP01 (phage cocktail)	beef	reduction of *E. coli* O157:H7 counts less than 10 CFU/ml at 37°C after 1h	(39)
	BEC8	Leafy green vegetables	reduction of 10^6 CFU/ml of *E. coli* O157:H7 with simultaneous treatment of BEC8 (approx. 10^6 PFU/leaf), and trans-cinnamaldehyde (TC) (0.5% v/v) at both 23°C and 27°C after 24 h	(40)
	BEC8	Materials typically used in food processing surfaces	more than 3 log CFU reduction at temperatures above 12°C within 10 min on all 3 surfaces	(41)
Salmonella enterica	F01-E2	Hot dogs, cooked and sliced turkey breast, mixed seafood, chocolate milk and egg yolk	no viable bacteria were detected after treatment at 8°C during storage 2-5 log suppression of *Salmonella* growth was suppressed at 15 °C during storage	(42)
	wksl3	chicken skin	2.5 log reduction in the number of bacteria from day2 to day7	(43)
	PhageA, PhageB	broccoli seeds	1.5 log suppression of *Salmonella* growth	(44)

	(phage cocktail)			
	PA13076, PC2184 (phage cocktail)	chicken breast, pasteurized whole milk and Chinese cabbage	(in all tested foods) reduction of 1.5–4 log CFU/sample	(45)
	UAB_Phi20, UAB_Phi78, UAB_Phi87	pig skin, chicken breasts, packaged lettuce, Fresh egg	(pig skin) 4 log reduction for *S.Typhimurium* and 2 log reduction for *S. Enteritidis* at 4°C for 7 days (chicken breasts) 2.2 log reduction for *S. Typhimurium* and 0.9 log reduction for *S.Enteritidis* at 4°C for 7 days (packaged lettuce) 3.9 log reduction for *S. Typhimurium* and 2.2 log reduction for *S.Enteritidis* at room temperature for 60 min (fresh egg) 0.9 log reduction for both *S. Typhimurium* and *S. Enteritidi*s	(46, 47)
	PC1	pig skin	reduction of 4.1–4.3 log10 CFU	(48)
	F1055S, F12013S	fertile eggs	reduction of the disease symptoms in the chicks	(49)
Campylobacter jejuni	phi2	chicken skin	more than 4 log reduction at 4°C	(50)
	phiCcoIBB35, phiCcoIBB37, phiCcoIBB12 (phage cocktail)	Administration to chicken	reduction of both *C. coli* and *C. jejuni* in feces by approximately 2 log10 CFU/g	(51)
	C220	Broiler Chicken	2 log CFU/g reduction in cecal colonization number of *C. jejuni*	(52)
	CP8, CP34	Broiler Chicken	0.5 to 5 log10 CFU/g reduction of cecal contents over a 5-day period	(20)
	NCTC12673	Chicken surface	95% reduction in the chicken portions at 4°C after 24h	(53)
	Phage Cj6	cooked and raw meat	(cooked meat) 2.8 log reduction in bacteria number (raw meat) 2.2 log reduction in bacteria number	(54)
Listeria monocytogenes	A511	(liquid foods) chocolate milk and mozzarella cheese brine (solid foods) hot dogs, sliced turkey meat, smoked salmon, seafood, sliced cabbage and lettuce leaves	(liquid foods) reduction below the detection level at 6°C after 6 day (solid foods) 5 log reduction at 6°C after 6 day	(55)
	A511	soft cheese	6 log reduction for 22 days	(56)
	P100	cheese	complete eradication or at least 3.5 log reduction for 22 day	(17)
	P100	melon slice	(melon slice)	(57)

		pear slice apple slice	1.5 log reduction in bacteria number , (pear slice) 1.0 log reduction in bacteria number (melon juice) 8.0 log reduction in bacteria number (pear juice) 2.1 log reduction in bacteria number (apple slice or juice) no significant reduction in bacteria number	
	FWLLm1	ready-to-eat chicken breast roll	2.5 log reduction and no re-growth at 5°C over 21 days	(58)
	LM103, LMP-102 (Phage cocktail)	honeydew melon, golden delicious apple	(honeydew melon) 2.0 to 4.6 log reduction (golden delicious apple) 0.4 log reduction	(59)
	LMP-102	honeydew melon	reduction of viable cell number to non- detectable levels immediately after treatment and suppressed growth of the pathogen at 10°C throughout the storage period of 7 days	(60)
Staphylococcus aureus	IPLA35, IPLA88	fresh type cheese, hard type cheese	(fresh type cheese) 3.8 log CFU/g reduction in 3 h, viable cell counts were under the detection limits after 6 h (hard type cheese) 4.6 log CFU/g reduction during ripening period and only 1.2 log CFU/g of viable cell was detected at the end of ripening period	(61)
	two kinds of phage cocktails (TEAM/P68/L H1-MUT and phi812/44AHJ D/phi2)	cheddar cheese	eradication of 10^6 CFU/g *S. aureus* at 4°C after 14 days	(62)
Cronobacter sakazakii	ESP 732-1, ESP 1-3	reconstituted infant formula	(phage ESP 732-1) eradicating *C.sakazakii* at 12°C, 24°C, and 37°C (phage ESP 1–3) eradicating *C. sakazakii* at 24°C.	(63)
	CR5	reconstituted infant formula	reduction of 10^2 CFU/ml with an MOI of 10^5 of phage for 10 h.	(64)
Vibrio spp.	VPp1	oyster rearing system	2.35–2.76 log CFU/g reduction of *V. parahaemolyticus* numbers in oysters at 16 °C within 36 h	(65, 66)
	Vpms1	brine shrimp	effectively prevent vibriosis in brine shrimp even with and MOI of 0.45.	(67)
	pVp-1	oysters	reduction of 3.3 log CFU/g	(68)

VII-4-1-1. *E. coli* O157:H7

   E. coli O157:H7 belongs to the Shiga toxin producing *E. coli* (STEC), a major food-borne pathogen causing hemolytic uremic syndrome and acute renal failure with an extremely low dose (about 10^1 cells) (69, 70). It is generally ingested by consumption of contaminated, undercooked beef and sometimes fresh fruit juices (30, 71). Therefore, control of *E. coli* O157:H7 in foods is important for prevention of food-borne outbreaks.

E. coli O157:H7-targeting phages and their host inhibition activities have been reported. Phage FAHEc1 (10^7 PFU/ml) applied to *E. coli* O157:H7 and sliced meat piece demonstrated 4 log reduction at 5°C and 2-3 log reductions at 37°C, respectively (33). To enhance the host growth inhibition and lysis activities, phage cocktails were prepared with phages DT1 and DT6. This phage cocktail treatment showed 6.3 log reduction of *E. coli* O157:H7 and minimized the appearance of Bacteriophage Insensitive Mutants (BIMs). Application of this phage cocktail or DT6 to a beef sample at 24°C for 6 h revealed that the phage cocktail treatment (2.58 log reduction) was more effective than DT6 treatment alone (0.74 log reduction), suggesting that phage cocktail is more effective for control of food-borne pathogen than a single phage (34). Comparative phage experiments with a phage cocktail and a single phage DT1 with *E. coli* O157:H7-contaminated milk samples support the efficiency of the phage cocktail (35). Further phage cocktail experiment with eight different virulent phages (BEC8) and 123 different *E. coli* O157:H7 strains showed that >94% of the tested strains were inhibited in the host range tests (41), indicating that virulent phages could inhibit the growth of *E. coli* O157:H7 effectively. This suggests the effectiveness of phage treatment to control *E. coli* O157:H7 in foods.

For phage applications to *E. coli* O157:H7 in foods, phage should be stable under various stress conditions including temperature, pH, water activity, and salt stress. Two different phages e11/2 and e4/1c were tested under these stress conditions, showing that they were stable under pH 4-10, -22°C to 60°C, and 1-2.5% NaCl concentration. Interestingly, phage stability test under various water activity levels showed that phage e4/1c was more stable than phage e11/2 (38). *E. coli* O157:H7-targeting phages showed that they are highly stable for survival under various food conditions and can effectively control the pathogen in foods with a form of phage cocktail.

VII-4-1-2. *Salmonella enterica*

Salmonella can cause disease so-called non-typhoidal salmonellosis, the most common food-borne disease with symptoms like common gastroenteritis, enteric fever and ulceration (30). *Salmonella* has been widely detected in various animal-based foods (72). However, it is very difficult to control in the food environment. Although natural and chemical food preservatives have been used for prevention of food-borne pathogen contaminations, they are not specific for *Salmonella*. As previously explained, phages have been approved as novel type food preservatives by the US FDA (31) and the characteristics of phages are high host specific with lysis activities (73). Therefore, phages have been interesting subjects for biocontrol of *Salmonella* in foods (74).

S. Typhimurium-targeting phage F01-E2 was tested for food applications including hot dogs (Wiener sausages), cooked and sliced turkey breast (deli meat,

cold cuts), mixed seafood (cooked and chilled cocktail of shrimps, shellfish, and squid), chocolate milk (whole milk with cocoa and sugar added), and egg yolk (pasteurized). Interestingly, this phage (3 x 10^8 PFU/gram of each food sample) was treated to *S.* Typhimurium-contaminated Ready-To-Eat foods and then they were stored at 8°C for 6 days. After storage, no bacterial host was detected in all RTE foods, indicating that a single treatment of phage may be enough to reduce *S.* Typhimurium in RTE foods during storage even at low temperature (42). In addition, the broad host range phage wksl3 (10^7 PFU/ml) was treated to chicken skin contaminated with *S.* Enteritidis (10^3 CFU/cm^2 skin) at 8°C for 7 days, showing 2.5 log reduction (43). Its genome analysis revealed that it does not have toxin, virulence factors, food allergen-related proteins, as well as lysogen-related gene clusters, suggesting that this phage should be safe for food and human trials. Administration of high dose phage (10^{11} PFU/kg mouse body weight) to mice showed that no death or clinical pathogenicity signs were observed (43).

To enhance the host lysis activity, *Salmonella* phage cocktails were prepared and tested. A single phage and a phage cocktail of two virulent phage, PA13076 and PC2184, were applied to three different foods (chicken breast, pasteurized whole milk, and Chinese cabbage) contaminated with *S.* Enteritidis, showing that a phage cocktail treatment exhibited more effective host lysis activity (4 log reduction) than a single phage treatment (2-3 log reductions) in milk (45). Furthermore, *Samonella* phage cocktails containing three phages (UAB_Phi20, UAB_Phi78, and UAB_Phi87) and four phages (Felix01, phiSH19, phiSH17, and phiSH18) also showed high host lysis activities to control *Samonella* in foods and animals (46, 48, 75).

VII-4-1-3. *Campylobacter jejuni*

      C. jejuni is one of leading causes of zoonotic diseases over the world and about 400–500 million cases of diarrhea are reported each year (76). Major sources of *C. jejuni* are poultry-originated foods (77). To control them, phage treatment has been proposed   (53, 78).

      Since poultry-originated foods are recognized as a reservoir of *C. jejuni*, most of the phage applications have been focused to reduce bacterial contamination of poultry skin and to inhibit bacterial colonization in poultry intestines. When *C. jejuni* C222 (10^4 CFU/cm^2)-contaminated chicken skin was treated with a single *C. jejuni* phage NCTC 12673 (10^6 PFU/cm^2), 95% of the contaminated *C. jejuni* was reduced. (53). In addition, application of *C. jejuni* phage phi2 on the chicken skin showed 2 log reduction. Interestingly, phage phi2 was able to survive on the chicken skin for 10 days, indicating that this phage is very stable and suitable to control *C. jejuni* on poultry (50).

      Treatment of the phage cocktail (CP8 and CP34) revealed 5 log reduction in *C. jejuni* colonization in bird intestines (79). In addition, a cocktail of three phages (phiCcoIBB35, phiCcoIBB37and phiCcoIBB12) was administered to chicken containing *C. jejuni* and *Campylobacter coli* by oral gavage and feeding, resulting in a 2 log reduction in the fecal sample (51). Interestingly, the cocktail of three phages had a broad lytic spectrum against *Campylobacter*, because three phages showed different and complementary lytic spectra against *C. coli* and *C. jejuni* strains (51).

VII-4-1-4. *Listeria monocytogenes*

The primary source of *Listeria* are ready-to-eat (RTE) foods, generally preserved in refrigerators, because it can survive and grow in the cold environment. In particular, *L. monocytogenes* is associated with listeriosis outbreaks over the world (80). Although listeriosis is not as common as other food-borne illness, its relatively high fatality rate (about 45%) is a major concern worldwide (81). Therefore, *L. monocytogenes* should be controlled in foods.

L. monocytogenes phage A511 was treated to liquid foods (chocolate milk and mozzarella cheese brine) as well as solid foods (hot dogs, sliced turkey meat, smoked salmon, seafood, sliced cabbage and lettuce leaves). Interestingly, phage A511 treatment to liquid foods reduced the bacterial cells below the detection limit at 6°C for 6 days and the treatment to solid foods also showed 5 log reductions in the same conditions (82). Moreover, A511-like phage FWLLm1 also showed 2.5 log reduction, suggesting that these phages have strong host lysis activity (58). After the FDA approval of phage application in foods (31), several commercial phage products for food applications were introduced. Listex P100 (Micreos Food Safety) was evaluated to treat cheese during ripening. While this treatment resulted in 3.5 log reduction, high multiplicity of infection (MOI) treatment ($>10^8$) was demonstrated to completely eradicate *L. monocytogenes* in cheese (83). In addition, an animal study confirmed that Listex P100 had no toxic effect in animals, suggesting its high safety (83). A cocktail containing two phages, LM103 and LMP-102, exhibited 2 to 4.6 log reductions in honeydew melon. However, the treatment on apple slices did not show reduction in *L. monocytogenes* and this implies that biocontrol of *L. monocytogenes* using phage cocktails may depend on the kinds of foods despite high

2 8 6

host specificity and host lysis activity (57).

VII-4-1-5. *Staphylococcus aureus*

S. aureus is generally found in various foods including sliced meat, salads, pastries, unpasteurized milk, and cheese products. It has been known that this bacterium produces heat stable enterotoxins causing food poisoning such as nausea, vomiting, stomach cramps, and diarrhea (84). Moreover, emergence of multidrug-resistant *S. aureus* (MRSA) suggests that alternative biocontrol agent need to be developed to replace the use of antibiotics for *S. aureus* treatment (84, 85).

Interestingly, *S. aureus* phage K is capable of replicating in heat-treated milk but not in raw milk. This is due to heat-labile immunoglobulins preventing adsorption of phage to *S. aureus* in raw milk. Thus, it is suggested that *S. aureus* phages should be applied after heat treatment of milk and milk-associated products (85). In addition, treatment with a cocktail containing two phages (IPLA35 and IPLA88) on fresh and hard cheeses during curdling process resulted in 3.83 and 4.64 log reductions of *S. aureus*, respectively (61). Despite the cocktail's effectiveness in controlling *S. aureus*, it had no effect on cheese starter strains nor did it alter the chemical properties of cheeses (61). Furthermore, two kinds of phage cocktails (TEAM/P68/LH1-MUT and phi812/44AHJD/phi2) were treated on cheddar cheese curd samples. Interestingly, both phage cocktails completely eradicated a 10^6 CFU/g of *S. aureus* population at all MOI levels tested (15, 45, and 150) without phage titer reduction. Furthermore, there was no stress-induced enterotoxin C overproduction by *S. aureus* upon phage treatment, implying that phage cocktail application has

potential as a *S. aureus*-targeting biocontrol strategy in foods (62).

VII-4-1-6. *Cronobacter sakazakii* and *Vibrio* spp.

      C. sakazakii is often detected in infant milk powder and is well-known to cause bacteremia, meningitis, and necrotizing enterocolitis. In general, newborn infants are highly susceptible to *C. sakazakii* infection with high fatality rate (86). To evaluate food applications of two different *C. sakazakii* phages, ESP 732-1 and ESP 1-3, were treated to the infant milk formula at three different temperatures (12, 24 and 37°C). Interestingly, phage ESP 732-1 at MOI of 10^7 eliminated 10^2 CFU/ml of *C. sakazakii* strain at all tested temperatures, while phage ESP 1-3 inhibited only at 24°C (63). This indicates that each *C. sakazakii* phage may have different optimum temperatures. In addition, phage CR5 could completely inhibit both clinical and food *C. sakazakii* isolates with a MOI of 10^5 (64).

      Vibrio infection is usually associated with eating undercooked seafoods such as oysters (87). Symptoms of vibriosis include watery diarrhea, abdominal cramps, nausea, or fever (87). To control this food-borne pathogen, *Vibrio* phages have been tested in seafood samples. Treatment of *V. parahaemolyticus* phage VPp1 at MOI of 0.1 in the oyster depuration caused 2.35-2.76 log reductions (65, 66). In addition, phage pVp1 at MOI of 10^4 was effective to control *V. parahaemolyticus* on the surface of oysters with 6 log reductions (68).

VII-4-2. Phage endolysin

Phage endolysin are peptidoglycan hydrolases that play a role in host lysis after phage replication and propagation. Therefore, it has been suggested as a novel biocontrol agent as well as natural food preservative to control food-borne gram-positive pathogens. In this section, general features and various food applications of endolysins will be explained and discussed. Various endolysin applications in foods are summarized in Table 7.2.

Table 7.2. Examples of endolysins used in studies related to pathogen reduction in foods

Target host	Endolysin	Applications	Results	reference
Listeria monocytogenes	Ply500	iceberg lettuce	silica nanoparticles (SNPs)-conjugated Ply500 showed 4 log reduction	(88)
	LysZ5	soya milk	4 log reduction within 3 h at 4°C	(89)
Staphylococcus aureus	LysH5	milk	cell number reduction to under the detected level at 37 °C after 4 h	(90)
	LysH5	milk	active in milk when secreted by *Lactococcus lactis*	(91)
	LysH5	milk	synergistic inhibition effect combination treatment with nisin	(92)
	Ply187AN-KSH3b	milk	immediate eradication of all CFUs (about 100 CFU) at time zero and remained undetectable throughout the 3 h period	(93)
	λSA2-E-Lyso-SH3b, λSA2-E-LysK-SH3b	cow milk	strong activity as lysostaphin reduction in bacterial numbers was maintained during 3 h	(94)
	HydH5Lyso, HydH5SH3b, CHAPSH3b	milk	significantly enhanced lytic activities when compared with parental protein HydH5, CHAPSH3b showed strong lytic activity in both whole and skim milk (pasteurized) under both at 25°C and 37°C	(95)
Clostridium perfrigens	Ctp1L	cow milk	moderate host lysis activity	(96, 97)

VII-4-2-1. General features

After replication and propagation of the *Caudovirales* phages in the host cells, assembled phages are released upon breakdown of bacterial cell wall caused by phage encoded endolysins. Endolysin has a specific activity to hydrolyze peptidoglycan of the cell wall and holin are known to help endolysin to cross the bacterial membrane to reach cell wall (98). Therefore, endolysin has potential as a biocontrol agent against various Gram-positive food-borne pathogens in the food industry. Generally, endolysin targeting gram-positive bacteria has two conserved protein domains, N-terminal enzymatic activity domain (EAD) and C-terminal cell wall binding domain (CBD). It has been reported that there are five types of EAD according to the cleavage sites: *N*-acetylmuramidases (lysozymes), *N*-acetyl-*β*-D-glucosaminidases (glycosidases), *N*-aycetylmuramoyl-L-alanine amidases, L-alanoyl-D-glutamate endopeptidases, and interpeptide bridge-specific endopeptidases (7). Since endolysins specifically target the peptidoglycan layer in the bacteria, they have been considered safe for humans without any immunological responses (99). Furthermore, no studies on the emergence of endolysin resistance strains has been reported to date (100). Therefore, endolysin may be a good candidate for biocontrol of food-borne pathogens in foods without harming humans. However, most of endolysins are limited to control of gram-positive bacteria and endolysin studies are still at the preliminary stage. To utilize this advantage of endolysins for food applications, further efforts and studies need to be conducted on various food-borne pathogens.

VII-4-2-2. Endolysin applications

The endolysin LysZ5 from a *L. monocytogenes* phage FWLLm3 can specifically inhibit the host growth up to 4 log CFU in soya milk within 3 h at 4°C, suggesting that LysZ5 has high host specificity and host lysis activity at refrigerator condition (89). However, listericidal peptidase, Ply500 showed a broad activity spectrum within the genus *Listeria* (101). Interestingly, silica nanoparticles (SNPs)-conjugated Ply500 showed 4 log reduction of *L. innocua* on iceberg lettuce (88). It is noteworthy that this SNPs-conjugated Ply500 revealed significant enzyme stability (retaining >95% of initial host lysis activity) even after 15 days incubation at 25°C, while native endolysin was completely inactivated under the same condition (88). This highlights effectiveness of enzyme immobilization to sustain the activity and stability of the endolysin in food applications.

The endolysin LysH5 from a *S. aureus* phage vB_SauS-phiIPLA88 was demonstrated to inhibit the growth of a broad range of clinical Staphylococcal strains including *S. aureus* and *S. epidermidis* (90). Interestingly, this endolysin could also control biofilm-forming *S. aureus* and *S. epidermidis* strains as well as rifampicin and ciprofloxacin-persister bacteria (102). To verify endolysin LysH5's activity in food applications, pasteurized milk containing *S. aureus* was treated with a single endolysin LysH5 or a combination of LysH5 and nisin (92). A synergistic inhibition effect of LysH5 and nisin was observed and this synergistic effect may be associated with these two substances taking different approaches to exert antimicrobial effect (92). Nisin exhibits antimicrobial activity by forming pores in the host membrane, while LysH5 hydrolyzes peptidoglycan. In addition, the phage vB_SauS-phiPLA88 has a highly thermostable HydH5, a peptidoglycan hydrolase domain, to lyse *S.*

aureus. Interestingly, HydH5 has two conserved protein domains, N-terminal CHAP domain and a C-terminal LYZ2 domain, but it does not have CBD (103). To enhance the host specificity and binding activity, three fusion proteins were constructed: complete HydH5+SH3b domain of lysostaphin (HydH5SH3b), CHAP+SH3b (CHAPSH3b), complete HydH5+complete lysostaphin (HydH5Lyso). Comparative host lysis analysis revealed that the fusion protein containing the CHAP domain of HydH5 and the SH3b domain of lysostaphin showed the strongest host lysis activity in pasteurized milk, suggesting that construction of fused-endolysins through genetic engineering may be necessary to enhance the host lysis activity (103). Endolysin LysK from *S. aureus* phage K has a high host lysis activity with broad host spectrum including general and clinical *S. aureus* strains and even MRSA (104). However, endolysin Ply187 from *S. aureus* phage 187 showed relatively weak host lysis activity, probably due to an inhibitory domain at the C-terminal (93). To enhance the host lysis activity of Ply187 without inhibition of host lysis activity, EAD of Ply187 (Ply187AN) was fused to CBD of LysK (KSH3b) to generate a chimeric Ply187AN-KSH3b enzyme. Interestingly, host lysis activity of the fusion protein (Ply187AN-KSH3b) was better than the parental endolysins (Ply187AN and LysK), probably due to removing the inhibitory domain from Ply187 upon fusion. Host lysis test of the contaminated milk samples using the fusion protein (Ply187AN-KSH3b) resulted in no detection of *S. aureus* after 3 h, supporting the advantage of the genetically engineered fusion endolysin (93).

The endolysin Ctp1L from *Clostridium* virulent phage phiCTP1 showed host lysis activity against *C. tyrobutyricum* and *C. sporogenes* in cow milk. However, this endolysin showed less host lysis activity in milk sample than in broth condition,

indicating effects of food components on endolysin activity should be considered for competitive endolysin application (96, 97).

While many endolysins were screened and characterized, further optimization of their host specificity and host lysis activity is required to maximize the activities. Generation of fused endolysin using genetic engineering may be one of good approaches to achieve this.

VII-5. Phage Rapid Detection of Food-borne Pathogens in Foods

VII-5-1. CBD-based rapid detection in foods

To detect various food-borne pathogens in food samples, three microbiological and molecular methods, such as culturing method using the specific selective media, PCR-based, and antibody-based detection methods, have been generally used. However, these detection methods have some limitations including long incubation time, requirement of expensive molecular techniques, and low sensitivity and stability of antibodies (105, 106). Therefore, development of simple, rapid, and sensitive method for food-borne pathogen detection is required.

As previously explained, endolysin has two conserved protein domains: N-terminal enzymatic activity domain (EAD) and C-terminal cell wall binding domain (CBD). While EAD is associated with host cell lysis, CBD plays a role in specific host recognition and binding. Due to high host specificity and host-specific binding, CBD can be used to replace antibody for rapid detection of specific bacteria (107, 108). Antibodies have been widely used for rapid detection and concentrating specific food-borne pathogens through specific binding. However, they have high detection limit, their binding specificity for pathogens may sometimes be low (14), and their production cost is high. Therefore, development of an inexpensive novel material for specific detection and concentration of food-borne pathogens in foods is highly required. The size of CBD (usually 10-20 kDa) is much smaller than that of antibodies (usually 150 kDa) and the number of CBD binding sites on a bacterial

cell is reported to be at least 10^7 suggesting that CBD might be a good candidate to substitute antibodies (109). CBD also has advantage of easy construction of fusion proteins containing various fluorescent proteins or other functional domains because it is expressed in bacteria.

Fluorescence-labelled CBD is a good tool to detect specific food-borne pathogen (110). To date, fused CBDs with various fluorescent proteins were developed to target and detect several gram-positive food-borne pathogens including *L. monocytogenes, S. aureus, B. cereus* (111-113). However, CBD has a critical limitation as it cannot detect gram-negative food-borne pathogens because of the outer membrane. By using different colored-fluorescent tags, CBD cocktail can identify multiple food-borne pathogens simultaneously present in a food sample. For example, different serovar groups of *Listeria* were identified by a multiplex decoration with different CBDs. Three CBDs, CBD-P35 and CBD500 tagged with different fluorescent markers (RedStar and GFP) were able to distinguish different *Listeria* strains in both milk and camembert cheese samples (18).

CBD can also be used for concentration of specific food-borne pathogens in foods. For example, CBD118 and CBD500 from *L. monocytogenes*-targeting endolysins Ply118 and Ply500 were coated on paramagnetic beads and the CBD-coated beads were evaluated for concentration of bacterial cells in various *L. monocytogenes*-contaminated food samples (47). The concentration of *L. monocytogenes* using these CBD-coated beads showed >90% recovery rate in culture condition. Moreover, these CBD-coated beads captured up to 1-100 CFU/gram of *L. monocytogenes* in various food samples including turkey breast, ground meat, salmon, cheese, iceberg lettuce, and milk (47). Furthermore, CBD from

S. aureus-targeting endolysin plyV12 was used to concentrate the host cell via immunomagnetic separation method and demonstrated that these CBD-coated beads could detect up to 400 CFU of *S. aureus*-contaminated milk in 1.5 h (109). These findings suggest that these bacterial concentration and detection methods of various food-borne pathogens could be implemented for food applications (109).

To summarize, CBD could be a great candidate to replace antibodies in rapid detection and concentration of pathogens, because it can overcome limitations of antibody with higher specificity and binding activity for gram-positive pathogens.

VII-5-2. Reporter phage-based rapid detection of live bacteria in foods

Although CBD has high host specificity and binding activity, it is not able to differentiate between live and dead cells. Furthermore, it is impossible to distinguish cell-bound CBD from its free form after CBD treatment without washing step, and it is even very difficult to wash food samples after CBD treatment. To overcome these limitations of CBD, reporter phage has been proposed to detect food-borne pathogens.

Reporter phage is a genetically engineered phage that contains fluorescence-emitting or color-developing gene clusters encoding bacterial luciferase, green fluorescence protein (GFP), and β-galactosidase (19). After recognizing its specific host strain, reporter phage infects the host and injects its genomic DNA (22, 23). Then, phage DNA is inserted into the host genome and fluorescent or colorimetric signals would be emitted for detection (16). The

advantage of reporter phage is that it can only emit the detection signal after host infection, indicating that reporter phage detection is restricted to live host cells. Another advantage is that reporter phage emits the detection signal when it infects the specific host, indicating that no washing step is required. Therefore, reporter phage could overcome the limitations of CBD, even though CBD is still a good material for rapid and specific detection of food-borne pathogens in foods.

Bacterial *lux* gene operon consists of *luxCDE* encoding fatty acid reductase complex containing reductase, synthetase, and transferase for biosynthesis of fatty aldehydes as substrates, and *luxAB* encoding luciferase α- and β-subunits for luminescence reaction with the substrates (114, 115). Based on this *lux* gene operon, a recombinant reporter phage containing only *luxAB* of *V. harveyi* was constructed (Fig. 7.1A). Interestingly, this reporter phage was able to detect six different *L. monocytogenes* strains up to 500-1,000 cells without enrichment step, suggesting high sensitivity and detection ability (116). Although this reporter phage could detect low level of *L. monocytogenes* in various food samples including hamburger, liverwurst, shrimp, pasteurized milk, cheeses, and cabbage, it does not contain *luxCDE* for biosynthesis of fatty aldehydes as substrates for luminescence reaction (117). In addition, *S.* Enteritidis-targeting reporter phage containing only *luxAB* (P22$_{luxAB}$) was able to detect up to 63 CFU/egg sample, indicating the requirement of substrate supply for detection (118). Therefore, for easy detection of *E. coli* in food samples without substrate supply, the reporter phage containing *luxI* gene (λ$_{luxI}$ phage) and the modified bioluminescent reporter strain containing complete *luxABCDE* operon as well as *luxR* for regulation of operon gene expression (*E. coli* OHHLux) was constructed (Fig. 7.1B). After infection and genome integration of the reporter

phage, *luxI* gene was expressed followed by the production of an autoinducer protein (N-3-(oxohexanoyl)-L-homoserine lactone; OHHL). This OHHL can be diffused within bacterial population including the reporter strains. After accepting OHHL, LuxR binds to OHHL and the complex activates the transcription of *luxABCDE* in the operon for luminescence reaction (119). While this detection system can detect up to 1 CFU/ml of *E. coli* in pure culture, it can detect up to 130 CFU/ml in the contaminated lettuce rinsate, suggesting that its sensitivity and detection ability works well even in food samples (119). Furthermore, the other reporter phage/strain detection system (PP01$_{luxI}$/*E. coli* OHHLux) targeting *E. coli* O157:H7 could detect up to 1 CFU/ml in pure culture as well as in the food/water samples (apple juice, spinach rinsate, and tap water). However, this system did not work well in the ground beef sample because this sample already had a small amount of OHHL. Therefore, careful selection of food samples for detection is necessary before applying this reporter phage/strain detection system (115). Although this reporter/strain system does not need a supply of substrate, it still needs the reporter strain for detection. To avoid this inconvenience, a new-type *S*. Typhimurium-targeting reporter phage containing a complete set of *luxABCDE* operon (SPC32H-CDABE) was constructed (17) (Fig. 7.1C). This reporter phage could detect up to 20 CFU/ml of *Salmonella* in pure culture. In addition, its food applications showed that it could detect 22 CFU/g of *Salmonella* in iceberg lettuce, 37 CFU/g of *Salmonella* in sliced pork, and 700 CFU/g of *Salmonella* in milk (17). This reporter phage would be useful for monitoring and rapid detection of *S*. Typhimurium in food sample without the supply of substrates or reporter strain for detection.

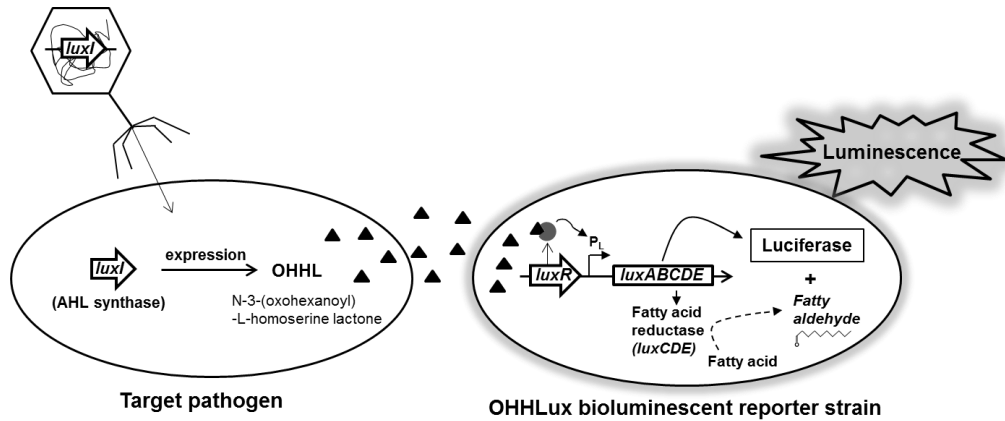In addition to the luciferase-based reporter phage systems, a GFP-based

reporter phage (PP01-GFP) targeting *E. coli* O157:H7 was constructed (120). Although its sensitivity and host range were determined, it was not used for food applications because many food components can be fluorescent. Another reporter phage system was constructed with *lacZ* gene, which encodes β-galactosidase. Interestingly, this *lacZ*-based reporter phage targeting *E. coli* O157:H7 needs specific substrates including chlorophenol red β-D-galactopyranoside (CPRG) for colorimetric reaction and Beta-glo^{TM} luminescent substrate (Promega, USA) for luminescence reaction (121). This reporter phage system was utilized for construction of integrated assay with swab for sampling, immunomagnetic beads for separation, *lacZ*-based reporter phage with specific substrates. Its application to beef slice samples showed that its detection limit was up to 10^3 CFU/100 cm^2 using colorimetric method and up to 10^1 CFU/100 cm^2 using luminescence method, suggesting that it is a good combined rapid detection approach using sampling, separation, and rapid detection (121).

However, it is still necessary to construct various reporter phages because a few food-borne pathogens are detectable using this reporter phage system. Although this reporter phage system can detect only live bacterial cells with high host specificity and low detection limit, construction and development of novel reporter phages are still difficult. Therefore, more study should be needed for development, optimization, and various food application of the reporter phage systems.
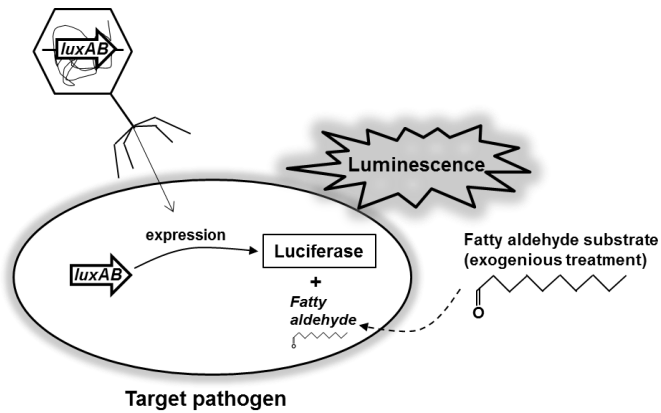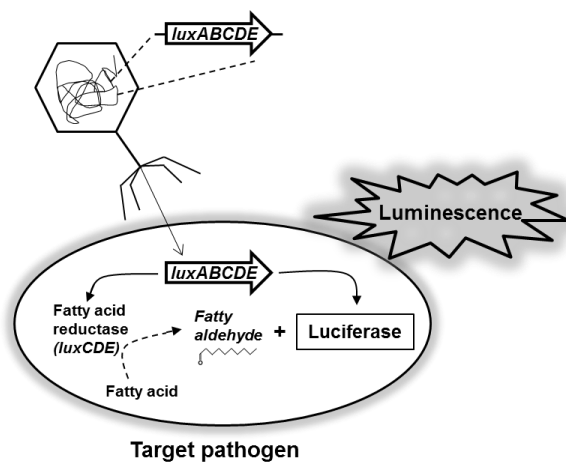
A. LuxAB system

B. LuxIR/LuxABCDE system

C. LuxABCDE system

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Figure 7.1. Luciferase-based reporter phage systems: (A) LuxAB system, (B) LuxIR/LuxABCDE system, and (C) LuxABCDE system.

VII-6. Conclusion

During the last century, bacteriophages have been screened and utilized for the purpose of therapy for various diseases caused by pathogens. Since the discovery of antibiotics in Western Europe, the use of phage for therapeutic purposes was reduced and restricted in the area of Eastern Europe. However, emergence of antibiotic-resistant strains, this phage therapy has recently been revisited and reissued. While many phages have been isolated and characterized for application of phage therapy, they also have been considered as novel biocontrol agents to control various food-borne pathogens. For food applications, phages are considered as natural food preservatives as well as rapid detection tools of food-borne pathogens. Numerous studies have been reported showing that phages may be useful for controlling specific food-borne pathogen with high safety for humans. Although phages are very useful and safe for food applications, they are not widely used because they still need some time to make the customers understand the advantage of phages as natural food preservatives. Because of this, well-designed experimental clinical studies should be performed to convince the customers that phage is highly safe and no harm to humans. To overcome the low preference to phage applications, endolysin with high host specificity and lysis activity has been suggested and developed. However, this enzyme still needs to be optimized experimentally and

enhanced by molecular protein engineering. For rapid detection of food-borne pathogens in foods, PCR- and antibody-based methods are generally used. However, these detection methods still have some problems including long reaction time, no knowledge of molecular techniques, high detection limit, etc. Based on the host specificity of phages, CBD from endolysin and reporter phage system have been suggested to detect food-borne pathogens for rapid detection without additional equipment or reagents, suggesting the next-generation rapid detection system of pathogens. Furthermore, CBD has similar characteristics including high specificity and binding activity to antibodies. Therefore, CBD has recently been suggested to be an alternative material of antibodies because CBD is much cheaper to be produced using *E. coli* overexpression/ purification system with much higher host specificity and binding activity than antibody. Therefore, antibody in some parts of the market may sooner or later be replaced by CBD. Although reporter phage construction is not simple at this time, one it is obtained, its detection is quite quick with low detection limit and very simple. In addition, reporter phage can detect only live pathogens. Therefore, reporter phage detection system may be suitable for development of commercial rapid detection kit in the future.

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국문 초록

살모넬라 타이피뮤리움(*Salmonella* Typhimurium)은 그람 음성 간균의 편모 세균으로 사람을 비롯한 대부분의 온혈 동물에서 발견되는 병원성 세균이다. 주요 식중독 원인균인 살모넬라균은 감염 시 메스꺼움, 구토, 복부 경련, 설사, 발열과 두통 등의 증상을 동반하는 위장염을 유발한다. 건강한 사람에게는 살모넬라균에 의한 감염 증상이 상대적으로 가볍게 나타나지만 유아, 노인과 환자의 경우, 면역력의 저하로 인해 살모넬라균 감염에 의해 사망에까지 이르게 된다. 특히, 항생제 저항성 비 장티푸스 살모넬라균 등의 항생제 내성 세균의 등장으로 인하여 박테리오파지(bacteriophage, 파지)와 엔도라이신(endolysin) 기반의 생물 방제제(biocontrol agent) 개발이 주목받고 있다.

살모넬라 타이피뮤리움에 대한 새로운 생물 방제제를 개발하기 위해 살모넬라 타이피뮤리움을 숙주로 사용하여 특이적으로 감염하는 26개의 새로운 파지를 분리하고 특성을 분석하였다. 숙주 수용체 분석을 통해, 살모넬라 타이피뮤리움 파지가 수용체로 활용하는 편모, O-항원, BtuB, LPS 코어 올리고당 (OS) 영역 및 OmpC 를 포함하는 다섯 종의 세포벽 수용체를 확인하였다. 파지와 숙주세균간의 상호 작용을 깊이 이해하고자 선별된 파지들의 전체 유전체를 비교 분석하였으며, 그 결과, 파지 유전체 중 꼬리(tail) 및 꼬리 섬유(tail fiber) 구조가 파지의 숙주

범위뿐만 아니라 숙주 수용체를 결정하는 데 중요한 요소임을 확인하였다.

파지의 숙주 수용체 연구를 바탕으로, 편모, O-항원과 BtuB 의 서로 다른 수용체를 각각 표적으로 하는 세 종류의 파지 BSPM4, BSP101 와 BSP22A 를 선별하였다. 세 종류의 파지의 유전체 분석을 실시한 결과, 이들 파지는 모두 용균/용원성 결정 유전자군과 독소 형성 유전자가 전혀 존재하지 않음을 확인하였으며, 따라서 이들은 생물학적 방제제로 개발하여 활용하기에 안전할 것으로 판단되었다. 이에 세 종류 파지로 구성된 신규한 파지 칵테일을 구축하였으며, 이의 항균력을 확인하였다. 그 결과, 새롭게 구축한 파지 칵테일 처리 시, 파지 저항성 균주의 발생률이 유의적으로 감소하였음을 확인하였다. 한편, 살모넬라균에 의한 식중독 발생은 다양한 신석 식품에서 발생한다고 다수 보고되어 있다. 따라서 신선 식품에 오염되어 있는 살모넬라균에 대한 파지 칵테일의 항균 효과를 확인하기 위해, 두 종류의 신선 채소로 양배추와 오이를 모델 식품으로 선정하여 연구하였다. 그 결과, 파지 칵테일을 처리한 경우, 두 종류의 식품 모두에서 유의적인 살모넬라균 감소 효과를 보였으며, 12 시간 까지도 그 활성이 지속되었다. 이러한 결과는 세 개의 서로 다른 숙주 수용체를 표적으로 하는 파지 혼합 기술을 통하여 새롭게 구축한 파지 칵테일의 항균 물질 개발 가능성을 보였으며, 이는 향후 새로운 생물방제제를 개발하는데 효과적인 전략이 될 수 있음을 시사하였다. 더불어 본 연구에서 새롭게 개발한 파지 칵테일은

살모넬라균이 오염된 신선 식품 내에서도 효과적인 숙주 생장 저해능을 보였으므로 향후 신선 채소의 안전성을 높일 수 있는 생물 방제제 후보 물질임을 시사하였다.

엔도라이신은 파지가 숙주에 감염 후 복제, 증식된 후 숙주의 펩티도글리칸층을 용해하는 효소로, 이의 강한 용균 활성과 숙주 박테리아에 대한 높은 특이성으로 인해 생물방제제로의 활용이 주목받고 있다. 한편, 그람 음성균의 경우 외막(outer membrane)의 존재로 인해 엔도라이신이 펩티도글리칸층에 쉽게 접근하여 작용하기 어렵다는 한계점이 있어, 현재까지 엔도라이신에 대한 연구는 대부분 그람 양성균에만 제한적으로 수행되어 왔다. 이에 그람 음성균을 제어하는 엔도라이신을 활용한 신규 항균제제 개발에 관한 활발한 연구가 필요하다.

이를 위해, BSPM4 파지의 유전체 분석 결과를 바탕으로 M4LysA 엔도라이신이 기존에 보고된 엔도라이신 및 용균 단백질과 상동성이 없어 신규성을 가짐을 확인하였으며, 이를 분리 정제한 후, 특성을 분석하였다. M4LysA 단백질에 대한 추가적인 유전체 분석 결과, 특이하게도 이는 기존에 알려진 파지 유래 펩티도글리칸층 분해 관련 도메인을 전혀 갖지 않음을 확인하였다. 하지만 M4LysA 단백질을 세포 내에서 발현시켰을 때 매우 빠르게 세포가 용해되는 현상을 확인하였으며, 발색반응을 수행한 결과 M4LysA 는 엔도펩티데이즈 활성을 가짐을 확인하였다. 이러한 결과는 BSPM4LysA 단백질이

새로운 종류의 항균 단백질 이라는 사실을 시사하였다. 흥미롭게도, M4LysA 단백질은 막관통영역(transmembrane domain; TMD)을 갖는 막단백질로 예측되었다. BSPM4LysA 엔도라이신 C-말단의 막관통영역 제거 시, 단백질의 peptidoglycan 용균 활성은 남아있는 한편, 단백질의 수용성이 증가하였는데, 이러한 결과는 M4Lys 이 peptidoglycan 을 녹임으로써 세포를 용해시킨다는 것을 시사하였다. 특히, M4LysA 는 C-말단에 막관통영역을 갖고 있어, 현재까지 보고된 엔도라이신과는 달리 Sec-translocase pathway 를 통하지 않고도 세포질(periplasm)로 secretion 될 수 있음을 확인하였다. 실제로, M4LysA 의 세포질로의 이동에 막관통영역이 중요하게 작용하는 것을 확인 하였다. 나아가, M4LysA 의 용균 활성 범위는 그 유래 파지인 BSPM4 보다 넓었으며, M4LysA 엔도라이신이 그람 음성균을 제어할 수 있는 새로운 종류의 항균제로써 개발 가능함을 뒷받침하였다.

한편, 엔도라이신의 많은 장점에도 불구하고 그람 음성균에 존재하는 외막(outer membrane)으로 인해 그 활용에 제한이 있다. 따라서 본 연구에서는 이를 극복하기 위한 방법으로 엔도라이신을 함유하는 리포솜을 제조하는 것을 최초로 제시하였다. 수용성이 강하며 아미데이즈 활성을 띄는 BSP16Lys 엔도라이신을 지질 소포(lipid vesicle)에 감싸(encapsulation) 리포솜을 구축하였으며, 이의 항균 활성을 평가한 결과, 흥미롭게도 외막 투과성 처리제(outer membrane permeabilizer)의 전처리 없이 그람 음성균 외부에서 리포솜 처리 시

한 시간 이내에 3 로그 이상의 살모넬라 타이피뮤리움이 빠르게 사멸되었음을 확인하였다. 또한 라이소자임을 함유하는 리포솜의 경우에도 외막 투과제 처리 없이 단독 처리시 유의적인 세균 수 감소가 나타났다. 이는 그람 음성균 제어 엔도라이신의 새로운 활용 가능성을 제시하였을 뿐만 아니라 향후 엔도라이신 함유 리포솜이 그람 음성균을 우수하게 사멸시킬 수 있는 우수한 항균 제제로 활용될 수 있다는 가능성을 시사하였다.

본 연구를 통해 살모넬라 타이피뮤리움 균을 표적으로 하는 다양한 종류의 파지와 엔도라이신의 생물방제제로써의 활용 방안 및 가능성을 다양한 측면에서 제시하였으며, 이는 파지 생물학에 대한 깊은 이해와 새로운 파지 유래 항균제제 개발에 대한 유용한 정보를 제공할 것이다.