A Thesis for the Degree of Master of Science

Isolation and Characterization of Bacteriophage SSU5 Specific for *Salmonella enterica* serovar Typhimurium Rough Strain

살모넬라 티피뮤리움 **rough strain**을 특이적으로 감염시키는
박테리오파지 **SSU5**의 분리 및 특성분석

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ABSTRACT

Salmonella sp. is a major food-borne pathogen causing a variety of diseases. Use of bacteriophage for control of foodborne pathogen is a newly promising way as an alternative antibiotic therapy. Previously, our group has isolated several phages specific for *S.* Typhimurium to develop phage cocktail for *Salmonella* biocontrol. However, most of them bind only one of 3 types of the cell surface structure: BtuB, O-antigen (O-Ag) and flagella. Using the *⊿btuB* and the *⊿btuB ⊿rfbP* double mutant strains of *Salmonella* as a host, I isolated SSU5 phage that does not use BtuB, O-antigen, or flagella as a receptor. TEM analysis showed that SSU5 belongs to the family *Siphoviridae* and genomic analysis revealed that SSU5 contains a linear dsDNA consisting of 103, 229 bp with a G+C content of 51.11%. The genome of SSU5 showed high homology to that of cryptic plasmid pHCM2 harbored by *Salmonella* Typhi strain CT18, and 72 out of 130 predicted ORFs were annotated as hypothetical proteins, supporting the novelty of this phage. The inhibition of SSU5 adsorption to the periodate treated-bacteria allowed me to hypothesize that the rough lipopolysaccharide (LPS) may be the host receptor for SSU5, and it

was verified by a spotting assay on the mutants that have various truncations in their core LPS. Analysis of the receptor of SSU5 revealed that this phage would be a promising tool as a phage cocktail component to control rough strains of *S.* Typhimurium generated by mutation or phase variation.

Keywords: Bacteriophage, *Salmonella* Typhimurium, phage receptor, core-oligosaccharide

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Ⅰ. INTRODUCTION

Infection of *Salmonella* is an important worldwide health problem. *Salmonella* is a foodborne pathogen causing severe illnesses including typhoid, enterocolitis, bacteremia and chronic asymptomatic carriage (Coburn, Grassl et al. 2007). Infants, the older people and people who are immunocompromised or have chronic illnesses could be more at risk than others who don't require antibiotic treatments for *Salmonella* infection. (Kennedy, Villar et al. 2004). Each year in the United States, salmonellosis has been estimated over 1.4 million illnesses, approximately 17,000 hospitalizations and 600 deaths (Mead, Slutsker et al. 1999). Also, multi-drug resistant *Salmonella* was obtained from food animals and humans, indicating increase of antimicrobial resistance rates in isolates and those isolates were resistant to cephamycins and extended-spectrum cephalosporins as well as tetracycline, chloramphenicol, streptomycin, and sulfisoxazole (Winokur, Brueggemann et al. 2000). Therefore, alternative treatments of bacterial infections have been required to control pathogens effectively. Among these alternative therapies, a bacteriophage therapy has risen to kill or inhibit bacteria.

Bacteriophages are viruses that specifically infect and lyse bacterial

cells taking over metabolic machinery of the host cell in the lytic cycle, indicating the possibility of phages as antimicrobial agents (Sulakvelidze, Alavidze et al. 2001). Phages are ubiquitous in environments and they have long history of safe use suggesting the convincing safety of phage therapy comparable to other chemotherapies. In contrast to antibiotics, they have highly specific antimicrobial activity to their host, such that they believed to do not disturb normal flora. With this specificity, they also easily handles, proposing the benefits of phage usage for application in the food safety (Garcia, Martinez et al. 2008). On account of these features, phages are predicted to control foodborne and antibiotic resistant pathogens effectively with safety.

Many researches have been proved their ability as biocontrol agents to control various foodborne pathogens including *Salmonella*. For example, *Salmonella* phage FelixO1 inhibited *Salmonella* growth on chicken frankfurters contaminated with 300 CFU of *Salmonella* Typhimurium with levels of about 2-log units (Whichard, Sriranganathan et al. 2003). *Salmonella enterica* serovar Typhimurium phage P7 showed significant reduction of host cells at high host cell density and MOI conditions (Bigwood, Hudson et al. 2008). *Salmonella* Enteritidis phage SJ2 reduced *Salmonella* in Cheddar cheese made from both raw and pasteurized milk

(Modi, Hirvi et al. 2001).

However, the emergence of phage-resistant bacteria could be a drawback of phages as biocontrol agents. Phage cocktail including phages which use different bacterial receptors has been suggested to overcome this problem delaying or eliminating phage-resistant cell (Levin and Bull 2004). To develop phage cocktails containing phages with various receptors, it is important to analyze mechanisms of phage adsorption, the first step of phage infection recognizing the receptor on the surface of host cell. Interaction between phage and bacterial cell determines the host specificity of the phage. The phage receptors in Gram negative bacteria comprised LPS, outer membrane proteins and flagella (Rakhuba, Kolomiets et al. 2010). In the case of *Salmonella* phages, they bind different structure of cell surface for adsorption, such as FhuA for ES18 (Killmann, Braun et al. 2001), OmpC for Gifsy-1 and Gifsy-2 (Ho and Slauch 2001), BtuB for SPC35 (Kim and Ryu 2011), flagella for Φχ (Meynell 1961) and LPS for various *Salmonella* phages including P22, SP6 and Felix O1 as a common receptor (Hudson, Lindberg et al. 1978, Steinbacher, Miller et al. 1997, Molineux 2001).

Although diverse receptors were identified for *Salmonella* phages, most of *Salmonella* phages previously isolated by our group used O-antigen, outer membrane protein BtuB or flagella as a receptor (Shin, Lee et al. 2012).

In this study, to purify phages which utilize other receptors, *S.* Typhimurium defective in O-antigen and BtuB was used as host bacteria. As a result, the novel bacteriophage SSU5 was isolated and was characterized for its receptor. The receptor of SSU5 is core-oligosaccharide suggesting that this phage could infect rough *S.* Typhimurium strains, and it would be a valuable component for effective phage cocktail.

Ⅱ. MATERIALS AND METHODS

2.1. Bacterial strains and growth condition

Bacterial strains used for the host range test and the receptor determination are listed in Table2 and 3, respectively. Prophage-cured *Salmonella enterica* serovar Typhimurium LT2 strain (designated as LT2(c)) from Cancer Reasearch Center and its derivatives were used to isolate and propagate bacteriophage. All bacterial strains were aerobically grown at 37°C in Luria-Bertani (LB) broth or agar that was added 1.5% agar to the LB broth.

2.2. Bacteriophage Isolation and purification

Sewage, slurry and fecal samples for screening of *Salmonella*-specific bacteriophage were collected from Seoul, Yangju and Suwon in South Korea. Twenty-five grams of each solid sample was homogenized in 225 ml sterile Butterfield's phosphate-buffered dilution water (0.25 M KH_2PO_4 adjusted to pH 7.2 with NaOH) in sterile bags, then 25 ml of each suspension was added to 25 ml of 2X LB broth and incubated for 24 h with uniform shaking at 37 °C. Chloroform (1%, final concentration) was supplemented to the culture and further incubated for 5 min at 37 °C with

shaking. After removing of bacterial debris by centrifugation at 9,000 × g , 4 °C for 10 min and filtration using 0.22 μm pore size filters (Millipore, Billerica, MA), 10-ml of each filtrate was mixed with 50 ml LB broth containing 1 % overnight culture of appropriate host *Salmonella* mutant starin (final concentration) and then the mixture was incubated at 37 °C for 12 h with shaking. The culture was centrifuged at 9,000 × g for 10 min and the supernatant containing phages was filtered using 0.22μm pore size filter (Millipore) to remove bacterial debris.

This supernatant was used to confirm the presence of bacteriophage by dotting on molten 0.4% LB soft agar containing 1 % host *Salmonella* strain (final concentration). The plates were incubated overnight at 37 °C and suspected for plaque formation. Each single plaque was picked with sterile tip and eluted in SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgSO4). This step was repeated at least three times to purify single bacteriophage.

The culture of appropriate host *Salmonella* mutant strain (OD_{600} = 0.5) was infected with phage at a multiplicity of infection (MOI) of 1 and incubated at 37°C for 4h. Cell debris was removed by subsequent centrifugation at 9,000 g for 10 min and filtration using 0.22 μm pore size filters, then phage particles in the filtrate were precipitated by mixing with

10% (wt/vol) polyethylene glycol (PEG) 6,000 (Sigma) in 1M sodium chloride. Finally, CsCl density gradient ultracentrifugation (himac CP 100β, Hitachi, Japan) with different CsCl steps (step density = 1.3, 1.45, 1.5 and 1.7 g/ml) at 78,500 × g for 2 h was carried out at 4°C. Viral particles were recovered and dialyzed using standard dialysis buffer (5 M NaCl, 1 M $MgCl_2$ and 1 M Tris·HCl at pH 8.0) and stored at 4°C.

2.3. Bacteriophage host range

A hundred microliter of each test bacterial culture was added to 5 ml of the molten 0.4% LB agar and the mixture was overlaid on the 1.5% LB agar plate. After solidification, 10 µl of each serially diluted SSU5 phage suspension from 10^1 to 10^9 was spotted on the overlaid plates and these plates were incubated at 37°C. The sensitivity of test bacteria to SSU5 phage was examined by degrees of transparency in the spots of bacterial lysis.

2.4. Transmission electron microscopy

CsCl-purified high titer bacteriophages in SM buffers were examined by TEM. Each phage sample was put on carbon-coated copper grids and negatively stained with 2 % aqueous uranyl acetate (pH 4.0) for 1 min. Electroscope microscopy was carried out using a transmission electron

microscope (LIBRA 120, Carl Zeiss) at 80 kV. Bacteriophages were identified and classified into its relative family according to the guidelines of the International Committee on Taxonomy of Viruses on the basis of the morphology of phages (Fauquet and Fargette 2005).

2.5. Construction of gene deletion mutants

Salmonella strain LT2(c) with deletion(s) of specific gene was constructed by lambda Red recombination system (Datsenko and Wanner 2000). The kanamycin resistance (Kan^R) cassette from plasmid pKD13 was amplified using primers which included 40 nucleotide homology of each deletion-target gene and 20 nucleotide priming sequences of pKD13, and the resulting PCR product was used to transform the wild-type or *rfbP* gene deletion strain (Kim and Ryu 2012) containing the plasmid pKD46. Finally, the Kan^R cassette was removed from transformants using pCP20 plasmid (Datsenko and Wanner 2000).

2.6. Bacteriophage adsorption assay

Bacteriophage adsorption assay with various *S.* Typhimurium strains was performed according to the previous study (Kim and Ryu 2012) with some modifications. *S.* Typhimurium strain culture was harvested at OD_{600} =

1.0, re-suspended and 10-fold diluted with LB broth. SSU5 phage was added at a MOI of 0.01, and the adsorption was proceeded at 37°C for 15min. The samples were collected at 0, 1, 5, 10 and 15 minutes, centrifuged at 16,000 x g for 1min and filtered using 0.22 μm pore size filters (Millipore) to obtain free phage in supernatant. The filtrates were used for counting unadsorbed phages by serial dilution and standard plate counting using LT2(c) with a deletion of *rfbP* gene. The initial phage titer was based on SSU5 only in LB broth. The ratio between initial titer and each sample titers after adsorption was calculated to determine adsorption rate of SSU5 to each strains by expressing an adsorption constant (*k*). $k = -\ln (P_t/P_0)/Nt$, where P_t = phage titer at the time t (PFU ml^{-1}), P_0 = initial phage titer (PFU ml^{-1}), N = bacterial density (cfu ml^{-1}) and *t* = time (min).

2.7. Bacteriophage bacterial challenge test

LB broth (50 ml) including 1% bacterial overnight culture (final concentration) was incubated aerobically at 37°C with shaking When OD_{600} reaches to 0.5, phages were added at a MOI of 0.1. During the further incubation at the same conditions, the culture samples were collected at every hour. OD_{600} was measured to observe bacterial growth. SM buffer instead of phage SSU5 was used as a negative control.

2.8. Periodate orProteinase K treatments

Periodate or proteinase K was treated to LT2(c) with a deletion of *rfbP* gene to examine the effect of the treatments on SSU5 adsorption. These treatments were carried out with some modification as previously described (Kiljunen, Datta et al. 2011). Briefly, when OD_{600} of the bacterial cell culture reaches to 1.0, 1ml of the cell culture was collected by centrifugation at 16,000 x g for 1 min and washed with 1 ml fresh LB broth. Proteinase K (0.2 mg ml^{-1}) was added to the prepared sample and was incubated at 37°C for 2 h. For periodate treatment, 2 ml of the same culture was centrifuged at 16,000 × g for 1 min, and the pellet was treated with 1 ml sodium acetate (50 mM; pH 5.2) or sodium acetate containing either 10 or 100 mM periodate for 2 h in the dark. After the treatments, samples were washed at least three times with 1 ml of LB broth, OD_{600} was adjusted to 0.1 and the phage adsorption assay was performed as described above.

2.9. Isolation of P22H5-resistant S. Typhimurium mutants

Culture of LT2(c) with *Salmonella* phageP22H5 at a MOI of 1 was incubated for 24 h at 37°C with shaking to isolate the P22H5-resistant *Salmonella* mutants. The loop-full P22H5-challenged culture was streaked on a fresh LB plate and incubated at 37°C. To purify the P22H5-resistant

colonies, a single colony was sequentially streaked on LB plates at least 3 times.. The putative P22H5-resistant colonies were subjected to the spotting assay to confirm the resistance to P22H5 and susceptible to SSU5.

2.10. LPS extraction and analysis from P22H5-resistant S. Typhimurium mutants

The modified phenol-water extraction method (Kim and Ryu 2012) was used for LPS extraction from P22H5-resistant *S.* Typhimurium mutants. Bacterial overnight cultures (1.5 ml) were collected by centrifugation at 16,000 x g for 1min, washed using 1 ml DPBS (PBS containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$), and re-suspended in 300 μl ddH_2O. The equal volume of preheated (68°C) phenol solution was added and the mixtures were incubated at 68°C for 15 min with vortexing at every 5 min. The samples were placed on ice for 5 min, and the aqueous phase was separated by centrifugation at 10,000 x g, 4°C for 5 min. After transfer of the aqueous phase into conical tube, 300 μl of ddH2O was added into the remaining phenol phase and repeated the extraction steps. 1M sodium acetate was added to the obtained aqueous phases at a final concentration of 0.5 M, 95% ethanol was added more than 10 times of sample volume, and the mixture was incubated overnight at -20 °C. The samples were centrifuged at 16,000 x

g, 4°C for 5 min to extract the crude LPS, re-suspended in 100 μl ddH2O, and ethanol precipitation was repeated again. Finally, the precipitated LPS was re-dissolved in 50 ml ddH2O and stored at -20°C.

The extracted LPS were analyzed by DOC-PAGE on a 15% acrylamide gels (Reuhs, Geller et al. 1998). Briefly, the separating gel (15%) was prepared with 5 ml of monomer stock solution [30% (w/v) acrylamide, 0.8% (w/v) N,N′-methylenebisacrylamide], 2 ml of resolving gel buffer solution (1.875 M Tris base, adjust to pH 8.8), 3 ml of ddH2O, 17.5 μl of 10% ammonium persulphate and 8.75 μl of N,N,N′,N′-tertramethylethylenediamine (TEMED). The stacking gel (4%) putting onto the solidified resolving gel was composed of 0.33 ml of monomer stock solution, 0.5 ml of stacking gel buffer solution (0.635 M Tris base, adjust to pH 6.8), 1.67 ml of ddH2O, 12.5 μl of 10% ammonium persulphate and 6.25 μl of TEMED. The gels were pre-run with a running buffer (290 mM glycine, 37 mM Tris base and 6 mM sodium deoxycholate) for 10 min at 15 mA using Bio-Rad Mini-PROTEAN® Tetra Cell before sample loading. Five-microliter of extracted LPS was mixed with equal volumes of sample buffer (2 ml of stacking gel buffer solution, 1 ml of glycerol, 2.5 mg of bromophenol blue and made up to 10 ml with ddH2O), and the mixtures were loaded onto the gels and electrophoresis was performed at 15 mA for

~60min. The gels were fluorescently stained using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes, Cat. No. P20495; Eugene, OR, USA) based on the manufacturer's instructions. The stained sample was visually detected using the 300 nm UV by the Gel Doc EZ imager (Bio-Rad)

2.11. Bacteriophage DNA purification

Bacteriophage genomic DNA was extracted from the CsCl-purified bacteriophage as previously described by Wilcox et al. (Wilcox, Toder et al. 1996). Phage lysate was treated with DNase I and RNaseA at 37 °C for 1 h to remove bacterial DNA and RNA prior to purification of SSU5 genomic DNA. Lysis buffer (1 % of Sodium dodecyl sulfate (SDS), 0.5 mol/l of EDTA and 10 mg/ml of proteinase K) was treated to the samples for 2 h at 56 °C. A manual phenol-chloroform DNA purification and ethanol precipitation were proceded.

2.12. Full-genome sequencing of bacteriophage SSU5 and bioinformatic analysis

Purified SSU5 phage DNA was sequenced by a pyrosequencing using the Genome Sequencer FLX Titanium by Macrogen, Seoul, South

Korea. GS De Novo assembler (v. 2.60) was used to assemble quality filtered reads, and GeneMarkS (Besemer, Lomsadze et al. 2001), Glimmer 3.02 (Delcher, Bratke et al. 2007), and FgenesV (Softberry, Inc., Mount Kisco, NY) were used for prediction of open reading frames (ORFs). The predicted ORFs were annotated according to the results of BLSTP (Altschul, Gish et al. 1990), InterProScan (Zdobnov and Apweiler 2001), and NCBI Conserved Domain Database (Marchler-Bauer, Anderson et al. 2007). tRNAs were predicted by tRNAscan-SE (Lowe and Eddy 1997).

2.13. Nucleotide sequence accession number

The nucleotide sequences of bacteriophage SSU5 is available to GenBank under the accession number JQ965645.

Ⅲ. RESULT

3.1. Isolation and the receptor determination of Salmonella phages

The aim of this study is isolation of *Salmonella* bacteriophages with different receptor from previously isolated phages by our group to improve the phage cocktail efficiency. Using the prophage-cured *Salmonella enterica* serovar Typhimurium LT2 strain with either *rfbP* or *btuB rfbP* double deletion as a host, fourteen bacteriophages specific for *Salmonella* were isolated (Table 1). Spotting assay on the *rfbP*, *btuB* or *flgK* deletion mutants (defective in O-antigen, BtuB or flagella, respectively) revealed that most of isolated phages utilized O-antigen, BtuB outer membrane protein or flagella as a receptor. Interestingly, one of them, named as SSU5, formed clear plaques on the *rfbP* deletion strain and lysis zone on the other two mutant strains as well as wild-type strain, suggesting the receptor of SSU5 is different from other isolated phages. Therefore, with the probability that SSU5 as a candidate for novel phage cocktail component using other receptor, I further characterized the SSU5.

Table 1. Characteristics of newly isolated bacteriophages.

Bacteriophage	Sample source	Receptor	Plaque morphology
SSU2	Water	O-antigen	Small, clear
SSU3	Water	O-antigen	Large, clear
SSU4	Water	O-antigen	Large, clear
SSU5	Water	?	Small, clear
SSU6	Soil	flagella	Small, clear
SSU10	Water	flagella	Small, clear
SSU11	Water	flagella	Small, clear
SSU12	Water	flagella	Small, clear
SSU13	Water	flagella	Small, turbid
SSU14	Chicken fecal	O-antigen	Small, clear
SSU26	Water	BtuB	Large, clear
SSU27	Water	flagella	Small, clear
SSU28	Water	flagella	Small, clear
SSU31	Sludge	O-antigen	Large, clear

3.2. Bacteriophage SSU5 morphology and classification

SSU5 was morphologically identified by TEM analysis. TEM image of SSU5 revealed that it has an icosahedral head and non-contractile flexible tail, indicating that SSU5 belongs to the family of *Siphoviridae* (Fig. 1). A diameter of the head and length of tail were approximately 70 nm and 220 nm, respectively.

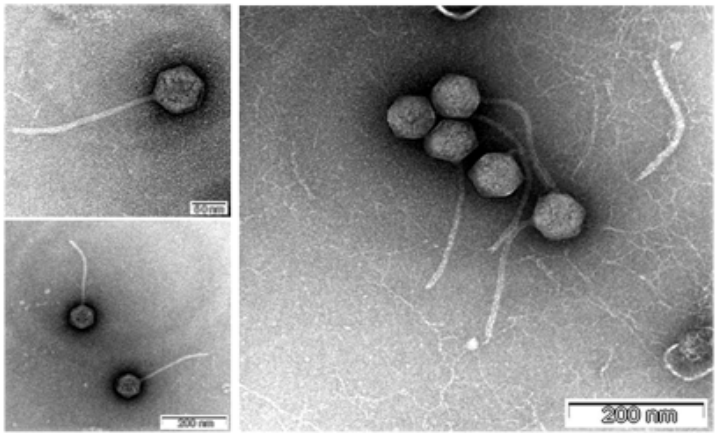


Figure 1. Transmission electron microscope image of phage SSU5. SSU5 was negatively stained by 2% uranyl acetate and observed by TEM. Each bar indicated at the bottom right-hand corner of the image represents 200 nm.

3.3. Host range of phage SSU5

SSU5 exhibited specific inhibition against Gram negative bacteria (Table 2). It generated clear plaques in five strains of *Salmonella* tested, and produced the zones of inhibition in other *Salmonella* strains. Interestingly, *Cronobacter sakazakii* ATCC 29544, *Shigella flexineri* 2a strain 2457T, and *E. coli* DH10B also infected by SSU5, suggesting that unknown common receptor for SSU5 are existed in these bacteria. Obviously, SSU5 formed clear plaques with the LT2(c) Δ*rfbP* mutant, which used as host bacteria in SSU5 screening, while it formed inhibition zone with the parental LT2(c) strain, implying that the receptor of SSU5 might be masked by O-antigens.

Table 2. Host range of phage SSU5

Strain	Plaque formation[a]
Salmonella ssp.	
S. Typhimurium ATCC 14028s	(+)
S. Typhimurium NCTC 12023	(+)
S. Typhimurium KCTC 1925	+
S. Typhimurium ATCC 12023	(+)
S. Typhimurium SL1344	(+)
S. Typhimurium LT2(c)[b]	(+)
S. Typhimurium UK1	(+)
S. Typhi Ty 2-b	-
S. Paratyphi A IB 211	-
S. Paratyphi B IB 231	(+)
S. Paratyphi C IB 216	(+)
S. Dublin IB 2973	(+)
S. arizonae KCCM 41035	+
S. arizonae KCCM 41036	+
S. arizonae KCCM 41037	-
S. indica KCCM 41759	+
S. houtenae KCCM 41760	+
S. diarizonae ATCC 41761	(+)
S. salamae KCCM 41762	(+)
Escherichia coli	
E. coli MG1655	(+)
E. coli DH5α	-
E. coli DH10B	+
E. coli O157:H7 ATCC 43890	-
Other Gram negative bacteria	
Cronobacter sakazakii ATCC 29544	+
Shigella flexineri 2a strain 2457T	+

	Shigella boydii 1B 2474	(+)

a, +, single plaque; (+), inhibition zone; -, no plaque
b, *S.* Typhimurium LT2(c) : prophage-cured *Salmonella enterica* serovar Typhimurium LT2

3.4. Bacteriophage SSU5 genomic analysis

The genome of SSU5 which was a linear double-stranded DNA consists of 103,229 bp with a G+C composition of 51.11 % (Fig. 2). This phage genome predicted total 130 ORFs and one tRNA for asparagine. Over half of ORFs (72 ORFs) were annotated as hypothetical proteins, suggesting the novelty of SSU5. Other ORFs encoded proteins associated with DNA packing/morphogenesis (terminase large subunit, major capsid protein, minor tail protein, etc.), DNA replication/repair (DNA helicase, primase, ligase, DNA polymerases, exonucleases, recombinase, etc.), host lysis (holin, endolysin, and Rz1), lysis/lysogeny switch (phage repressor and anti-repressor), and other functions (repressor of phase I flagellin,selenium-binding protein YdfZ, etc.). Interestingly, the genome of SSU5 showed a high degree of homology to that of cryptic plasmid pHCM2 (106,516 bp; NC_003385) harbored by *Salmonella* Typhi strain CT18 (Kidgell, Pickard et al. 2002). The SSU5 genomic analysis compared to the genome of pHCM2 revealed that all ORFs except 22 ORFs of SSU5 showed an identity up to 100 % with ORFs of pHCM2 at the protein level. The 22 ORFs showing no homology to ORFs of pHCM2 were related with some key elements for phage lifecycle (i.e., receptor-recognizing phage tail fiber adhesin, superinfection exclusion protein, and phage repressor), whereas a putative

phage integrase (NP_569492.1) was found in pHCM2 but not in SSU5, implicating that the cryptic plasmid pHCM2 might be originated from the phage SSU5 or its ancestors.

Figure 2. SSU5 genome analysis. Yellow arrows and a red arrow indicate ORF and tRNA, respectively. The genome of SSU5 is a linear dsDNA consisting of 103,229 bp with a G+C contents of 51.11%. Total 130 ORFs and one tRNA for asparagine were predicted.

3.5. Identification of phage SSU5 receptor

Since the receptor of SSU5 might be blocked by O-antigens as described above, we hypothesized that one of outer membrane proteins was a receptor of SSU5. To confirm this, the genes associated with synthesis of variable outer membrane proteins that used as receptors by other phages were deleted on the Δ*rfbP* background, and these mutants were subjected to the SSU5 spotting assay (Table 3). The fact that flagella or BtuB was not the receptor for SSU5 was also verified again with double deletion mutant strains *ΔflgKrfbP* and *ΔbtuBrfbP*. . Interestingly, SSU5 produced single clear plaques on all of these mutants, suggesting that, unlike our hypothesis, SSU5 didn't use outer membrane proteins tested as a receptor.

The other candidate for SSU5 receptor is core-oligosaccharides in LPS, because this structure also blocked by the O-antigens. To clarify whether the receptor of SSU5 was the core-oligosaccharides or the other outer membrane protein, SSU5 adsorption assay was performed with periodate or proteinase K treated *ΔrfbP* mutant. The proteinas K was used to digest cell surface proteins, such as outer membrane proteins, whereas the periodate degrades carbohydrate structures containing a 1,2-diol motif, such as LPS. The measurement of the residual phage particles in the supernatant after adsorption assay revealed that the adsorption of SSU5 to *Salmonella*

was inhibited by the treatment of periodate but not proteinase K (Fig. 3). This result suggested that the SSU5 receptor was not a protein structure but the carbohydrate structure.

Table 3. Confirmation of a SSU5 phage receptor using LT2(c) strains with deletion of gene(s) involved in synthesis of receptor candidates. All genes were related to synthesis of outer membranes protein except for *rfbP* (O-antigen) and *flgK*(flagella).

Host	Defective structure(s)	Plaque formation of SSU5
LT2(c)	-	(+)
Δ btuB	BtuB	(+)
Δ rfbP	O-antigens	+
Δ flgK	flagella	(+)
Δ btuBrfbP	BtuB and O-antigens	+
Δ flgKrfbP	flagellar and O-antigens	+
Δ lamBrfbP	LamB and O-antigens	+
Δ ompArfbP	OmpA and O-antigens	+
Δ ompCrfbP	OmpC and O-antigens	+
Δ ompFrfbP	OmpF and O-antigens	+
Δ ompWrfbP	OmpW and O-antigens	+
Δ phoErfbP	PhoE and O-antigens	+
Δ rfbPtolC	TolC and O-antigens	+

+, single plaque; (+), inhibition zone; -, no plaque

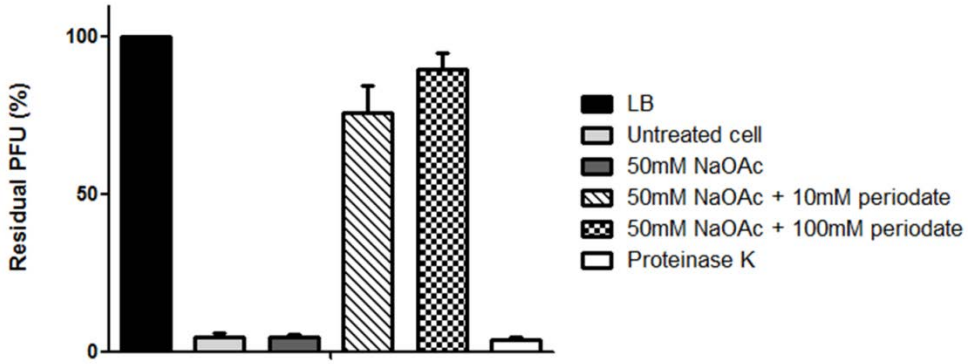
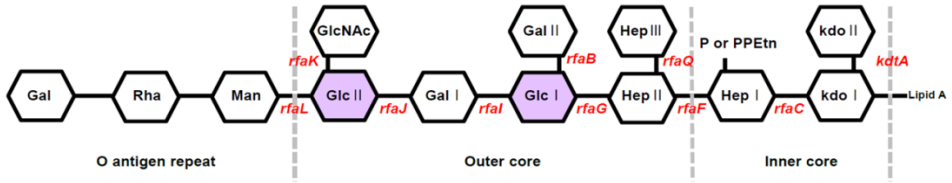


Figure 3. Effect of Periodate and Proteinase K treatments on SSU5 adsorption. SSU5 adsorption assay was performed with the periodate- or proteinase K-treated LT2 (c) Δ*rfbP* strain. The non-treated strain was used as the control (untreated cell). SSU5 titer in LB broth was considered as 100 % control and the residual phage titer in each sample after 15 min adsorption at 37 °C was relatively represented in percentage.

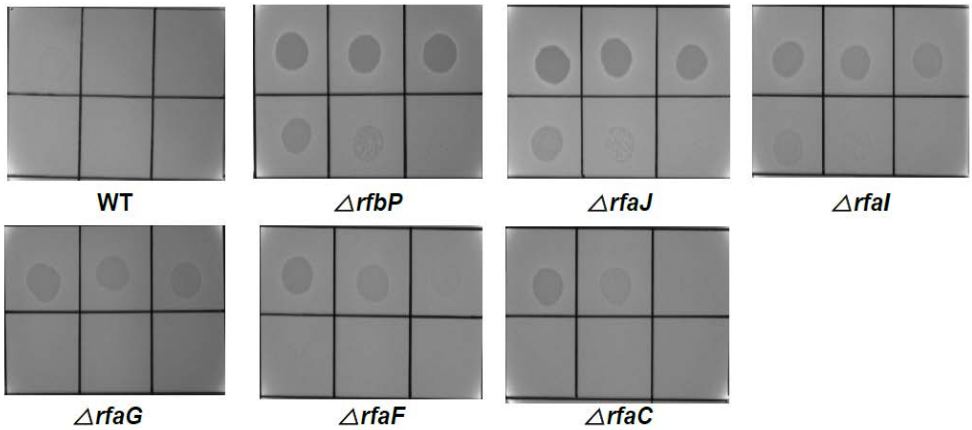
3.6. Effect of various LPS core truncation on SSU5 adsorption

Genes involved in LPS core biosynthesis were deleted to identify which moiety of core-oligosaccharide was the SSU5 receptor. *rfaJ*, *rfaI*, *rfaG*, *rfaF* and *rfaC* gene deletion mutants, that were predicted to have various truncation in LPS core-oligosaccharide (Fig. 4A), were constructed. Spotting assay on wild type and mutant strains showed that SSU5 infectivity was gradually decreased as the core residue was shortened. Especially, the EOPs of *ΔrfaG*, *ΔrfaF* and *ΔrfaC* strains were significantly reduced compared to wild type (Fig. 4B). Adsorption assay with wild type and these mutants were also performed to support the result of spotting assay. Deep rough strains as well as wild-type strain exhibited a very slow SSU5 adsorption rate (Fig. 4C), and the fastest SSU5 adsorption rate was expressed with *ΔrfbP* strain. The adsorption constant (*k*) was calculated based on the ratio between initial titer and each sample titers after adsorption for 15 min adsorption. Compared to *ΔrfaF* which had the lowest adsorption constant, *ΔrfbP* had approximately 17-fold higher adsorption constant, suggesting that the outer core-oligosaccharide might be important for SSU5 adsorption.

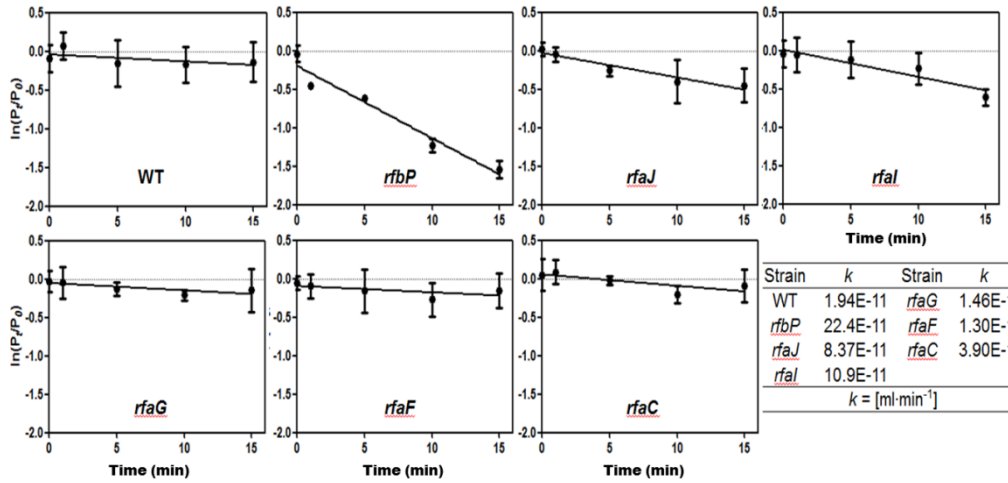
(A)



(B)



(C)



Strain	*k*	Strain	*k*
WT	1.94E-11	*rfaG*	1.46E-11
rfbP	22.4E-11	*rfaF*	1.30E-11
rfaJ	8.37E-11	*rfaC*	3.90E-11
rfaI	10.9E-11		

k = [ml·min^{-1}]

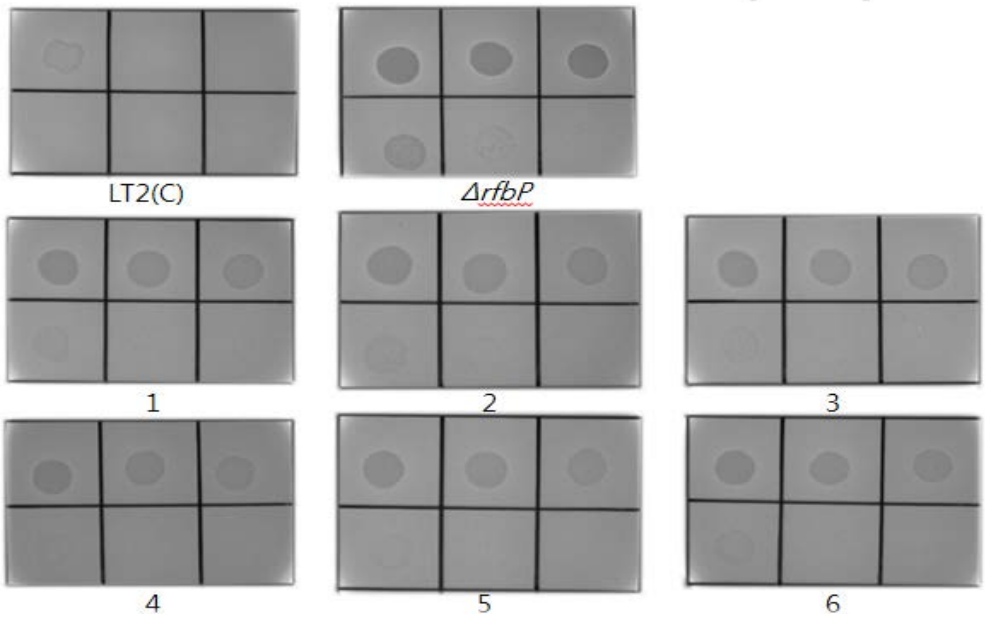
Figure 4. The relation between various LPS core truncation and SSU5 adsorption. (A) LPS structure of *S.* Typhimurium. Red letters indicate the relevant genes involved in the biosynthesis of the core oligosaccharide. (B) SSU5 spotting assay on mutants with truncation in LPS core-oligosaccharides. (C) Adsorption assay of SSU5 to these mutants and cognate adsorption constant (*k*). Adsorption constant was calculated by the following formula: constant $k = -\ln (P_t/P_0)/Nt$; P_t = phage titer at the time *t* (PFU ml^{-1}), P_0 = initial phage titer (PFU ml^{-1}), N = bacterial density (cfu ml^{-1}) and *t* = time (min).

3.7. SSU5 infection to P22H5 resistant mutants

When bacteria suffered from phage infections, they often lost or altered phage receptor on the cell surface to be resistant to phage infection (Labrie, Samson et al. 2010). For example, infection of O-antigen-specific phages, such as P22H5, would result in the selection of O-antigen deficient bacterial mutants. Since SSU5 recognized core-oligosaccharide, I hypothesized that SSU5 might infect mutants which were resistant to P22H5. To elucidate this hypothesis, P22H5-resistant *Salmonella* mutants were isolated by P22H5 challenging to LT2(c) strain. After purifying the resistant mutants, diluted SSU5 lysates were spotted on the mutant lawns. The result showed that SSU5 formed plaques on some of isolated mutant lawns with similar level of EOP compared to *ΔrfbP* mutant strain (Fig. 5A). To confirm the deficiency of O-antigens in these P22H5-resistant mutants, their LPS structure was analyzed by deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE). As shown in the figure 5B, the wild type LT2(c) strain had a complete LPS structure (ladder of various lengths of O-antigens with core-oligosaccharides) resembled to the standard *Salmonella* LPS, and *S.* Typhimurium KCTC 1925 as well as *ΔrfbP* strains had outer core- and inner core-oligosaccharides without the O-antigens. Expectedly, all P22H5-resistant mutants lost their O-antigens and had only the inner-core

oligosaccharides, supporting that SSU5 can infect the O-antigen-specific phage-resistant *Salmonella* via binding to core-oligosaccharide region.

(A)



(B)

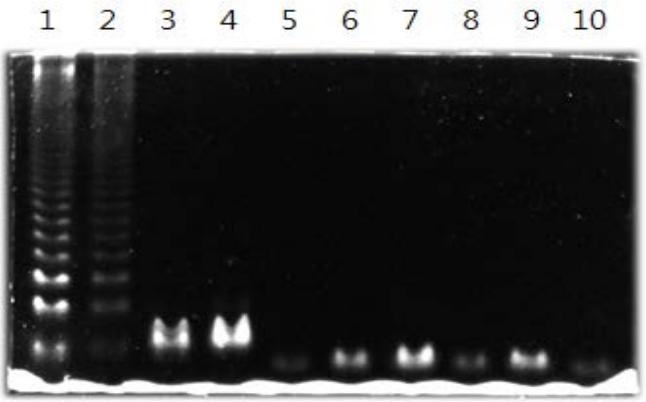


Figure 5. SSU5 infect the P22H5-resistant mutants that lost their O-antigens. (A) SSU5 were spotted on to the lawns of isolated P22H5-resistant mutants. (B) DOC-PAGE of P22H5-resistant mutants. LPS extracted by hot-phenol extraction method were electrophoresed on the 15% polyacrylamide gel. Gels were stained using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit prior to detection under UV light. Each lane contains LPS of following strains: Lane 1, *Salmonella* standard LPS; lane 2, LT2(c); lane 3, *S.* Typhimurium KCTC 1925; ;ane 4, Δ*rfbP*/LT2(c); lane 5~10, P22H5-resistant mutants 1~6.

3.8. Bacterial challenge tests with SSU5 and phage cocktail.

Considering the ability of SSU5 that can infect O-antigen deficient *Salmonella*, SSU5 might overcome the emergence of phage-resistant mutants if it composes the phage cocktail with other phages using O-antigens as the host receptor. To determine this ability of SSU5, phage cocktail including SSU5 and SSU14 was tested. Phage SSU14, which also isolated in this study, formed clear plaques on *Salmonella* lawns utilizing O-antigens as a receptor (Table 1), and inhibited the growth of host *Salmonella* for a long time compared to other isolated phages (data not shown). Each phage or both phages were added to the exponentially growing *S*. Typhimurium LT2(c) and OD_{600} was measured every hour. The result showed that SSU5 seemed to do not control the growth of LT2(c). However, phage cocktail consisting with SSU5 and SSU14 delayed the appearance of phage-resistant bacterial mutant by approximately 2 hours than the SSU14 only treated group (Fig. 6), suggesting that SSU5 have a potential as a phage cocktail component to inhibit *S*. Typhimurium efficiently and prolong the appearance of phage-resistant mutants.

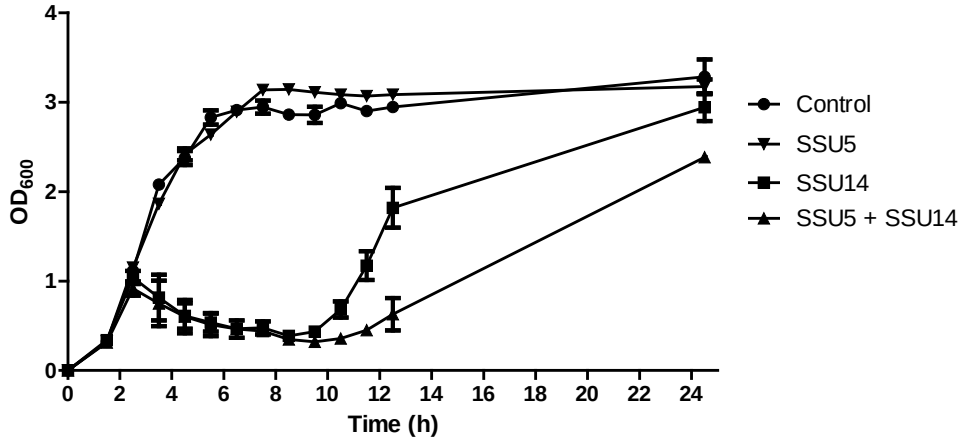


Figure 6 Bacterial challenge test with SSU5, SSU14 or phage cocktail with both phages. Phages were infected at 1.5 h after bacterial inoculation, and the OD_{600} was measured for the monitoring of bacterial growth. The phage lysate was replaced by SM buffer in LT2(c) culture to use as a negative control. The means with SD of three independent experiments were represented.

Ⅳ. DISCUSSION

Bacteriophage adsorption to host bacteria is an important step to infect their prey successfully. This step is achieved by specific recognition of host cell surface receptors by phages, determining the host specificity of phages. The interaction between host bacteria and phage has been studied in many researches, and consequently, various phage receptors were identified in Gram positive and Gram negative bacteria. LPS, a component of outer membrane in Gram negative bacteria, was composed of monosaccharides and fatty acids, and was used by phages as a common receptor. Complete LPS structure, called as S (smooth)-type, consisted with O-antigen repeat, core-oligosaccharide and lipid A, while R (rough)-type LPS structure consisted only with core region and lipid A without O-antigen (Wilkinson 1996). Some phages specific for Gram negative bacteria use different parts of this LPS region for adsorption. For example, ε^{15} and P22 utilize O-antigens as a receptor whereas ΦX174, S13, 6SR, T3, T4 and T7 bind to core-antigen (Rakhuba, Kolomiets et al. 2010).

The study of phage receptors is also important for overcome of a major drawback of phage application: the emergence of phage-resistant

mutants. One of resistant mechanisms of bacterial cells against phage infection is preventing phage adsorption by modifying or losing phage receptors, and producing extracellular matrix and competitive inhibitors to mask/inhibit the receptor (Labrie, Samson et al. 2010). Therefore, development of phage cocktail containing various phages that target different receptors would be effective for control of pathogens. By doing this, each phage in cocktail could inhibit the growth of resistant mutants against other phages, resulting in more efficient control of target bacteria (Levin and Bull 2004). One practical example was reported recently: phage cocktail consisted of three different phages (GH-K1, GH-K2 and GH-K3) exhibited the efficient control of *Klebsiella pneumonia* and the reduced mutation frequency in that pathogen (Gu, Liu et al. 2012). Notably, each phage was isolated by 'SBS methods,' which sequentially used a specific phage-resistant mutant as host bacteria during the isolation of other phage(s), and thus, they might recognize different cell surface components as receptors.

In this study, *Siphoviridae* family phage SSU5 was isolated from sewage using O-antigen- and/or BtuB-deficient *Salmonella* mutant as host bacteria, accordingly this phage didn't bind to O-antigen and outer membrane protein BtuB (Table 1). Interestingly, SSU5 formed inhibition zone on most tested strains but clear plaques on *S.* Typhimurium with a

deletion of *rfbP* gene (Table 2 and 3), suggesting that the receptor of SSU5 is blocked by O-antigens. Previously, it was reported that long O-antigen chains in *E. coli* strains could be a barrier to access outer membrane protein receptors of phages (Vanderley, Degraaff et al. 1986). However, SSU5 could infect the mutants lacking some representative outer membrane proteins implying that the receptor of SSU5 might not be a protein structure (Table 3). Indeed, adsorption assay with periodate- or proteinase K-treated strains revealed that SSU5 receptor is a carbohydrate moiety rather than the protein structure (Fig. 3). The major carbohydrate component of the cell envelope in Gram negative bacteria is LPS, and thus, SSU5 might recognize a part of LPS.

Considering that SSU5 didn't infect wild–type *Salmonella* possessing the intact smooth-type LPS (Table 3 and Fig. 4), O-antigens of LPS were not the receptor of SSU5. Similar to SSU5, some phages that specifically infect rough-type strains lacking O-antigens but not smooth strains had been reported (Lindberg 1973). From the results of spotting assay on mutants harboring deletion in various genes involved in the LPS outer core and inner core synthesis, it was revealed that SSU5 recognize the core region of LPS as a receptor (Fig. 4B). The significant reduction of SSU5 adsorption with mutants possessing the inner core region only indicated that

the outer core region is the most important part for SSU5 adsorption (Fig. 4C).

In accordance with this, SSU5 could not inhibit the growth of wild-type LT2(c) strain that contains an intact LPS structure (Fig. 6). Although this feature seemed to make SSU5 to be worthless in biocontrol application, SSU5 would be a useful component of phage cocktail if it was used together with other phages that utilizing O-antigens as receptor. Indeed, LPS analysis of resistant *Salmonella* mutants against O-antigen-specific phage P22H5 showed the defective LPS structure (Fig. 5B), and thus, all of these mutants were susceptible to SSU5 infection (Fig. 5A). The result of bacterial challenge test with phage cocktail including SSU5 and SSU14 also revealed the ability of SSU5 to inhibit the growth of P22H5-resistant mutants (Fig. 6): SSU5 delayed and reduced the appearance of phage-resistant mutants for approximately 22 hours after infection. It was reported that the *Vibrio cholera* phage K139 using O1-antigen as a receptor selected the phage-resistant mutants that were defective in synthesis of O1-antigen or core-oligosaccharide (Nesper, Kapfhammer et al. 2000). Similar to this, the resistant mutants from the SSU14-treated culture are expected to lack the O-antigens or core-region. Remarkably, the mutants lacking O-antigens or core-oligosaccharides had been revealed as avirulent and were rapidly removed by

antibody and complement in the human body (Roantree 1967, Vishwanath and Hackstadt 1988). In case of *Yersinia pestis*, most resistant *Y. pestis* mutants against LPS-specific phages showed an attenuation in mice, supporting a value of using phages which recognize a part of LPS structure (Filippov, Sergueev et al. 2011).

Additionally, rough-specific phages have another merit in the biocontrol application: providing broader host range than O-antigen-specific phages. In many species, the kind of O-antigen serotype is highly diverse while that of core-oligosaccharide is well conserved. There are 170 O serotypes in *E. coli* and more than 46 serogroups in *S. enterica* by modification, whereas only five core types are exhibited by *E. coli* and two core types of them are similar to that of *Salmonella* (Raetz and Whitfield 2002). The conserved core-oligosaccharides among some genus in *Enterobacteriaceae* family, including *E. coli*, *Salmonella* ssp, *Cronobacter* sp, and *Shigella* sp., might allow SSU5 to infect *Cronobacter sakazakii* ATCC 29544 and *Shigella flexineri* 2a strain 2457T as well as *Salmonella* (Table 2), suggesting the possibility of SSU5 to control various pathogens other than *Salmonella*.

The necessity of rough strain specific-phages for the *Salmonella* biocontrol was also strongly supported by the fact that the modulation of O-

antigen length in *Salmonella* is phase variable. *S.*Typhimurium 798 isolated from a pig has two phenotypes by phase switching: nonadhesive phenotype containing short O-antigen and adhesive phenotype containing long O-antigen. These phase variable phenotype was considered as the effective method to maintain an *in vivo* population yet not enough to cause disease(Kwan and Isaacson 1998). Recently, the phase variable expression of STM2209-STM2208 genes in *Salmonella enterica* was revealed, and was responsible for the phase variable alteration of O-antigen chain length. Notably, clones with short O-antigens by phase variation of these genes were resistant to O-antigen-specific phage P22 (Cota, Blanc-Potard et al. 2012). These reports implied that the rough strains also should be eliminated as the smooth strains, because the avirulent rough strain can revert to virulent smooth strains by phase variations. Obviously, rough-specific phages such as SSU5 would control the potentially virulent rough strains.

In conclusion, newly isolated *Salmonella* phage SSU5 has an originality compared to the previously isolated *Salmonella* phages by our group. It recognized the LPS core region for adsorption, providing SSU5 infectivity against rough strains. Furthermore, the results indicated that SSU5 can overcome the emergence of phage-resistant mutants if it used as a phage cocktail with other phages that utilize O-antigens as a receptor. Along with

phage SSU5, other phages that specific to rough *Salmonella* strains also might be good candidates of phage cocktail component to control *S.* Typhimurium.

Ⅴ. REFERENCES

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). "Basic local alignment search tool." Journal of molecular biology **215**(3): 403-410.

Besemer, J., A. Lomsadze and M. Borodovsky (2001). "GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions." Nucleic Acids Research **29**(12): 2607-2618.

Bigwood, T., J. A. Hudson, C. Billington, G. V. Carey-Smith and J. A. Hememann (2008). "Phage inactivation of foodborne pathogens on cooked and raw meat." Food Microbiology **25**(2): 400-406.

Coburn, B., G. A. Grassl and B. B. Finlay (2007). "Salmonella, the host and disease: a brief review." Immunol Cell Biol **85**(2): 112-118.

Cota, I., A. B. Blanc-Potard and J. Casadesus (2012). "STM2209-STM2208 (opvAB): a phase variation locus of Salmonella enterica involved in control of O-antigen chain length." PLoS One **7**(5): e36863.

Datsenko, K. A. and B. L. Wanner (2000). "One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products." Proceedings of the National Academy of Sciences **97**(12): 6640-6645.

Delcher, A. L., K. A. Bratke, E. C. Powers and S. L. Salzberg (2007). "Identifying bacterial genes and endosymbiont DNA with Glimmer." Bioinformatics **23**(6): 673-679.

Fauquet, C. and D. Fargette (2005). "International Committee on Taxonomy

of Viruses and the 3,142 unassigned species." Virol J **2**: 64.

Filippov, A. A., K. V. Sergueev, Y. He, X. Z. Huang, B. T. Gnade, A. J. Mueller, C. M. Fernandez-Prada and M. P. Nikolich (2011). "Bacteriophage-resistant mutants in Yersinia pestis: identification of phage receptors and attenuation for mice." PLoS One **6**(9): e25486.

Garcia, P., B. Martinez, J. M. Obeso and A. Rodriguez (2008). "Bacteriophages and their application in food safety." Letters in Applied Microbiology **47**(6): 479-485.

Gu, J., X. Liu, Y. Li, W. Han, L. Lei, Y. Yang, H. Zhao, Y. Gao, J. Song, R. Lu, C. Sun and X. Feng (2012). "A method for generation phage cocktail with great therapeutic potential." PLoS One **7**(3): e31698.

Ho, T. D. and J. M. Slauch (2001). "OmpC is the receptor for Gifsy-1 and Gifsy-2 bacteriophages of Salmonella." J Bacteriol **183**(4): 1495-1498.

Hudson, H. P., A. A. Lindberg and B. A. D. Stocker (1978). "Lipopolysaccharide Core Defects in Salmonella-Typhimurium Mutants Which Are Resistant to Felix O-Phage but Retain Smooth Character." Journal of General Microbiology **109**(Nov): 97-112.

Kennedy, M., R. Villar, D. J. Vugia, T. Rabatsky-Ehr, M. M. Farley, M. Pass, K. Smith, P. Smith, P. R. Cieslak, B. Imhoff, P. M. Griffin and G. Emerging Infections Program FoodNet Working (2004). "Hospitalizations and deaths due to Salmonella infections, FoodNet, 1996-1999." Clin Infect Dis **38 Suppl 3**: S142-148.

Kidgell, C., D. Pickard, J. Wain, K. James, L. T. Diem Nga, T. S. Diep, M. M. Levine, P. O'Gaora, M. B. Prentice, J. Parkhill, N. Day, J. Farrar and G. Dougan (2002). "Characterisation and distribution of a cryptic Salmonella typhi plasmid pHCM2." Plasmid **47**(3): 159-171.

Kiljunen, S., N. Datta, S. V. Dentovskaya, A. P. Anisimov, Y. A. Knirel, J. A. Bengoechea, O. Holst and M. Skurnik (2011). "Identification of the Lipopolysaccharide Core of Yersinia pestis and Yersinia pseudotuberculosis as the Receptor for Bacteriophage φA1122." Journal of bacteriology **193**(18): 4963-4972.

Killmann, H., M. Braun, C. Herrmann and V. Braun (2001). "FhuA barrel-cork hybrids are active transporters and receptors." J Bacteriol **183**(11): 3476-3487.

Kim, M. and S. Ryu (2011). "Characterization of a T5-like coliphage, SPC35, and differential development of resistance to SPC35 in Salmonella enterica serovar typhimurium and Escherichia coli." Appl Environ Microbiol **77**(6): 2042-2050.

Kim, M. and S. Ryu (2012). "Spontaneous and transient defence against bacteriophage by phase-variable glucosylation of O-antigen in Salmonella enterica serovar Typhimurium." Molecular Microbiology.

Kwan, L. Y. and R. E. Isaacson (1998). "Identification and characterization of a phase-variable nonfimbrial Salmonella typhimurium gene that alters O-antigen production." Infect Immun **66**(12): 5725-5730.

Labrie, S. J., J. E. Samson and S. Moineau (2010). "Bacteriophage resistance mechanisms." Nat Rev Microbiol **8**(5): 317-327.

Levin, B. R. and J. J. Bull (2004). "Population and evolutionary dynamics of phage therapy." Nat Rev Microbiol **2**(2): 166-173.

Lindberg, A. A. (1973). "Bacteriophage receptors." Annu Rev Microbiol **27**: 205-241.

Lowe, T. M. and S. R. Eddy (1997). "tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence." Nucleic

Acids Research **25**(5): 0955-0964.

Marchler-Bauer, A., J. B. Anderson, M. K. Derbyshire, C. DeWeese-Scott, N. R. Gonzales, M. Gwadz, L. Hao, S. He, D. I. Hurwitz and J. D. Jackson (2007). "CDD: a conserved domain database for interactive domain family analysis." Nucleic Acids Research **35**(suppl 1): D237-D240.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin and R. V. Tauxe (1999). "Food-related illness and death in the United States." Emerg Infect Dis **5**(5): 607-625.

Meynell, E. W. (1961). "A Phage, Phichi, Which Attacks Motile Bacteria." Journal of General Microbiology **25**(2): 253-&.

Modi, R., Y. Hirvi, A. Hill and M. W. Griffiths (2001). "Effect of phage on survival of Salmonella enteritidis during manufacture and storage of cheddar cheese made from raw and pasteurized milk." J Food Prot **64**(7): 927-933.

Molineux, L. J. (2001). "No syringes please, ejection of phage T7 DNA from the virion is enzyme driven." Molecular Microbiology **40**(1): 1-8.

Nesper, J., D. Kapfhammer, K. E. Klose, H. Merkert and J. Reidl (2000). "Characterization of Vibrio cholerae O1 antigen as the bacteriophage K139 receptor and identification of IS1004 insertions aborting O1 antigen biosynthesis." Journal of Bacteriology **182**(18): 5097-5104.

Raetz, C. R. and C. Whitfield (2002). "Lipopolysaccharide endotoxins." Annu Rev Biochem **71**: 635-700.

Rakhuba, D. V., E. I. Kolomiets, E. S. Dey and G. I. Novik (2010). "Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell." Pol J Microbiol **59**(3): 145-155.

Reuhs, B. L., D. P. Geller, J. S. Kim, J. E. Fox, V. S. Kolli and S. G. Pueppke (1998). "Sinorhizobium fredii and Sinorhizobium meliloti produce structurally conserved lipopolysaccharides and strain-specific K antigens." Appl Environ Microbiol **64**(12): 4930-4938.

Roantree, R. J. (1967). "Salmonella O antigens and virulence." Annu Rev Microbiol **21**: 443-466.

Shin, H., J. H. Lee, H. Kim, Y. Choi, S. Heu and S. Ryu (2012). "Receptor diversity and host interaction of bacteriophages infecting Salmonella enterica serovar Typhimurium." PLoS One **7**(8): e43392.

Steinbacher, S., S. Miller, U. Baxa, A. Weintraub and R. Seckler (1997). "Interaction of Salmonella phage P22 with its O-antigen receptor studied by X-ray crystallography." Biol Chem **378**(3-4): 337-343.

Sulakvelidze, A., Z. Alavidze and J. G. Morris, Jr. (2001). "Bacteriophage therapy." Antimicrob Agents Chemother **45**(3): 649-659.

Vanderley, P., P. Degraaff and J. Tommassen (1986). "Shielding of Escherichia-Coli Outer-Membrane Proteins as Receptors for Bacteriophages and Colicins by O-Antigenic Chains of Lipopolysaccharide." Journal of Bacteriology **168**(1): 449-451.

Vishwanath, S. and T. Hackstadt (1988). "Lipopolysaccharide Phase Variation Determines the Complement-Mediated Serum Susceptibility of Coxiella-Burnetii." Infection and Immunity **56**(1): 40-44.

Whichard, J. M., N. Sriranganathan and F. W. Pierson (2003). "Suppression of Salmonella growth by wild-type and large-plaque variants of bacteriophage Felix O1 in liquid culture and on chicken frankfurters." J Food Prot **66**(2): 220-225.

Wilcox, S., R. Toder and J. Foster (1996). "Rapid isolation of recombinant

lambda phage DNA for use in fluorescence in situ hybridization." Chromosome Research **4**(5): 397-404.

Wilkinson, S. G. (1996). "Bacterial lipopolysaccharides - Themes and variations." Progress in Lipid Research **35**(3): 283-343.

Winokur, P. L., A. Brueggemann, D. L. DeSalvo, L. Hoffmann, M. D. Apley, E. K. Uhlenhopp, M. A. Pfaller and G. V. Doern (2000). "Animal and human multidrug-resistant, cephalosporin-resistant salmonella isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase." Antimicrob Agents Chemother **44**(10): 2777-2783.

Zdobnov, E. M. and R. Apweiler (2001). "InterProScan–an integration platform for the signature-recognition methods in InterPro." Bioinformatics **17**(9): 847-848.

국문 초록

살모넬라 티피뮤리움은 대표적인 식중독을 일으키는 원인균으로 발병 시에는 복통, 설사, 발열 증상과 함께 구토와 어지러움을 유발한다. 최근 항생제의 무분별한 사용으로 인해 항생제 저항성 살모넬라가 출현하게 되면서 이를 제어하기 위한 새로운 대안책으로 살모넬라를 특이적으로 감염하는 박테리오파지를 이용한 연구가 주목 받고 있다. 지금까지 본 저자가 소속된 연구실에서는 살모넬라를 제어하기 위한 파지 칵테일을 구축하기 위해 다양한 파지들을 분리하였으나, 오직 LPS의 O 항원, 외막단백질 BtuB, 또는 편모를 수용체로 하는 파지들만이 분리되었다. 이에 본 연구에서는 다른 수용체를 사용하는 살모넬라 파지를 분리하고 해당 파지 및 이를 포함하는 파지 칵테일의 살모넬라 제어능을 확인하고자 하였다. BtuB와 O항원이 각각 또는 둘 다 결여된 살모넬라 LT2(c) 돌연변이 균주들을 숙주 세균으로 사용하여, 하수 및 농장에서 채취한 시료들로부터 앞서 언급한 세 수용체를 사용하지 않는 새로운 파지 SSU5를 분리하였다. 전자현미경 분석 및 전체 염기서열 분석을 통해, SSU5는 정 20면체 머리와 비수축성 꼬리를 가지는 시포비리대 과의 파지이며 살모넬라 타이피 CT18 균주가 가진 pHCM2 플라스미드 유전체와 매우 높은 상동성을 보이는 유전체를 가지고 있음을 밝혔다. SSU5가 이용하는 숙주의 수

용체를 명확히 밝히기 위해 다양한 외막단백질 또는 지질다당류 및 그 core-올리고당들의 결여 돌연변이체들을 구축하고, 이를 이용해 파지 흡착 분석시험과 파지 닷팅 분석시험을 수행하였다. 그 결과 SSU5는 지질다당류의 core-올리고당, 특히 외부 core-올리고당을 인식하여 살모넬라를 감염시킬 수 있음을 알아내었다. 또한 O항원을 수용체로 사용하는 P22 파지의 저항성 균주가 O항원과 core-올리고당의 일부분이 결여되어 있음을 확인하였고 SSU5가 이러한 균주들을 감염시킬 수 있음을 확인할 수 있었다. 이를 통해 O항원을 수용체로 사용하는 SSU14과 칵테일로 SSU5를 사용하였고 SSU14을 단독으로 사용하였을 때보다 저항성 균주가 나타나는 시간이 늦추어지는 것을 확인하였다. 이처럼 SSU5는 돌연변이나 변이 다양성에 의해 생성되는 O항원-결여 살모넬라를 감염시킬 수 있으므로, O-항원을 수용체로 이용하는 파지들과 함께 파지 칵테일의 조성물로 사용함으로써 살모넬라를 효과적으로 제어할 수 있을 것이라 기대된다.

주요어 : 박테리오파지, 살모넬라 티피뮤리움, 파지 수용체, core-올리고당