A Thesis for the Degree of Doctor of Philosophy

Molecular Analysis of the Bacteria-Bacteriophage Interactions to Improve Phage Biocontrol

파지 응용 생물방제의 향상을 위한 세균−박테리오파지 상호작용의 분자 수준 분석

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Abstract

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Bacteriophages (phages) are bacterial viruses that specifically infect and kill the host bacteria. Recently, the widespread emergence of antibiotic-resistant bacteria has led the reconsideration of phage's potential as an alternative biocontrol agent. I isolated nine phages specific for *Salmonella* ssp. or *Escherichia coli* from various chicken fecal and intestinal organ samples to investigate their potentials as alternative agents to antibiotics. Among them, a virulent phage SPC35, which infect both *S.* Typhimurium and *E. coli*, was further studied. Morphological analysis by transmission electron microscopy and analysis of its 118,351-bp genome revealed that SPC35 is a T5-like phage belonging to the family *Siphoviridae*. BtuB, the outer membrane protein for vitamin B_{12} uptake, was turned out to be a host receptor for SPC35. Interestingly, resistant mutants of both *E. coli* and *S.* Typhimurium were developed faster than my expectation when the cultures were infected with SPC35. Investigation of the *btuB* gene revealed that an insertion sequence 2 (IS*2*) element disrupted the *btuB* of the most of resistant *E. coli* mutants. In contrast, the resistant *S.* Typhimurium mutants contain no *btuB* mutations, and

they regained SPC35-susceptibility easily in the absence of SPC35, suggesting a phase variable phage resistance.

Although the alteration of host receptors, such as the disruption of BtuB, is one of the most common bacterial defense mechanism against phage infection by completely blocking phage attachment, it comes at a potential fitness cost to the bacteria. In this respect, I elucidated that *S*. Typhimurium can develop the cost-free, transient SPC35-resistance through a phase variable modification of the O-antigen. Phage SPC35 typically requires BtuB as a host receptor but also uses the *Salmonella* O12-antigen as an adsorption-assisting apparatus for the successful infection of *S*. Typhimurium. The α-1,4-glucosylation of galactose residues in the O12-antigen by phase variably expressed O-antigen glucosylating genes, designated the ^{LT2}*gtrABC1* cluster, blocks the adsorption-assisting function of the O12-antigen. Consequently, it confers transient SPC35-resistance to *Salmonella* without any mutations in the *btuB* gene. This temporal switch-off of phage adsorption through phase variable antigenic modification might be widespread among several Gram-negative pathogen-phage systems, because the antigenic variations also known to confer advantages in pathogenic virulence. These results suggest that a cocktail of phages which target different receptors of the pathogen would be more effective than a sole phage for successful biocontrol.

Lysogenic infections developed by temperate phages are switched to lytic mode in response to the host SOS response. The ultimate factor that governs this switch is a phage repressor, which has been generally identified as a host RecA-dependent autocleavable protein. In an effort to reveal the mechanism underlying the

phenotypic differences between the podoviral *Salmonella* temperate phages SPC32H and SPC32N, which has a single nucleotide polymorphism (SNP) in only two locations in the whole genome, I identified a new class of anti-repressor of the phage lytic switch. The SPC32H repressor (Rep) was not cleaved by the SOS response but, instead, was inactivated by a small anti-repressor protein (Ant), the expression of which is negatively controlled by host LexA. A single-nucleotide mutation in the consensus sequence of LexA-binding site that overlap with the *ant* promoter results in constitutive Ant synthesis and consequently, inclines SPC32N to enter the lytic cycle. The amino acid sequence analysis indicated that Ant might structurally distinct from the previously reported *Sipho-* and *Myoviridae* phage anti-repressors, and numerous Ant homologues were observed in a variety of putative prophages and temperate *Podoviridae* phages in the database, demonstrating that the induction mediated by anti-repressor may be widespread among temperate phages in the order *Caudovirales* for prudent prophage induction. The engineering methods used in this study as well as the engineered mutant temperate phages that biased to the lytic cycle could be applied to the fields of alternative antibiotics. In conclusion, the virulent phages as well as the temperate phages isolated in this study can be practically used as the novel biocontrol agents for *Salmonella* control. In addition, the elucidated mechanisms of bacteria-phage interactions, including the phage-resistance mechanisms in host bacteria and the lytic switch mechanism of prophage, would be the basis for the development of advanced strategies using bacteriophages to combat with the life-threatening pathogens.

Keywords: bacteriophage, phage biocontrol, *Salmonella* spp., bacteria-phage interaction, phage-resistance, anti-repressor.

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Chapter I.

Introduction

I-1. Bacteriophage

Bacteriophages (also called as phages) are bacterial viruses that specifically infect and replicate within the host bacteria. The term is derived from the Greek word meaning ‘devourer of bacteria,’ because these viruses eventually lyse the infected bacteria to release their replicated progenies to the environment. They are known as the most abundant living entities (approximately ~10^{31} phages) on earth, being outnumber their host bacteria by 10-fold (Brüssow and Hendrix, 2002). Phages have been found wherever potential host cells are thrive; e.g., in soils, rivers, lakes, sea, sewage, feces of animals and human, and even in deep thermal vents (Guttman *et al.*, 2005; Liu and Zhang, 2008). In 1915, Frederick W. Twort firstly described the possibility of presence of bacterial virus, based on his odd observations with the propagatable “glassy transformaion” of micrococci colonies (Twort, 1915). Two years later, Felix d’Herelle, who conceived the name of *bacteriophage*, also quite independently discovered the invisible microbes that infected bacteria and multiplied indefinitely when living host cells are supplied (d'Herelle, 1917). Since these discoveries, phages have been deeply studied chemically, biologically, and genetically by many laboratories.

Bacteriophages have simple but elaborately constructed structures consisting with double-stranded or single-stranded deoxyribo- or ribonucleic acids as genetic

elements and protein coats (called as capsids) that encapsulated the nucleic acids. According to their morphology and state of nucleic acid, bacteriophages, including archaeal viruses as well as bacterial viruses, are classified by the International Committee on Taxonomy of Viruses (ICTV) into one order, 14 families, and 37 genera with at least five other potential families awaiting classification (Ackermann, 2009). Phage particles are majorily tailed (more than 96% of phages previously reported) and minorily polyhedral, filamentous, or pleomorphic. Tailed phages constitute the order *Caudovirales*, and further classified into three families: family *Siphoviridae* have a long, non-contractile tail, while family *Myoviridae* possesses a long, contractile tail; phages in family *Podoviridae* are characterized by a short tail. The heads of *Caudovirales* phages are morphologically icosahedra or closely related bodies, and filled with a double-stranded DNA (dsDNA). Considering the overall structure, these tailed phages are reminiscent of the spaceships.

As spaceships land on the planet firstly to settle at new world, phages also initially attach to the host bacteria to begin their new life-cycle. This initial attachment step is called as “adsorption.” Phage particles moving in random Brownian motion collide with host bacterial cells, and subsequently bind to its specific host receptors using their receptor-binding proteins (RBPs). The apparatuses that recognized by phages as host receptors are usually exposed on the

host cell surface. Intensive studies have been revealed that many cell surface components of Gram-negative and Gram-positive bacteria are utilized as phage's receptor, including lipopolysaccharides (LPS), teichoic acids, outer membrane proteins such as OmpC, LamB, BtuB, FhuA, etc., and even flagella (Lindberg, 1973; Rakhuba *et al.*, 2010). Since each RBP specifically recognizes and binds to its correspondent host receptor, this adsorption step determines the host range of individual phages. The specific binding between host receptors and phage RBPs triggers the next stage of phage life cycle, the penetration of phage nucleic acids into the grabbed host cells. In case of phages belongs to family *Myoviridae*, they contract their long tail sheaths to inject their genetic materials into the cell, similar to syringe motion. By contrast, *Podoviridae* phages (and rarely some *Siphoviridae* phages possessing a long, flexible tail ending with a base plate-like appendage) degrade a portion of host cell membrane (e.g., O-polysaccharide chains) by using of their tailspikes containing enzymatic activity, such that inject its genome (Eriksson *et al.*, 1979; Wollin *et al.*, 1981). While the empty shells of phage virion are extracellularly remain, the penetrated phage DNA are immediately transcribed by taking advantage of bacteria's transcriptional machineries to start a replication of their progenies. Subsequently, bacterial ribosomes translate these viral mRNAs into several phage proteins, including inhibitors for bacterial DNA/RNA/protein synthesis, phage structural proteins, and proteins involved in host cell lysis, etc.

Meanwhile, phage genomes are also replicated into hundreds of copies. After one copy of replicated phage genome is packaged into a pre-assembled phage capsid (procapsid), virion assembles are sequentlially finished making complete infectious particle. As a last step of phage life-cycle, newly synthesized phage progenies are released from the host via cell lysis. Enzymes called as holin and endolysin are role in this step by drilling inner membrane and degrading peptidoglycan layers of host cell, respectively. Released phage particle infects a new host again if specific host bacteria cells are presented at around, and repeat this life-cycle continuously.

For some classes of phages, however, an optional stage of their life-cycle is existed. After the internalization into host cytosol, the genome of this class phage stands at the crossroads of choice: lytic or lysogenic development. The formal one is pathways described above - lysing the host cells and releasing progenies. In contrast to this, the lysogenic development did not initiate the lytic mode. Instead, phage genome is integrated into the host genome (or sometimes, maintained as a plasmid) as a quiescent state (Ravin, 2011). The phage in this condition called as a prophage, and the host cell contains prophage(s) in its genome called as a lysogen. Prophages are capable to continuously replicate their copies without any harm to host cells, since they automatically replicated into two copies as the host genome is replicated and divided into two daughter cells. At certain conditions, however, the prophages come out of their silent state and enter the lytic development. Mostly,

conditions that threat the very survival of host cells alarm prophages to induce the lytic mode. For example, an irradiation of UV light to the lysogen induces a prophage excision from the lysogen's genome, resulting in production of infectious phage particles in the cell culture. Based on their relatively mild character, phages in this class are called as temperate phages. By contrast, the other phages that can only multiply their copies by the lytic development are called as virulent phages.

I-2. Phage therapy and phage biocontrol

A historical perspective

d’Herelle’s pioneering works on phages led two areas of phage research: the biological nature and the practical application of phages. As described above, the nature of phages were deeply characterized in chemical and genetical as well as biological aspects, and it was clearly revealed that phages are the elaborately organized infectious particles that are obligate intracellular parasites. More interests were, however, focused on the therapeutic potentials of phages. This was based on the d’Herelle’s findings that the infectious dysentery or typhoid were recovered along with the increasing of phage titers in patients (d'Herelle, 1917). He also tested a prophylactic ability of phages by treating chickens with phages prior to inoculation with *Bacillus gallinarum*. Obviously, phages highly protected the treated chickens from avian typhosis (d'Herelle, 1926). Since the formal results evidently showed the therapeutic and prophylactic effectiveness of phages, the experiments were extended to himself and his families to determine the safety of his phage preparations, and were demonstrated the safety of phage treatments to human (d'Herelle, 1926).

Inspired from d’Herelle’s studies, many microbiologists, especially in the formal Soviet Union and Eastern Europe, had been researched the practical applications of

bacteriophages. With the developments of penicillin and other chemical antibiotics in the 1940's, however, researches on such phage applications were abandoned in Western countries. A major reason for demise in West was an insufficient understanding on phage biology since poor science at that time. It was also due to the fact that defined chemical antibiotics were more effective to cure the illnesses, simple to use and allowable to the public than phage preparations. This antibiotic era had been maintained for about 40 years with developments of enhanced or novel antibiotics, but faced a difficult situation: the emergence of antibiotic-resistant pathogenic bacteria. As antibiotics were widely used in many areas, bacteria started to develop resistant mechanisms against these life-threatening chemical agents, and even exchanged the genes related with antibiotic-resistance with each other. Although novel antibiotics have been developed and deployed to control these resistant mutants, resistances against virtually every new class of antibiotics were emerged (Clatworthy *et al.*, 2007). As a result, so called 'super bacteria' exhibiting resistance against multiple antibiotics was reported in several pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Falagas et al., 2006; Livermore, 2012; Moellering, 2012), and tremendously threatens public health. With this antibiotic's limitation, phage therapy and phage biocontrol were reevaluated and received renewed interests in recent years.

Practical phage applications

Compared to the chemotherapies, applications of phages considered to contain many advantages like followings. Firstly, they have a high specificity to the target bacteria. The mode of action of chemical antibiotics is diverse but commonly targets the essential metabolism(s) of prokaryotes, such that they can eliminate the natural normal bacterial flora as well as the unwanted pathogenic bacteria. Phages, however, only kill the target bacteria leaving other bacteria that alone, because they do not adsorb to and infect non-host bacteria. Therefore, some possible side effects, such as a temporary diarrhea, that derived from the absence of normal microflora would not occur with phage therapy. Secondly, phages can be auto-dosing while antibiotics require continuous sequential treatments to completely eliminate the target. As obligate parasitic entities, bacteriophages can infinitely multiply their copies so long as their host are existed, and will be removed if host are completely disappeared. It implies that frequent treatments are unnecessary, such that phages have advantages than the antibiotics in aspects of practical convenience as well as financial terms. The relatively lower development costs for phage system than that for novel antibiotics is the third merits for phage applications (Matsuzaki *et al.*, 2005). Lastly, resistant bacterial mutants against phages can be rapidly overcome with simultaneous phage mutants since phages themselves are also able to actively

mutate with their host. In contrast, new antibiotics that can kill the resistant mutants against the present antibiotics have to be passively developed through intensive and time-consuming researches. Since this development is getting worse as more and more pathogens rapidly obtain the resistance to the developed antibiotics, phages that co-evolved with their host steal the limelight from antibiotics.

With these advantages that revealed along with an improvement of our knowledge in molecular genetics and basic cell biology, phage applications were resumed in several areas, including medical practice and food and agricultural industries. Series of studies performed in the 1980s by William Smith and his colleagues are considered as a blasting fuse of rekindled interests in phage therapy in the West. In 1982, they reported a successful use of *E. coli*-specific phages to treat diarrhea-causing *E. coli* infections in mice. In this study, Smith *et al*. showed that a single dose of phage is more effective than several doses of antibiotics such as tetracycline, ampicillin and chloramphenicol, etc. for treating mice experimentally infected with pathogenic *E. coli* (Smith and Huggins, 1982). The similar results were also observed with the *E. coli*-infected calves, lambs and piglets in subsequent studies (Smith and Huggins, 1983; Smith *et al.*, 1987a, b), reevaluating the efficacy of phage applications in a veterinary context. Since then, many reports describing the usefulness of phage treatment against experimental

animal infections by other pathogens including *Salmonella* spp. (Atterbury *et al.*, 2007; Bardina *et al.*, 2012; Borie *et al.*, 2008; Borie *et al.*, 2009; Fiorentin *et al.*, 2005), *Pseudomonas aeruginosa* (Fukuda *et al.*, 2012; Heo *et al.*, 2009; McVay *et al.*, 2007; Wang *et al.*, 2006), *Acinetobacter baumannii* (Soothill, 1992) and *Vibrio vulnificus* (Cerveny *et al.*, 2002) were published.

Not only animals but the human was also subjected to the bacteriophage therapies. In 1981 to 1986, Slopek and colleagues performed clinical challenges of phages to patients with pathogenic bacterial infections by staphylococci, *Klebsiella*, *Pseudomonas*, *E. coli* and *Salmonella* (Slopek *et al.*, 1984; Slopek *et al.*, 1983a, b; Slopek *et al.*, 1985a, b, c; Slopek *et al.*, 1987). Total 550 patients, including 518 ones who had previously undergone unsuccessful treats with antibiotics, were ingested phage suspensions or dressed with phage-soaked bandages if they had wound infections. Overall, 94% of cases were successfully recovered from infections, and the success rate was ranged from 75% to 100% dependent to pathogen. Another example for phage applications to the human was a clinical study of *Klebsiella* infections; total of 109 patients who treated with *Klebsiella*-specific phage preparation were effectively recovered without toxic side effects (Bogovazova *et al.*, 1992).

Since the costly outbreaks of foodborne diseases have been continuously occurring in many countries, applications of phages have also been extended to the

fields of food industries. The concept is simple: pathogens that harm to crops or livestock or that spoil foodstuffs could be controlled with appropriate phage(s). In situations where the use of antibiotics was prohibited to prevent a spreading of antibiotics-resistances, an establishment of food safety by use of phages as novel biocontrol agents is a noticeable challenge. This novel approach is further supported by an increasing public awareness that chemical preservatives in food are not good for health. As extensions of classical phage therapy, several studies firstly focused on a control of livestock-associated pathogens in the animals before slaughter. For example, bacteriophages specific for toxigenic *E. coli*, *Salmonella* or *Campylobacter* reduced the levels of each pathogen significantly in cattle or broilers (Atterbury *et al.*, 2007; Fiorentin *et al.*, 2005; Sheng *et al.*, 2006; Wang *et al.*, 2006). Phages were also directly applied to the food stuffs, including fruits and vegetables, fresh produce, beef and infant formula. *Listeria* phages P100 and A511 reduced 4-log units of *Listeria* count on cabbage and iceberg lettuce stored at 6°C for 6 days (Guenther *et al.*, 2009). Another study, fresh-cut iceberg lettuce and cantaloupe were contaminated with *E. coli* O157:H7, and subsequently treated with three lytic phages targeting this pathogen (Sharma *et al.*, 2009). It resulted in significant reductions of *E. coli* cells after incubation at 4°C for 2 or 7 days. This toxigenic *E. coli* was also eliminated by three specific phages from the experimentally contaminated beef surfaces incubated at 37°C for 1 hr (O'Flynn *et*

al., 2004). *Cronobacter sakazakii* (formally known as *Enterobacter sakazakii*) contamination in prepared infant milk formula was also controlled by phage ESP 732-1 treatments, in dose-dependently (Kim *et al.*, 2007).

In recent years, phage biocontrol began to step forward to the areas of hygiene. Yang *et al*. isolated and characterized a virulent *A. baumannii* phage AB1 and, they suggested the application of phage AB1 as a non-toxic sanitizer in public place, e.g., hospital, for controlling *A. baumannii* contamination (Yang *et al.*, 2010).

Problems to overcome

Although phages contain high potentials as attractive alternative agents to antibiotics, there still are at least two problems to overcome to be widely accepted by the public: (i) immunological perspectives of phage applications and (ii) emergence of phage-resistant bacterial mutants.

The intake of phages has been considered as to be innocuous because they are commonly existed in everywhere including foods that we regularly eat everyday. However, the consumption of phage preparations is a different matter: huge number of phage particles that entered simultaneously into the body might provoke abnormal immune responses, e.g., allergies. Another possible problem is a development of neutralizing antibodies against phages, which would result in a diminished effectiveness of phage treatment (Sulakvelidze *et al.*, 2001). Especially,

the target bacteria-lysing effect of successive treatments using identical phage preparations to the initial treatment would significantly affected by the already developed phage-neutralizing antibodies. Very few cases of side effects of phage applications, however, were revealed in the extensive experiments performed in the former Soviet Union and Eastern Europe (Hanlon, 2007) and, reduction of therapeutic effect with multiple administrations has not been reported (Matsuzaki *et al.*, 2005). Recently, it was also revealed that phage preparations do not disturb bactericidal activities of human granulocytes and monocytes (Kurzepa-Skaradzinska *et al.*, 2013). Nevertheless, more careful studies considering human body have to be conducted to clearly address the concerns on safety as well as immunological aspect of phage applications.

Studies plan to solve the second problem of phage applications -phage resistant mutant- also must be conducted. Like as the case of antibiotics, the development of resistant mutants against phages is unquestionable since, identical to antibiotics, phages also threaten the survival of bacteria. To date, various phage-resistance mechanisms have been reported: the alterations of host receptors to prevent phage adsorption, restriction of penetrated phage nucleic acids, and activation of phage abortive infections (Labrie *et al.*, 2010). Among them, altering or modifying of host receptors is one of the most common strategies to defense phage attacks. Various host receptors for phages, including lipopolysaccharides (LPS), flagella, and outer

membrane proteins, are blocked, modified, or even eliminated to avoid the grabs by the killer (Allison and Verma, 2000; Nesper *et al.*, 2000; Nordstrom and Forsgren, 1974). Although pathogens eventually developed phage-resistances in this ways, solutions for this problem were also proposed. Since mutations that simultaneously alter several types of host receptors on one cell are unlike to occur, the use of different phages in cocktail was suggested (Gill and Hyman, 2010; Goodridge, 2004; Hagens and Loessner, 2007; Levin and Bull, 2004), and practically used (O'Flynn *et al.*, 2004; Sharma *et al.*, 2009; Tanji *et al.*, 2004), to reduce the frequency of emergence of phage-resistant pathogens. In addition, as phages are mutable predators of bacteria, they also co-evolved along with the evolution of their prey, such that the escape phage mutants that can infect the phage-resistant mutants could be easily isolated. Subsequently, they would be used as a component of phage cocktail. Indeed, T-even phage Ox2 did evolve to alternatively use OmpC, OmpX, and even LPS as host receptor instead of the original receptor OmpA (Drexler *et al.*, 1991; Drexler *et al.*, 1989; Montag *et al.*, 1987). On the other hand, there are some optimistic views on the phage-resistant mutants because the mutations that confer resistance against phages are too costly for pathogens to normally survive in human body and cause infectious diseases (Hagens and Loessner, 2007; Hyman and Abedon, 2010). Even though the proposed methods or opinion described above sound convincing, more intensive

researches on phage resistance are necessary to validate these methods, and consequently to guarantee the safety of phage therapy and biocontrol. With the real circumstances of somewhat negative results by phage resistances (Carey-Smith *et al.*, 2006; Fischer *et al.*, 2004; Kocharunchitt *et al.*, 2009), the understanding of underlying mechanism(s) in phage resistance and investigation of other ways to overcome this problem are inevitably required for successful phage applications.

I-3. *Salmonella,* a problematic foodborne pathogen

The bacterial genus *Salmonella* comprises the Gram-negative, rod-shaped, facultative anaerobes with multiple flagella projecting in all directions. As a member of the family *Enterobacteriaceae, Salmonella* causes variety of diseases including mild diarrhea, enteric fever, and bacteremia in both worm- and cold-blooded animals. It suggested that *E. coli* and *Salmonella* spp. derived from a common ancestor about 120-160 million years ago because these two bacteria differ by only 10% in their genome sequences (Alff-Steinberger, 2000).

Since first identified in 1885 by the US Bureau of Animal Industry (Salmon and Smith, 1885), Salmonellae has been classified with complex nomenclatures (Brenner *et al.*, 2000). There are two distinct species in the genus *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. The formal one includes all medically important salmonellae, and is subdivided into six subspecies (*S. enterica* ssp. *enterica*, *S. enterica* ssp. *salamae*, *S. enterica* ssp. *arizonae*, *S. enterica* ssp. *diarizonae*, *S. enterica* ssp. *houtenae*, and *S. enterica* ssp. *indica*) based on their biochemical differences. These subspecies are further classified into more than 50 and 2400 serovars based on the somatic (O) antigen and flagellar (H) antigen, respectively, by use of scheme suggested by Kauffman and White (de Jong *et al.*, 2012). However, designation for each serovar is commonly written in shortly rather

than full form, e.g., *S.* Typhimurium instead of *S. enterica* ssp. *enterica* serovar Typhimurium, for convenience and consistent with the previous literatures.

With regard to the salmonellae-causing diseases and their host, *Salmonella* serovars can be divided into two groups: invasive typhoidal serotypes and non-typhoidal *Salmonella* (referred as NTS) serotypes. The formal one includes *S.* Typhi and *S.* Paratyphi, and they are exclusively human-restricted. These serotypes cause a bacteremic illness known as typhoid fever (or enteric fever) but it shows different manifestations from other Gram-negative bacteremias (Tsolis *et al.*, 2008). The latter serotypes are represented by *S.* Typhimurium and *S.* Enteritidis, and characterized by their wide range of vertebrate host including rodents, cattle and mammals (Garai *et al.*, 2012). These NTS mainly cause a self-limiting diarrhea in healthy individuals and occasionally cause secondary bacteremia and focal infections in immunocompromised peoples. In developing countries, however, the bacterial bloodstream is continuously caused mostly by NTS and resulted in case fatality of 20-25% in Africa (Feasey *et al.*, 2012; Reddy *et al.*, 2010).

With these mild to severe diseases, *Salmonella* has been a huge global burden. In 2000, it was estimated that more than 21 million illnesses and 217,000 deaths were globally caused by typhoid fever, and 5.4 million illnesses by paratyphoid fever (Crump *et al.*, 2004; Crump and Mintz, 2010). The cases of non-typhoidal *Salmonella* gastroenteritis was exceed those of enteric fevers: approximately 93.8

million cases of gastroenteritis with 155,000 deaths were globally occurred in every year by *Salmonella* species, and about 85% cases of these were foodborne (Majowicz *et al.*, 2010). As contaminated foodstuffs, such as poultry products, raw eggs, milk, beef, and raw fruits/vegetables, are known as the primary common sources of *Salmonella* (Sanchez-Vargas *et al.*, 2011; Weber, 2009), dissemination of this pathogen is in rapid increasing through modern mass production and global distribution of foods. Therefore, the prevention of salmonellosis should be focuses on the food safety, including improvement of sanitation and ensuring the safety of raw food and water supplies in food processing (Crump and Mintz, 2010).

Symptoms of salmonellosis (diarrhea, nausea, fever and abdominal cramps) started to develop within 12 to 74 hr after infection, and usually resolve in 5 to 7 days in healthy peoples often without treatments other than oral fluids (Center for Disease Control and Prevention, 2010). Infants, the elderly and peoples in immunocompromised are at greatest risk for severe or complicated diseases, such that they are needed a more careful attention. In some cases (i.e., illness accompanying systemic infections and secondary bacteremia), however, antibiotics such as ampicillin, trimethoprim-sulfamethoxazole or ciprofloxacin are necessary.

I-4. Purpose of this study

Conventional method to control the pathogenic bacteria is use of antibiotics. These bactericidal chemicals target the vital processes of pathogens, such as synthesis of RNA (transcription), protein (translation) or cell wall (Clatworthy *et al.*, 2007). The misuse and/or abuse of antibiotics, however, led the emergence of antibiotic-resistant bacteria, and thus, the public health is facing a threat of drug-resistant pathogens.

Salmonella are no exception to this problem. *Salmonella* causes a huge burden of morbidity and mortality in worldwide, and costs enormous financial losses (Crump *et al.*, 2004; Majowicz *et al.*, 2010), such that antibiotics have been occasionally used to prevent or control *Salmonella* infections. Especially in livestock industries, these bactericidal agents were frequently used to promote the growth of food animals, such as poultry and pigs (Angulo *et al.*, 2000; Holmberg *et al.*, 1984). Antibiotics were also used as therapeutic purpose; ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole are used traditionally as first-line antimicrobial agents to treat enteric fevers caused by *S*. Typhi and *S*. Paratyphi (Crump and Mintz, 2010) and invasive NTS diseases (Guerrant *et al.*, 2001). In result, like as other pathogens, some *Salmonella* have become resistant to multiple antibiotics, and designated as MDR (multidrug resistant) *Salmonella*.

Although fluoroquinolones were recommended as the next-line antibiotics for drug-resistant *Salmonella*-causing diseases (Guerrant *et al.*, 2001), resistant salmonellae against these agents were also reported (Brown *et al.*, 1996) and susceptibility to this drug is gradually decreasing (Lynch *et al.*, 2009).

In this situation, bacteriophages, especially virulent phages, have drawn attention as alternatives to antibiotics. Several phages specific to *Salmonella* have been isolated from various sources (e.g., sewage, fecal samples and poultry farm), and were applied into experimentally contaminated foods to study their control ability against *Salmonella*. Most of studies showed a promising result: a cocktail of 4 phages reduced about 3.5-log unit of *S*. Enteritidis count on honeydew melon slices (Leverentz *et al.*, 2001); *S*. Typhimurium DT104 on chicken frankfurters was controlled by the virulent *Salmonella* phage Felix O1 and a related phage variant by 2-log unit (Whichard *et al.*, 2003); virulent phage FO1-E2 reduced *Salmonella* counts more than 3-log unit in various ready-to-eat foods (i.e., wiener sausages, turkey breast, mixed seafood, chocolate milk and egg yolk) (Guenther *et al.*, 2012).

Unfortunately, however, there are also several reports describing inefficient biocontrol ability of *Salmonella* phages. Carey-Smith *et al*. investigated an ability of novel phage FGCSSa1 in *Salmonella* growth inhibition, but this phage incompletely lysed *Salmonella* cultures (Carey-Smith *et al.*, 2006). *Salmonella* phages SSP5 and SSP6 belong to family *Myoviridae* and *Siphoviridae*, respectively,

also could not completely eliminate the *Salmonella* from experimentally contaminated alfalfa seeds (Kocharunchitt *et al.*, 2009). The above reports did not investigate the reasons for resistance, but inferred that phage resistant mutants that are already presented in the inoculum or suffer physiologic/genetic modifications during the phage challenge were the reason for incomplete control of *Salmonella*.

Accordingly, this study intends to (i) isolate and characterize novel phages specific to *Salmonella* spp. from various samples, (ii) reveal the mechanism(s) for resistance against these phages in their host bacteria and, (iii) elucidate a prophage induction mechanism and exploit the possibility of temperate phages in phage applications. The present study would be helpful to improve the phage biocontrols, providing comprehensive understandings on phage-host interactions and practical useful tools.

Chapter II.

Characterization of a T5-Like Coliphage SPC35 and Differential Development of Resistance to SPC35 in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*

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II-1. Introduction

Bacteriophages (phages) are bacterial viruses and exist everywhere their host thrives. It is estimated that there are at least 10 phage particles per bacterial cell (Skurnik and Strauch, 2006). Phages are highly specific for a bacterial species; therefore, they are able to target a pathogen without disrupting normal microflora (Hagens and Loessner, 2007; Matsuzaki *et al.*, 2005). As multidrug-resistant bacteria have become prevalence, phages have attracted attention as a potential alternative to antibiotics. Their advantages include lower developing cost compared to the traditional antibiotics (Matsuzaki *et al.*, 2005), ability to replicate in the presence of host bacteria, lack of side effects, and high host specificity. The clinical use of phages, known as 'phage therapy' (Matsuzaki *et al.*, 2005), has been reported for human and animal diseases caused by *Escherichia coli* (Barrow *et al.*, 1998; Chibani-Chennoufi *et al.*, 2004), *Pseudomonas aeruginosa* (Ahmad, 2002; Soothill, 1994), *Salmonella* spp. (Borie *et al.*, 2009; Toro *et al.*, 2005), and *Staphylococcus aureus* (Matsuzaki *et al.*, 2003).

Recently, the use of phages has also been extended to the field of food safety (Greer, 2005; Hagens and Loessner, 2007; Hudson *et al.*, 2005). The burden of foodborne illness on public health is tremendous and generates great economical cost. These diseases still occur despite dramatic improvements in hygiene and

sanitation in food processing. Phages have been used to control foodborne diseases caused by *Salmonella* ssp. (Goode *et al.*, 2003; Modi *et al.*, 2001), *Listeria monocytogenes* (Carlton *et al.*, 2005; Guenther *et al.*, 2009), *Campylobacter jejuni* (Goode *et al.*, 2003; Hwang *et al.*, 2009; Wagenaar *et al.*, 2005), and *E. coli* O157:H7 (O'Flynn *et al.*, 2004; Sharma *et al.*, 2009). Recently, the *L. monocytogenes*-specific phages obtained the status of generally recognized as safe (GRAS) and, was approved as a food preservative by the US Food and Drug Administration (Bren, 2007). Motivated by this situation, many researchers started to investigate the development of bacteriophages as biocontrol agents.

However, several limitations must be overcome before phages can be successfully applied as therapeutic and/or biocontrol agents. The first limitation is the narrow host range of phages. The high host specificity of most phages is a so called “double-edged sword”; high specificity is necessary for the specific targeting of unwanted pathogens, but the narrow host range renders phage biocontrol ineffective for non-host strains (Greer and Dilts, 1990). The use of broad host range phages which isolated by multiple-host enrichment methods (Bielke *et al.*, 2007; Carey-Smith *et al.*, 2006; Jensen *et al.*, 1998) or a phage cocktail consisted with multiple phage types (Kunisaki and Tanji, 2010; O'Flynn *et al.*, 2004; Zhang *et al.*, 2010) has been proposed and applied to overcome this problem. Another limitation is the rapid emergence of phage resistant mutants (Bastias *et al.*, 2010; Carey-

Smith *et al.*, 2006; Garcia *et al.*, 2009; Kocharunchitt *et al.*, 2009). A phage cocktail can delay or prevent the emergence of resistant mutants (Hudson *et al.*, 2005; Kunisaki and Tanji, 2010); however, some of the underlying mechanism(s) for the appearance of phage resistant mutants are not fully elucidated.

In this context, I isolated and characterized the novel lytic bacteriophage SPC35. This bacteriophage has a noticeable broad host range: it can infect both genus *Salmonella* and genus *Escherichia*, which are important foodborne pathogens of humans and animals (Buzby and Roberts, 2009; Newell *et al.*, 2010). Bacterial challenge assays revealed distinctive patterns of growth inhibition and emergence of phage SPC35 resistant mutants in these two genera.

II-2. Materials and Methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table II-1. All *S.* Typhimurium and *E. coli* mutants were derived from *S.* Typhimurium SL1344 and *E. coli* MG1655, respectively. *E. coli* isolates were kindly provided by Dong-Hyun Kang at Seoul National University, Korea. Bacteria were cultured at 37°C in Luria-Bertani (LB) broth and plates supplemented with 25 μg ml^{-1} of chloramphenicol where appropriate.

Plasmid construction. The plasmid used in the complementation assay was constructed by cloning the complete *btuB* gene and its promoter region from *S.* Typhimurium SL1344 into the low copy-number plasmid pACYC184. The *btuB* gene and its promoter region were PCR amplified using primer set btuB-CF-2 (5′-TTG TAG GGC ATG CTC AGT GGA TGT-3′) and btuB-CR-2 (5′-ATA CAA GCT TGG TGG GAC GTG GTT-3′). The resultant PCR product was digested with restriction enzymes SphI and HindIII and then inserted into the corresponding restriction sites of plasmid pACYC184. The DNA sequence was verified by sequencing, and the resultant plasmid was named pMS100.

Table II-1. Bacterial strains used in Chapter II and SPC35 host range

Strains (genotype)	Plaque formation[a]	Source or reference[b]
***Salmonella enterica* serovar Typhimurium**		
UK1	CC	Laboratory collection
LT2	CC	Laboratory collection
SL1344	CC	Laboratory collection
Δ*fhuA* (SL1344 Δ*fhuA*)	CC	(25)
Δ*fepA* (SL1344 Δ*fepA*)	CC	(25)
Δ*btuB* (SL1344 Δ*btuB*)	-	(25)
Δ*btuB* $_{(pMS100)}$ (Δ*btuB* harboring pMS100)	CC	This study
14028s	CC	Laboratory collection
ATCC 19586	CC	ATCC
ATCC 43174	CC	ATCC
DT 104	CC	Laboratory collection
***S.* Enteritidis**		
ATCC 13078	C	ATCC
***S. enterica* isolates**		
3068	CC	Laboratory collection
3605	TT	Laboratory collection
3792	CC	Laboratory collection
4509	CC	Laboratory collection

Escherichia coli

ATCC 25922	(+)	ATCC
B/1	(+)	*
B/4	-	*
BE4a	-	*
C600	-	*
DH5α	CC	Laboratory collection
DH10B	CC	Laboratory collection
FS575	T	*
GM1	CC	*
GM119	CC	*
HB101	(+)	*
JM109	CC	*
K-12 substr. MC4100	CC	Laboratory collection
K-12 substr. MG1655	CC	Laboratory collection
K-12 substr. W3110	T	*
K-12 2B	-	*
K802	(+)	*
LE392	-	*
RP1	C	*
SY327	CC	*
TGI	CC	*

***E. coli* isolates**

C-660	CC	*

E-2g	(+)	*
E-2j	-	*
E-7b	-	*
E-34	-	*
WADDL 2701	CC	*
WADDL 2735	-	*
WADDL 2983	-	*
WADDL 2902	-	*
WADDL 3463	C	*
WADDL 3476	(+)	*
WADDL 3502	-	*
WADDL 3811	-	*
WADDL 3977	-	*
WADDL 4036	-	*
WADDL 4064	(+)	*
WADDL 4083	CC	*
WADDL 4190	-	*
WADDL 4241	-	*

[a], CC, relatively more clear plaque; C, clear plaque; T, turbid plaque; TT, relatively more turbid plaque; (+), growth inhibition zone; -, no plaque.

[b], ATCC, American Type Culture Collection; *, Collection of Food Hygiene laboratory at Seoul National University, Korea.

Bacteriophage isolation. Ten samples of chicken feces and two samples of chicken internal organs were obtained from the Mo-ran traditional market, Seoul, Korea. The 25-g samples were homogenized in 225 ml sterile Butterfield's phosphate-buffered dilution water (312.5 μM KH_2PO_4 adjusted to pH 7.2 with NaOH) for 90 sec by stomacher (BacMixer® 400, Interscience Laboratory Inc., St Nom, France). The 10-ml samples were then mixed with 50 ml LB broth and incubated at 37°C for 24 h with constant agitation (220 rpm). After centrifugation of the culture (9000 x g, 10 min, 4°C) and filtration of the clear supernatant (0.22-μm pore filter, Millipore, Ireland), the filtrate (10 ml) was mixed with 50 ml LB broth and 500 μl overnight culture of *S*. Typhimurium SL1344. This bacteria-phage mixture was incubated at 37°C for 8 h (at 220 rpm), followed by centrifugation and filtration as described above. Ten-fold serial dilutions of these filtrates were subjected to the spotting assay with *S*. Typhimurium SL1344 or *E. coli* MG1655 as indicator strain to confirm the presence of bacteriophages. To isolate and purify the single bacteriophage, the overlay assay was carried out as previously described (Adams, 1959). Each plaque showing a unique morphology were picked with a sterile pipette tip, followed by elution with sodium chloride-magnesium sulfate buffer [SM buffer; 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgSO_4$]. This process was repeated at least three times.

Spotting assay and overlay assay. The host bacteria were cultured overnight and then 100 μl of them was inoculated into 5 ml of soft LB agar (0.4% agar), which had been heated to 42°C in a waterbath. After gentle vortexing, this mixture was poured into prepared LB agar plates (1.5% agar, with 25 μg ml^{-1} chloramphenicol if necessary) and allowed to solidify at room temperature for 30 min to produce bacterial lawns. Then, 10 μl phage stock dilutions (10-fold serial dilutions in SM buffer) were spotted onto the top agar layer, and the plates were dried at room temperature for 30 min. These plates were incubated overnight at 37°C and inspected the next day for single plaques or bacterial growth inhibition zones.

The overlay assay was carried out similarly but with some modifications. Briefly, phage stock dilutions (100 μl) were mixed with the same volumes of overnight bacteria culture in 5 ml soft LB agar (0.4% agar) and then poured into plates. After solidification for 30 min at room temperature, the plates were incubated at 37°C, and plaques were examined.

Propagation of SPC35. Bacteriophage propagation and concentration were carried out according to methods of Sambrook (Sambrook and Russell, 2001) with some modifications. Briefly, the lysate of a single plaque of phage SPC35 was added to *E. coli* MG1655 culture ($OD_{600\ nm}$ = 0.5–0.6), which was then incubated at

37°C for 4 h. The cleared culture with cell debris (host bacteria lysate) was treated with chloroform (1% of final volume), incubated at 37°C for 5 min, and then centrifuged (15,000 x g, 10 min, 4˚C). Bacteriophage particles in the filtered supernatant (0.22-μm pore filter) were precipitated with 10% (w/v) polyethylene glycol (PEG) 6000 in 1 M NaCl at 4°C for 10 h. After centrifugation (10,000 x g, 15 min, 4°C), precipitated phages were resuspended in SM buffer and separated by CsCl density gradient ultracentrifugation (78,500 x g, 2 h, 4°C). The phage band fraction was separated by puncturing centrifuge tube bottom with a heated needle and, dialyzed against 1 L of dialysis buffer [50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM $MgSO_4$] in a Spectra/Por® dialysis membrane (MWCO: 12,000-14,000, Spectrum Laboratories, Inc., US). The dialysis buffer was changed after one hour and the phage fraction was dialyzed for an additional one hour before it was transferred into a sterilized glass ampoule. The titers of this concentrated phage stocks were determined by the overlay assay and, the phage stocks were stored at 4°C until further use.

Morphological analysis by TEM. Drops of SPC35 stock dilution (4 μl, approximately $1x10^{10}$ PFU ml^{-1}) were placed on carbon-coated copper grids; after 1 min, the excess phage suspension was removed with filter paper. Equal volumes of 2% aqueous uranyl acetate (pH 4.0) were added for 20 sec to negatively stain the

phage particles, and excess solution was removed as above. Phages were examined by transmission electron microscopy (LEO 912AB, Carl Zeiss) at 80 kV accelerating voltage and images were scanned with a Proscan 1024 X 1024-pixel CCD camera at the National Academy of Agricultural Science (Suwon, Korea). Phages were classified according to the International Committee on Taxonomy of Viruses (ICTV) classification (Fauquet *et al.*, 2005).

Sequencing of SPC35 genomic DNA and genome analysis. Extraction of phage DNA was carried out using proteinase K and SDS as previously described (Sambrook and Russell, 2001). Whole genome sequencing of SPC35 was performed with a Genome Sequencer FLX Titanium (Roche, Mannheim, GE) and assembled with GS De Novo Assembler Software (Roche) at Macrogen Inc., Korea. Open reading frames (ORFs) of the SPC35 genome were determined using GeneMark.hmm (Lukashin and Borodovsky, 1998) and the BLAST algorithm (Altschul *et al.*, 1997). The tRNAscan-SE program (Lowe and Eddy, 1997) was used to search for tRNA-coding sequences. The SPC35 genome sequence was deposited to GenBank under the accession number of HQ406778.

Phage adsorption assay. Exponentially growing cells in early log phase (1 ml, $OD_{600\ nm}$=1.0–1.5) were harvested by centrifugation (13,000 x g, 1 min, 4°C),

washed with phosphate-buffered saline (pH 7.2), and re-suspended in 10 ml LB broth. This bacterial suspension was aliquoted into ten microtubes (1 ml suspension per 1.75-ml tube) and pre-incubated at 37°C for 5 min. Then, SPC35 was added (MOI = 0.01) to each tube with one minute intervals, and the tubes were incubated until 10 min incubation period for the first tube. Cells were immediately removed by centrifugation (13,000 x g, 1 min, 4°C) and filtration (0.22-μm pore), and the number of unbound free phage particles in each tube was determined by overlay assay. The baseline number was determined by adding the same concentration of SPC35 to the bacteria-free LB broth and incubating at 37°C for 10 min.

Bacterial challenge assay. For the bacterial challenge assay, 50 ml fresh LB broth was inoculated with an overnight culture of *S.* Typhimurium SL1344 or *E. coli* MG1655 (1% inoculum), followed by incubation at 37°C at 220 rpm until the $OD_{600\ nm}$ was 0.5. SPC35 stock dilutions (100 μl) were then added (MOI of 0.1, 1, or 10) to these cultures, which were incubated for another 24 h. Bacterial growth was monitored by measuring $OD_{600\ nm}$ at various time points. As a negative control, one bacterial culture was inoculated with 100 μl SM buffer instead of SPC35.

Isolation and sub-culturing of SPC35-resistant mutants. To isolate SPC35-resistant mutants, the high-titer overlay assay was performed as previously

described (Chibeu *et al.*, 2009; Johnson and Lory, 1987). Briefly, overnight cultures of the host bacteria were serially diluted (10-fold), and 100 μl of each dilution was added to 5 ml soft LB agar (0.4% agar). Then, 100 μl SPC35 suspension (about 2x 10^{10} PFU ml^{-1}) was added (MOI ≥10), and these mixtures were poured onto LB agar plates and incubated for 24 h at 37°C smilar to the standard overlay assay.

Five colonies from each resultant plate of *S.* Typhimurium SL1344 and *E. coli* MG1655 were picked with sterilized loops. Each colony was streaked onto a fresh LB agar plate and simultaneously inoculated into 3 ml LB broth, followed by overnight incubation at 37°C. The spotting assay was conducted to verify the phage resistance. These procedures were repeated at least three times to isolate a single colony and evaluate maintenance of phage resistance.

Amplification and DNA sequencing of *btuB*. The *btuB* gene of *E. coli* MG1655 and *S.* Typhimurium was amplified by PCR using the following primer sets: *E. coli* MG1655 (MG btuB-CF) 5′ -GCA TGC TCA TCA GAT GTC CAG ATC T-3′ and (MG btuB-CR) 5′-AAG CTT ACC AGC ACG GTG GGA C-3′; *S.* Typhimurium (btuB-CF) 5′-GCA TGC TCA GTG GAT GTC CAG C-3′ and (btuB-CR) 5′-AAG CTT ACC AGC ACG GTG GGA C-3′. PCR was carried out using a GeneAmp® PCR System 9700 (Applied Biosystems™, Foster City, CA, USA) with

the following steps; a pre-denaturation at 95°C for 7 min, followed by amplification for 30 cycles at 95°C for 45 sec, 60°C for 45 sec, and 72°C for 140 sec; and a final extension at 72°C for 7 min. The PCR products were analyzed by 1% agarose gel electrophoresis with 0.5X TAE buffer [20 mM Tris-acetate, 0.5 mM EDTA (pH 8.0)].

The *btuB* genes from SPC35-resistant *E. coli* mutants were sequenced by primer walking using the oligonucleotides SPC35-1F (5′-GGG TGA GTG GTT CTG CC-3′), SPC35-1R (5′-CCG TAG GAA GCA ATG AAG CG-3′), SPC35-2F (5′-CCC TGA ATC TCC AGA CAA CC-3′), and SPC35-2R (5′-GCT CTC TGC TAT TCC GTT ACTC-3′), and the previously described primers MG btuB-CF and MG btuB-CR. DNA sequencing was performed with an ABI PRISM® 3730XL DNA Analyzer (Applied Biosystems™) at Macrogen Inc., Korea.

II-3. Results

Isolation of SPC35 and host range determination. Nine bacteriophages that formed clear plaques on *S.* Typhimurium SL1344 were isolated. The ability of these phages to infect both *S.* Typhimurium SL1344 and *E. coli* MG1655 was confirmed by spotting phage lysate onto bacterial lawns. One phage, SPC35, formed clear plaques against both bacteria and was selected for further experiments. *E. coli* MG1655 was chosen to propagate SPC35 because plaques produced in this strain were larger and clearer than those of *S.* Typhimurium SL1344.

SPC35 stock (about 2.8 x 10^{11} PFU ml^{-1}) was diluted 10-fold in SM buffer and analyzed by the spotting assay to determine the host range. All of the tested seven *S.* Typhimurium strains, one *Salmonella* Enteritidis isolate, and four *Salmonella enterica* isolates were susceptible to SPC35. In addition, SPC35 produced single plaques or bacterial growth inhibition zones in tested 23 of 40 *E. coli* strains (Table II-1).

SPC35 morphology. Morphological analysis by TEM revealed that phage SPC35 has a head with icosahedral symmetry and a long tail without a contractile sheath, indicating that it belongs to the family *Siphoviridae*. The isometric head of SPC35 had a mean diameter of 70 nm and the long, non-contractile tail was 154

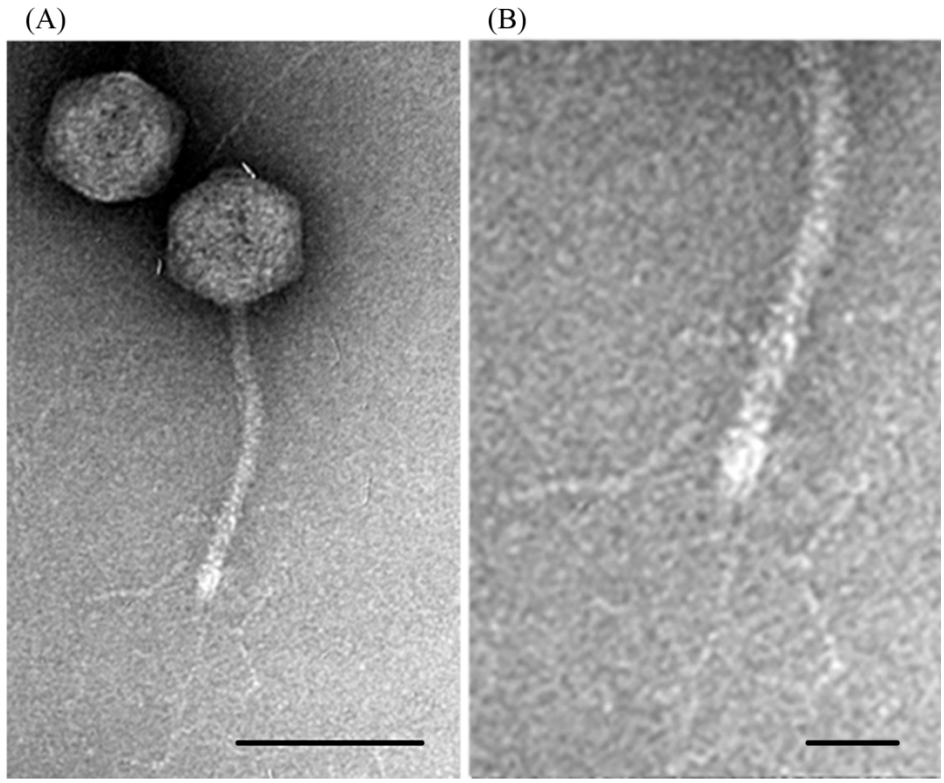

Fig. II-1. Transmission electron micrographs of phage SPC35. Whole phage particle is shown in panel A and the tail with tail fibers are enlarged in panel B. Scale bar is 100 nm for panel A and 25 nm for panel B, respectively.

nm x 10 nm (Fig. II-1), which closely resembled the morphology of bacteriophage T5 (head diameter of 60 nm and tail with 150 nm x 8 nm).

Sequencing and organization of the SPC35 genome. Only one contig of 108,778 bp (average GC content, 39.24%) was constructed with 99.97% of bases with a quality score ≥40. However, the 4,548 nucleotides at left-end of contig and the 5,025 nucleotides at right-end of contig were covered by an average read depth of 125.05, while the nucleotides in the other region were covered by an average read depth of 69.18, suggesting that SPC35 contains terminally redundant DNA at both ends of genome. Accordingly, SPC35 genome composed of 118,351 bp with terminally repeated 9,573 bp and exhibited an average GC content of 39.39%. One hundred thirty-seven of the 145 predicted putative ORFs were closely related to those of T5 or T5-like phages; thus, phage SPC35 was classified as a T5 group phage (Fig. II-2, II-3, and Table II-2). Similar to the genomes of these phages, the SPC35 genome was divided into pre-early, early, and late regions by gene function (Fig. II-2).

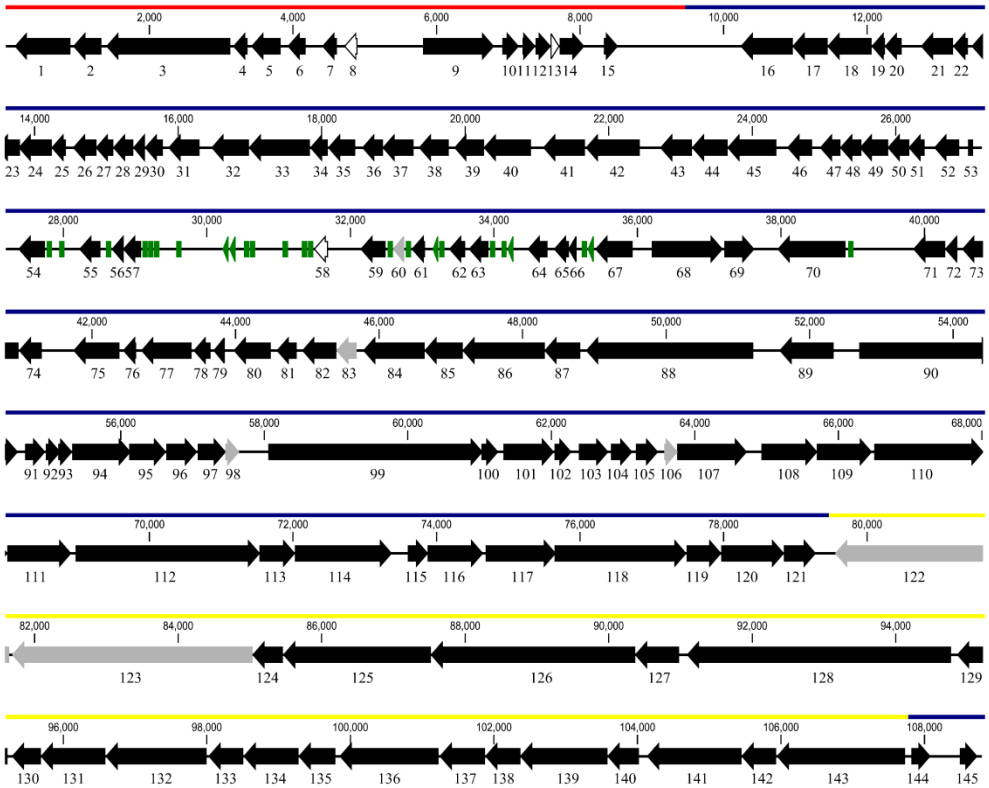

Fig. II-2. SPC35 genome map excluding the terminally redundant DNA sequence in right-end. The predicted ORFs with their direction of transcription and tRNA-coding sequences are indicated by colored arrows: black, homologous to T5 group phages (T5, BF23, H8, and EPS7); gray, homologous to other microorganisms; white, no homology; green, tRNA-coding sequences. Red, blue, and yellow lines above arrows indicate the pre-early, early, and late regions, respectively.

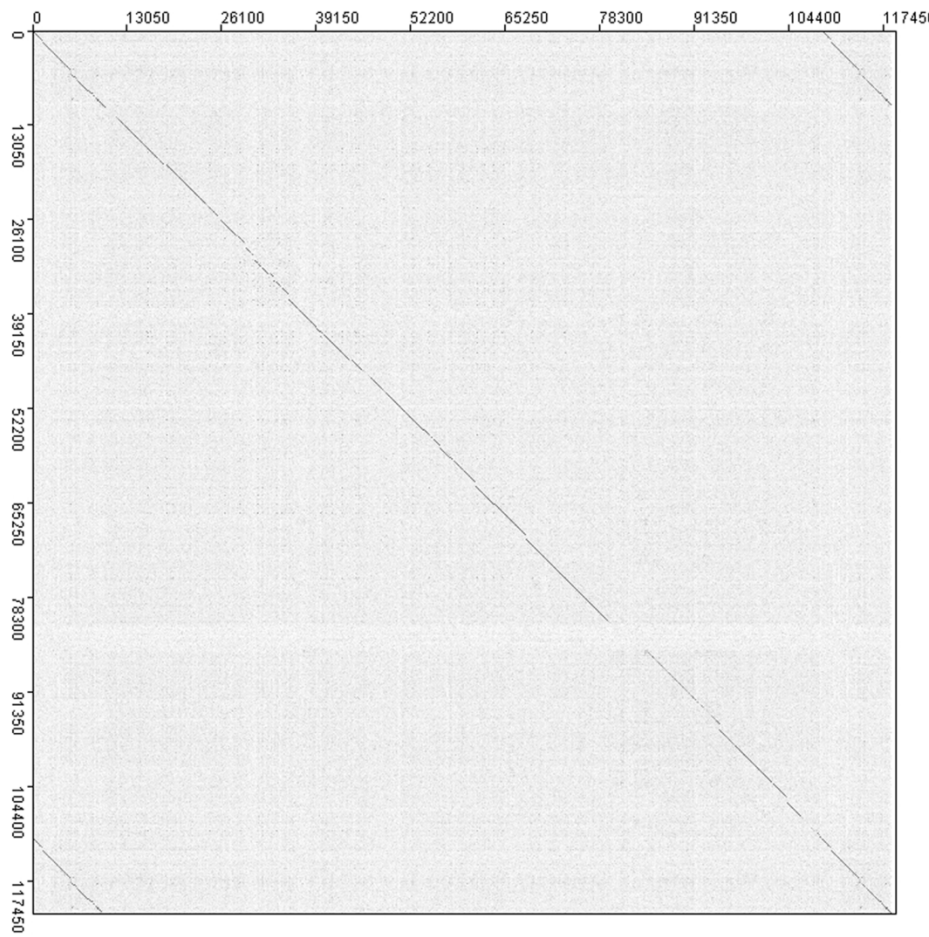

Fig. II-3. Dot-plot analysis. The SPC35 genome sequence (X-axis) and the T5 genome sequence (Y-axis, accession number NC005859) wer compared by using of JDotter (Brodie *et al.*, 2004).

Table II-2. Features of ORFs in the SPC35 genome

ORF	Strand	Start	End	Size (bp)	Orthologue [organism]	Score (E-value)	% ID/Sim
1	-	124	900	777	deoxynucleoside-5'-monophosphatase [Enterobacteria phage T5]	1253 (5E-136)	94/97
2	-	939	1331	393	hypothetical protein T5.002 [Enterobacteria phage T5]	598 (2E-60)	88/93
3	-	1400	3127	1728	A1 protein [Enterobacteria phage T5]	2792 (0)	96/98
4	-	3168	3365	198	probable A1 protein precursor [Enterobacteria phage T5]	316 (7E-28)	95/98
5	-	3421	3828	408	A2 protein [Enterobacteria phage T5]	666 (2E-68)	95/97
6	-	3927	4178	252	hypothetical protein T5.007 [Enterobacteria phage T5]	331 (1E-29)	92/96
7	-	4409	4612	204	hypothetical protein T5.008 [Enterobacteria phage T5]	303 (3E-26)	88/93
8	-	4714	4899	186	No hits found		
9	+	5812	6801	990	hypothetical protein T5.011 [Enterobacteria phage T5]	1089 (8E-117)	75/85
10	+	6917	7147	231	hypothetical protein T5.012 [Enterobacteria phage T5]	380 (3E-35)	93/97
11	+	7198	7377	180	hypothetical protein T5.013 [Enterobacteria phage T5]	161 (8E-10)	90/90
12	+	7380	7592	213	hypothetical protein T5.014 [Enterobacteria phage T5]	338 (2E-30)	91/96
13	+	7589	7714	126	No hits found		
14	+	7716	8060	345	hypothetical protein AGC_0013 [Enterobacteria phage EPS7]	542 (5E-54)	87/96

15	+	8334	8528	195	hypothetical protein T5.017 [Enterobacteria phage T5]	295 (2E-25)	88/95
16	-	10240	10965	726	hypothetical protein AGC_0018 [Enterobacteria phage EPS7]	580 (6E-58)	88/92
17	-	10962	11450	489	Putative ORF [Enterobacteria phage H8]	817 (7E-86)	96/98
18	-	11450	12064	615	hypothetical protein T5.019 [Enterobacteria phage T5]	799 (2E-83)	72/84
19	-	12064	12249	186	hypothetical protein T5.020 [Enterobacteria phage T5]	113 (3E-04)	96/100
20	-	12249	12482	234	hypothetical protein T5.021 [Enterobacteria phage T5]	254 (1E-20)	81/88
21	-	12753	13199	447	ORF022 [Enterobacteria phage T5]	801 (5E-84)	98/99
22	-	13201	14404	204	hypothetical protein T5.024 [Enterobacteria phage T5]	334 (6E-30)	94/99
23	-	13459	13797	339	hypothetical protein T5.025 [Enterobacteria phage T5]	491 (4E-48)	84/94
24	-	13778	14239	462	hypothetical protein T5.026 [Enterobacteria phage T5]	807 (9E-85)	97/99
25	-	14236	14436	201	hypothetical protein T5.027 [Enterobacteria phage T5]	319 (3E-28)	95/98
26	-	14536	14862	327	hypothetical protein T5.028 [Enterobacteria phage T5]	474 (4E-46)	83/92
27	-	14852	15097	246	hypothetical protein T5.029 [Enterobacteria phage T5]	384 (1E-35)	91/95
28	-	15094	15375	282	hypothetical protein T5.030 [Enterobacteria phage T5]	336 (4E-30)	85/90
29	-	15375	15536	162	hypothetical protein T5.031 [Enterobacteria phage T5]	111 (5E-04)	59/84
30	-	15536	15787	252	hypothetical protein T5.032 [Enterobacteria phage T5]	420 (7E-40)	100/100
31	-	15866	16297	432	hypothetical protein T5.033 [Enterobacteria phage T5]	752 (2E-78)	99/100

32	-	16465	16986	522	putative serine/threonine protein phosphatase [Enterobacteria phage T5]	864 (2E-91)	94/96
33	-	16986	17837	852	putative serine/threonine protein phosphatase [Enterobacteria phage EPS7]	1169 (4E-126)	76/85
34	-	17840	18085	246	hypothetical protein T5.036 [Enterobacteria phage T5]	300 (6E-26)	84/95
35	-	18090	18467	378	hypothetical protein AGC_0039 [Enterobacteria phage EPS7]	341 (1E-30)	80/92
36	-	18566	21856	291	putative thioredoxin [Enterobacteria phage T5]	488 (8E-48)	95/98
37	-	18849	19280	432	hypothetical protein T5.038 [Enterobacteria phage T5]	652 (8E-67)	100/100
38	-	19356	19772	417	hypothetical protein T5.039 [Enterobacteria phage T5]	579 (2E-58)	96/97
39	-	19850	20263	414	lysozyme [Enterobacteria phage T5]	480 (8E-47)	64/80
40	-	20260	20916	657	putative holin [Enterobacteria phage T5]	1092 (2E-117)	94/97
41	-	21088	21669	582	putative ATP-dependent Clp protease [Enterobacteria phage T5]	1017 (6E-109)	98/99
42	-	21682	22434	753	deoxynucleoside-5'-monophosphate kinase [Enterobacteria phage T5]	1319 (1E-143)	99/100
43	-	22718	23161	444	hypothetical protein T5.045 [Enterobacteria phage T5]	664 (4E-68)	94/97
44	-	23161	23658	498	H-N-H-endonuclease F-TflVI [Enterobacteria phage T5]	879 (4E-93)	99/99
45	-	23655	24335	681	hypothetical protein T5.047 [Enterobacteria phage T5]	671 (1E-68)	59/69
46	-	24490	24834	345	hypothetical protein T5p047 [Enterobacteria phage T5]	467 (3E-45)	81/91
47	-	24945	25229	285	hypothetical protein T5.049 [Enterobacteria phage T5]	434 (2E-41)	87/96
48	-	25226	25522	297	ORF047 [Enterobacteria phage T5]	449 (3E-43)	85/93

49	-	25500	25895	396	hypothetical protein AGC_0053 [Enterobacteria phage EPS7]	547 (1E-54)	89/91
50	-	25888	26187	300	hypothetical protein AGC_0054 [Enterobacteria phage EPS7]	459 (2E-44)	86/94
51	-	26180	26401	222	hypothetical protein T5.053 [Enterobacteria phage T5]	343 (5E-31)	92/93
52	-	26538	26885	348	hypothetical protein T5.054 [Enterobacteria phage T5]	467 (2E-45)	83/94
53	-	27007	27078	72	hypothetical protein [Bacteriophage 5]	100 (8E-03)	72/76
54	-	27376	27744	369	putative acetyltransferase-related protein [Enterobacteria phage T5]	607 (1E-61)	94/98
55	-	28224	28517	294	hypothetical protein [Bacteriophage 5]	477 (2E-46)	94/98
56	-	28676	28840	165	hypothetical protein [Bacteriophage 5]	262 (1E-21)	98/98
57	-	18849	19280	432	hypothetical protein T5.038 [Enterobacteria phage T5]	652 (8E-67)	100/100
58	-	19356	19772	417	hypothetical protein T5.039 [Enterobacteria phage T5]	579 (2E-58)	96/97
59	-	19850	20263	414	lysozyme [Enterobacteria phage T5]	480 (8E-47)	64/80
60	-	20260	20916	657	putative holin [Enterobacteria phage T5]	1092 (2E-117)	94/97
61	-	21088	21669	582	putative ATP-dependent Clp protease [Enterobacteria phage T5]	1017 (6E-109)	98/99
62	-	21682	22434	753	deoxynucleoside-5'-monophosphate kinase [Enterobacteria phage T5]	1319 (1E-143)	99/100
63	-	22718	23161	444	hypothetical protein T5.045 [Enterobacteria phage T5]	664 (4E-68)	94/97
64	-	23161	23658	498	H-N-H-endonuclease F-TflVI [Enterobacteria phage T5]	879 (4E-93)	99/99
65	-	34838	35044	207	hypothetical protein AGC_0079 [Enterobacteria phage EPS7]	293 (4E-25)	84/94

66	-	35037	35153	117	hypothetical protein [Enterobacteria phage BF23]	187 (7E-13)	97/100
67	-	35414	35932	519	hypothetical protein [Bacteriophage 5]	835 (7E-88)	100/100
68	+	36199	37188	990	hypothetical protein [Bacteriophage 5]	1354 (2E-147)	96/98
69	+	37210	37629	420	hypothetical protein [Bacteriophage 5]	235 (2E-18)	100/100
70	-	37954	38901	948	hypothetical protein [Enterobacteria phage BF23]	1286 (1E-139)	97/99
71	-	39845	40288	444	hypothetical protein AGC_0089 [Enterobacteria phage EPS7]	729 (9E-76)	93/98
72	-	40288	40458	171	hypothetical protein T5.082 [Enterobacteria phage T5]	268 (3E-22)	93/100
73	-	40527	40976	450	spore cortex-lytic enzyme precursor [Enterobacteria phage T5]	638 (4E-65)	96/97
74	-	40982	41299	318	hypothetical protein T5.084 [Enterobacteria phage T5]	541 (6E-54)	96/98
75	-	41742	42380	639	hypothetical protein T5.085 [Enterobacteria phage T5]	1062 (5E-114)	98/98
76	-	42433	42615	183	hypothetical protein T5.086 [Enterobacteria phage T5]	292 (5E-25)	97/98
77	-	42686	43387	702	putative metallopeptidase [Enterobacteria phage T5]	1214 (1E-131)	95/98
78	-	83416	43652	237	hypothetical protein T5.088 [Enterobacteria phage T5]	388 (4E-36)	95/96
79	-	43694	43849	156	hypothetical protein AGC_0098 [Enterobacteria phage EPS7]	175 (2E-11)	93/95
80	-	43975	44490	516	hypothetical protein T5.089 [Enterobacteria phage T5]	763 (2E-79)	91/94
81	-	44574	44852	279	hypothetical protein T5.090 [Enterobacteria phage T5]	466 (3E-45)	99/99
82	-	44929	45405	477	ribonuclease H [Enterobacteria phage T5]	847 (2E-89)	98/99

83	-	45405	45686	282	gp48 [*Vibrio cholerae* 1587]	230 (8E-18)	49/66
84	-	45780	46634	855	thymidylate synthase [Enterobacteria phage EPS7]	1431 (2E-156)	92/95
85	-	46634	47167	534	putative dihydrofolate reductase [Enterobacteria phage T5]	842 (1E-88)	90/92
86	-	47164	48309	1146	putative aerobic ribonucleoside diphosphate reductase, small subunit [Enterobacteria phage T5]	1993 (0)	98/99
87	-	48306	48803	498	putative H-N-H-endonuclease P-TflVIII [Enterobacteria phage T5]	311 (3E-27)	42/57
88	-	48885	51215	2331	putative aerobic ribonucleoside diphosphate reductase, large subunit [Enterobacteria phage T5]	3958 (0)	100/100
89	-	51581	52333	753	phosphate starvation-inducible protein [Enterobacteria phage T5]	1319 (1E-143)	99/100
90	+	52692	54566	1875	anaerobic ribonucleoside-triphosphate reductase [Enterobacteria phage T5]	3284 (0)	99/100
91	+	54666	54947	282	hypothetical protein AGC_0113 [Enterobacteria phage EPS7]	467 (3E-45)	95/97
92	+	54957	55160	204	hypothetical protein T5.101 [Enterobacteria phage T5]	300 (5E-26)	92/98
93	+	55129	55323	195	hypothetical protein AGC_0115 [Enterobacteria phage EPS7]	305 (2E-26)	91/100
94	+	55323	56138	816	putative Sir2-like protein [Enterobacteria phage T5]	1394 (3E-152)	94/98
95	+	56119	56631	513	hypothetical protein T5.105 [Enterobacteria phage T5]	808 (9E-85)	99/100
96	+	56634	57062	429	hypothetical protein T5.106 [Enterobacteria phage T5]	698 (4E-72)	92/97
97	+	57072	57464	393	hypothetical protein T5.107 [Enterobacteria phage T5]	637 (5E-65)	91/96
98	+	57457	57651	195	g20 [Cyanophage clone GS2633]	76 (5E00)	37/65

99	+	58057	61044	2988	putative replication origin binding protein [Enterobacteria phage T5]	4666 (0)	92/97
100	+	61028	61261	234	hypothetical protein T5.110 [Enterobacteria phage T5]	386 (6E-36)	96/100
101	+	61332	62036	705	D2 protein [Enterobacteria phage T5]	1224 (1E-132)	99/100
102	+	62047	62280	234	hypothetical protein T5.112 [Enterobacteria phage T5]	248 (6E-20)	86/92
103	+	62384	62794	411	D3 protein [Enterobacteria phage T5]	524 (6E-52)	98/99
104	+	62832	63128	297	hypothetical protein T5.114 [Enterobacteria phage T5]	421 (5E-40)	100/100
105	+	63179	63487	309	hypothetical protein T5.115 [Enterobacteria phage T5]	539 (1E-53)	100/100
106	+	63573	10898	189	putative glutamate formiminotransferase [*Aciduliprofundum boonei* T469]	79 (3E00)	40/66
107	+	63754	64725	972	NAD-dependent DNA ligase, subunit A [Enterobacteria phage T5]	1657 (0)	97/98
108	+	64928	65707	780	NAD-dependent DNA ligase subunit B [Enterobacteria phage T5]	1252 (7E-136)	97/98
109	+	65700	66467	768	D5 protein [Enterobacteria phage T5]	1174 (8E-127)	98/99
110	+	66499	68022	1524	putative replicative DNA helicase (D6) [Enterobacteria phage T5]	2539 (0)	99/100
111	+	68019	68909	891	putative DNA replication primase [Enterobacteria phage T5]	1561 (1E-171)	99/100
112	+	68972	71539	2568	DNA polymerase [Enterobacteria phage T5]	4569 (0)	99/100
113	+	71532	72029	498	hypothetical protein T5.123 [Enterobacteria phage T5]	851 (7E-90)	99/100
114	+	72026	43378	1353	putative ATP-dependent helicase (D10) [Enterobacteria phage T5]	2401 (0)	99/100
115	+	73599	73883	285	hypothetical protein T5.125 [Enterobacteria phage T5]	483 (4E-47)	99/100

116	+	73876	74649	774	D11 protein [Enterobacteria phage T5]	1324 (3E-144)	97/99
117	+	74686	75663	978	putative recombination endonuclease, subunit D12 [Enterobacteria phage T5]	1611 (3E-177)	97/98
118	+	75644	77482	1839	probable exonuclease subunit 2 [Enterobacteria phage T5]	2975 (0)	99/99
119	+	77486	77968	483	D14 protein [Enterobacteria phage T5]	845 (3E-89)	99/99
120	+	77968	78843	876	flap endonuclease [Enterobacteria phage T5]	1537 (9E-169)	98/100
121	+	78840	79286	447	putative deoxyUTP pyrophosphatase [Enterobacteria phage T5]	703 (1E-72)	95/98
122	-	79556	81643	2088	putative phage tail protein [*Salmonella* Typhi str. CT18]	490 (7E-47)	52/69
123	-	81687	85037	3351	gp25 [Enterobacteria phage N15]	469 (3E-44)	35/54
124	-	85037	85459	423	putative phage tail protein [Enterobacteria phage EPS7]	657 (3E-67)	88/94
125	-	85464	87521	2058	tail protein Pb4 [Enterobacteria phage EPS7]	2886 (0)	98/99
126	-	87521	90370	2850	tail protein Pb3 [Enterobacteria phage EPS7]	4500 (0)	89/96
127	-	90367	90981	615	hypothetical protein AGC_0150 [Enterobacteria phage EPS7]	930 (1E-98)	85/94
128	-	91091	94771	3681	pore-forming tail tip protein [Enterobacteria phage T5]	3597 (0)	68/78
129	-	94855	95223	369	hypothetical protein T5.142 [Enterobacteria phage T5]	561 (3E-56)	98/100
130	-	95285	95689	405	hypothetical protein T5.143 [Enterobacteria phage T5]	685 (1E-70)	96/99
131	-	95686	96585	900	minor tail protein gp24 [Enterobacteria phage BF23]	1178 (4E-127)	70/86
132	-	96590	97996	1407	major tail protein [Enterobacteria phage EPS7]	2147 (4E-127)	90/94

133	-	98023	98508	486	hypothetical protein T5.146 [Enterobacteria phage T5]	855 (3E-90)	99/100
134	-	98512	99279	768	hypothetical protein T5.147 [Enterobacteria phage T5]	1212 (3E-131)	96/98
135	-	99279	99791	513	hypothetical protein T5.148 [Enterobacteria phage T5]	869 (7E-92)	94/98
136	-	99851	101227	1377	major head protein precursor [Enterobacteria phage EPS7]	2113 (0)	95/98
137	-	101245	101877	633	putative prohead protease [Enterobacteria phage T5]	1114 (5E-120)	100/100
138	-	101881	102369	489	putative tail protein [Enterobacteria phage T5]	344 (5E-31)	98/100
139	-	102366	103583	1218	portal protein [Enterobacteria phage T5]	2114 (0)	98/100
140	-	103583	104020	438	hypothetical protein T5p149 [Enterobacteria phage T5]	585 (6E-59)	83/90
141	-	104135	105451	1317	terminase, large subunit [Enterobacteria phage T5]	2345 (0)	100/100
142	-	105451	105933	483	putative SciB protein [Enterobacteria phage T5]	646 (4E-66)	100/100
143	-	105944	107731	1788	receptor-binding protein [Enterobacteria phage BF23]	2715 (0)	84/91
144	+	107817	108083	267	receptor-blocking protein [Enterobacteria phage BF23]	461 (1E-44)	99/100
145	+	108492	108737	246	hypothetical protein T5.162 [Enterobacteria phage T5]	275 (5E-23)	89/94

The pre-early region of the SPC35 genome, which appears to be transferred into the host bacteria in the first-step transfer, contains 15 ORFs that encode various host enzyme inhibitors including inhibitors for host DNA, RNA, and protein synthesis, and gene products that degrade host DNA. ORF10, ORF11, ORF12, ORF14, and ORF15 showed high similarities to ORFs in the pre-early region of T5 and EPS7, which encode these inhibitory proteins (Hong *et al.*, 2008; Wang *et al.*, 2005). In addition, the gene products of ORF1, ORF3, ORF4, and ORF5 were highly homologous to deoxynucleoside-5′-monophosphatase (dmp, 97%), A1 protein (98%), probable A1 protein precursor (98%), and A2 protein (97%) of T5, respectively. These proteins are required for the second-step transfer of the T5 phage genome (Wang *et al.*, 2005).

The two-step genome transfer mechanism of T5 is facilitated by terminally redundant DNA sequences spanning the pre-early region, a common feature of T5-like phages (Hong *et al.*, 2008). Similar to T5 or T5-like phages, the terminally repeated 9,573 nucleotides of SPC35 genome spanned the pre-early region (Fig. II-2 and II-3), and appeared to facilitate the two-step transfer of SPC35 genome.

The early region of the SPC35 genome encodes a variety of proteins thought to participate in DNA replication, transcription, recombination, metabolism, signal transduction, and cell lysis; most of these genes are highly homologous to T5 genes. For instance, the putative gene products of ORF110, ORF111, and ORF112 are

replicative DNA helicase, DNA replication primase, and DNA polymerase, respectively, with 99% identity to the corresponding T5 genes. ORF39, ORF40, and ORF73 encode phage lysis-related proteins including lysozyme (endolysin, 80% similarity to the T5 gene), putative holin (97% similarity to the T5 gene), and spore-cortex-lytic enzyme precursor (97% similarity to the T5 gene), respectively. In addition, the repertory of 24 identified tRNA-coding sequences in the early region of the SPC35 genome was identical to that of the T5 genome, except that the tRNA-Val was absent and an additional tRNA-Lys was present in SPC35 genome. Because a codon usage is quite different between SPC32H and its host *Salmonella*, these tRNAs might present for the efficient translation of SPC35 mRNAs. I could not find any genes involved in lysogen formation.

The late region of the SPC35 genome was composed of 24 ORFs. The majority of these ORFs encode structural and morphogenesis proteins such as head and tail proteins, portal protein, and receptor-binding protein. The putative prohead protease, portal protein, terminase (large subunit), and putative SciB protein encoded by ORF137, ORF139, ORF141, and ORF142, respectively, were nearly identical to the corresponding T5 proteins, whereas tail proteins Pb3 and Pb4, major tail protein, and major head protein precursor encoded by ORF126, ORF125, ORF132, and ORF136, respectively, showed greater homology to EPS7 proteins. In addition, ORF143 encoding a receptor-binding protein and ORF144 encoding a

receptor-blocking protein were highly homologous to those of BF23 (84% and 99% identity, respectively), suggesting a common host receptor for BF23 and SPC35.

BtuB act as a receptor for SPC35. Because morphologic characteristics and genome of SPC35 were similar to those of T5 group phages, I investigated several known receptors of T5-like phages for SPC35 infection. *S.* Typhimurium SL1344 deletion mutants lacking *fhuA*, *btuB*, or *fepA* (Hagens and Loessner, 2007) were tested for susceptibility to SPC35 by the spotting assay. The only mutant SPC35 could not infect was the *btuB* deletion mutant, indicating a crucial role for BtuB in the infection process of SPC35. Complementation of the *btuB* mutation by pMS100 harboring the wild type (WT) *btuB* gene restored the susceptibility of SL1344 to SPC35.

The requirement of BtuB in the initial step of SPC35 infection was further assessed by the phage adsorption assay of WT, mutant, and complemented strains. As shown in Fig. II-4, phage adsorption of *S.* Typhimurium SL1344 was completely abolished by the *btuB* mutation. Complementation of the *btuB* mutation with pMS100 restored to infection to the levels seen with WT *btuB*, resulting in the gradual reduction of free phage particles. These results strongly suggest that BtuB is the host receptor for phage SPC35.

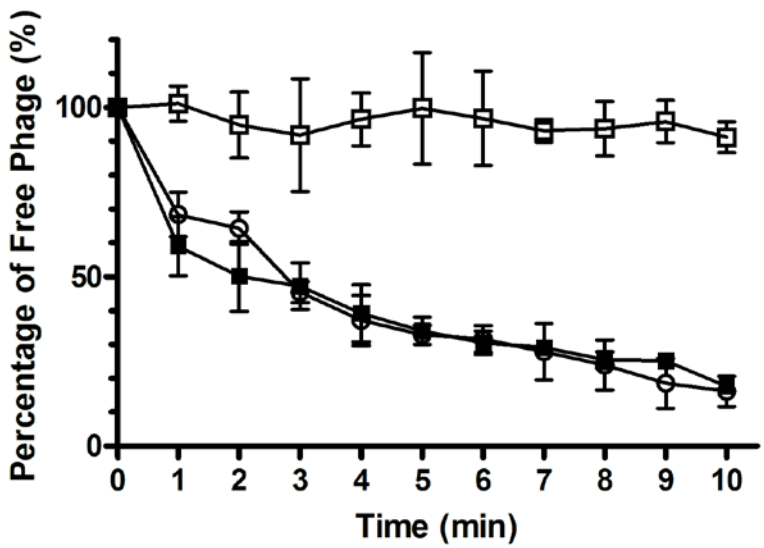

Fig. II-4. SPC35 adsorption assay. Exponentially growing WT *S.* Typhimurium SL1344 (open circle), Δ*btuB* mutant (open square), or *btuB* complementation strain (closed square) cells were infected with SPC35 (MOI of 0.01) and incubated at 37°C. After centrifugation and filtration, phage titer in the filtrate was determined by standard overlay assay. The percentage of free phage particles was calculated as: [(phage titer in the supernatant of SPC35-infected bacterial suspension)/(phage titer in the supernatant of SPC35-inoculated LB broth)x 100]. The results are expressed as mean and standard deviation of triplicate assays.

Differential responses of *S*. Typhimurium and *E. coli* to SPC35 infection. To assess the host bacteria lysing ability of SPC35 in broth, I evaluated the growth of host bacteria in the presence of SPC35. The effect of SPC35 infection on growth of *S*. Typhimurium SL1344 differed markedly from that of *E. coli* MG1655 (Fig. II-5). SPC35 caused growth retardation in *S*. Typhimurium SL1344 rather than growth inhibition. Infection of SL1344 at MOI of 0.1 produced only slight growth retardation, and MOI of 1 or 10 produced growth retardation of about 2 hours (Fig. II-5A). In *E. coli* MG1655, however, infection with SPC35 at MOI of 0.1 began to suppress the host growth significantly about 2 hours after infection. Growth inhibition was maintained until approximately 10 h after phage infection, after which bacterial growth eventually resumed (Fig. II-5B). Several isolates from this culture obtained after sequential streaking on fresh LB agar plates showed a SPC35-insensitivity in standard spotting assay (data not shown), indicating that phage resistant mutants were generated. Addition of SPC35 at MOI of 1 or 10 also suppressed *E. coli* MG1655 growth, but phage-resistant mutants appeared more rapidly at MOI of 10.

The differential responses to SPC35 infection by *S*. Typhimurium and *E .coli* were also observed on solid media. In the high-titer (ca. 2×10^{10} PFU ml^{-1}) overlay assay, colonies of various sizes within the overall confluent lysis zone were observed on the *E. coli* MG1655 plate. In contrast, the *S*. Typhimurium SL1344

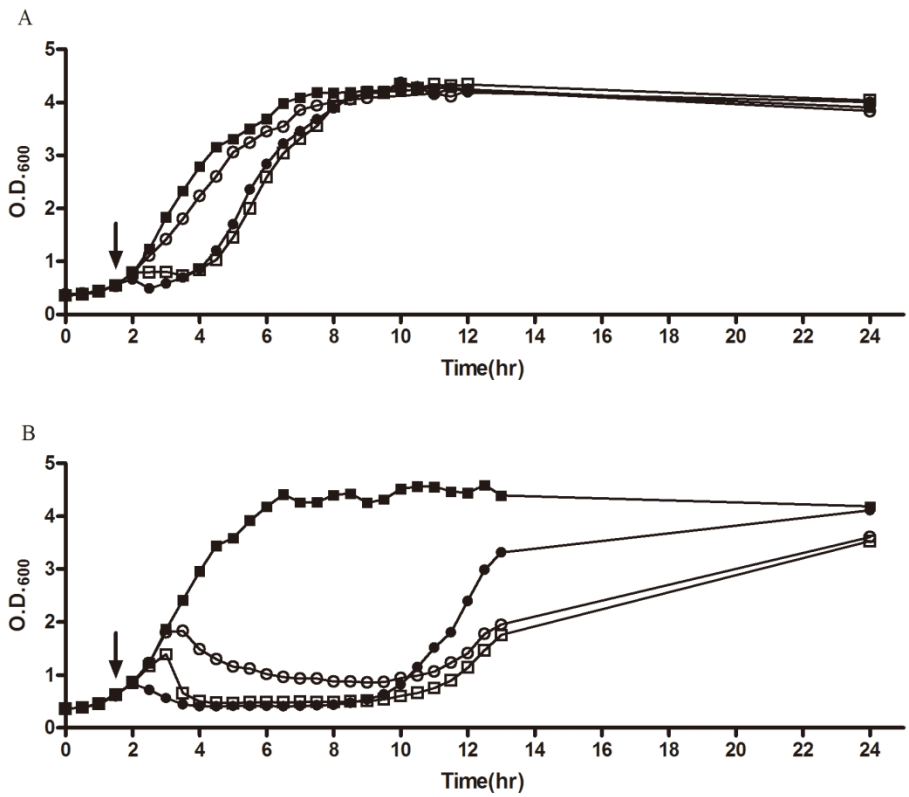

Fig. II-5. Bacterial challenge assay with SPC35. SPC35 (100 μl) was added at MOI of 0.1 (open circle), MOI of 1 (open square), or MOI of 10 (closed circle) to the *S.* Typhimurium SL1344 (A) or *E. coli* MG1655 (B) culture after 1.5 hours incubation (vertical arrows, $OD_{600\ nm}$=0.5–0.6). A bacterial culture inoculated with SM buffer instead of SPC35 was used as negative control (closed square).

plate produced a bacterial lawn without any visible lysis zones or plaques (data not shown). However, when 10-fold dilutions of *S.* Typhimurium were incubated with the same concentration of SPC35, several putative phage-resistant colonies were formed at MOI of 10^6.

SPC35 resistance is heritable in *E. coli* mutants but not in *S.* Typhimurium mutants. Five of the SPC35-resistant *S.* Typhimurium colonies and five of the SPC35-resistant *E. coli* colonies were picked from each of the high-titer overlay plates; these colonies were streaked three times onto fresh LB agar plates in the absence of SPC35 to isolate a purified single colony. At each streaking step, the newly formed colonies were tested by spotting assay to verify phage resistance. Interestingly, persistence of phage resistance differed between the putative *E. coli* and *S.* Typhimurium mutants. All five putative *E. coli* mutants maintained their resistance throughout the sequential streaking, resulting in no plaques in the spotted lawns. In contrast, all five putative *S.* Typhimurium mutants showed SPC35 sensitivity after the first streaking. However, SPC35 formed relatively turbid plaques in the spotting assay performed with colonies obtained after the first or second streaking, but formed clear plaques when the assay was performed with colonies obtained after the third streaking (Fig. II-6).

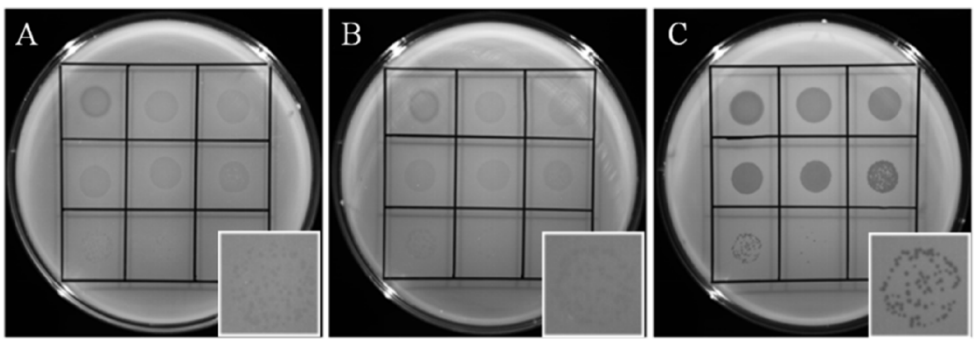

Fig. II-6. SPC35 spotting assay with SPC35-resistant *S*. Typhimurium isolates. Serially diluted (10-fold in SM buffer) phage SPC35 lysates were spotted onto plates of SPC35-resistant *S*. Typhimurium isolates which obtained after a single streaking (A), second streaking (B), and third streaking (C). Insets at the bottom right show individual plaques.

Disruption of the *btuB* gene by insertion sequence 2 results in heritable resistance to SPC35 in *E. coli.* To determine whether the SPC35-resistant *E. coli* had lost their ability to synthesize functional BtuB protein by mutation(s), I analyzed the *btuB* gene of these isolates by PCR amplification. Analysis of PCR products by agarose gel electrophoresis showed fragments of about 3,500 bp from all mutants except one mutant, SPC35^{R}MG-M2 (Fig. II-7). The size of the PCR product in WT *E. coli* MG1655 was about 2,200 bp; therefore, these results indicated that the *btuB* gene was mutated by insertion of a ~ 1,300-bp DNA fragment in mutants SPC35^{R}MG-L1, SPC35^{R}MG-M1, SPC35^{R}MG-S1, and SPC35^{R}MG-S2. DNA sequencing of these amplified fragments revealed an insertion sequence 2 (IS*2*) in the *btuB* gene of all four mutants; however, its orientation and insertion site are differed (Table II-3 and Fig. II-8). The IS*2* was oriented in the same direction as *btuB* transcription in SPC35^{R}MG-L1 and SPC35^{R}MG-M1, but the reverse orientation was observed in SPC35^{R}MG-S1 and SPC35^{R}MG-S2. The IS*2* insertion in SPC35^{R}MG-L1 was 901 nucleotides from the *btuB* start codon, but 1687, 1001, and 939 nucleotides from the start codon in SPC35^{R}MG-M1, SPC35^{R}MG-S1, SPC35^{R}MG-S2, respectively. In these four mutants, the flanking sequence of IS*2* insertion sites had a duplicated sequence of 5-bp long, but there is no common noticeable % GC difference in these flanking sequences (Table II-3). Complementation of one of the *btuB*::IS*2* mutant clones,

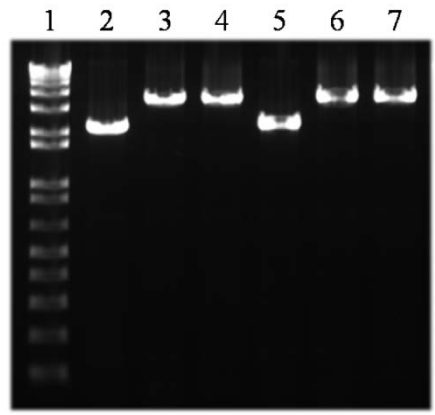

Fig. II-7. PCR analysis of the *btuB* gene in SPC35-resistant *E. coli* mutants. Lane 1, 1kb^{+} DNA ladder; lane 2, WT *E. coli* MG1655; lane 3, SPC35^{R}MG-L1; lane 4, SPC35^{R}MG-M1; lane 5, SPC35^{R}MG-M2; lane 6, SPC35^{R}MG-S1; lane 7, SPC35^{R}MG-S2.

Table II-3. DNA sequences at the regions of IS*2* insertion.

Strains or S*2*s	Flanking sequence[a] (% G+C)	Left end	IS*2*	Right end	Flanking sequence (% G+C)	IS*2* orientation[b]
E. coli MG1655	ATTACAACTACGATCCCCAT				TATGGTCGTTATGAT	
SPC35^{R} MG-L1	ATTACAACTACGATCCCCAT (40)	TAGACTGGCCCCCTG	……	ATAGGGGCAAATCCA	CCCATTATGGTCGTTATGAT (40)	+
SPC35^{R} MG-M1	ATCAAACCGTTAAAATGGGC (40)	TAGACTGGCCCCCTG	……	ATAGGGGCAAATCCA	TGGGCGGTGTGAGCTTGTGG (65)	+
SPC35^{R} MG-S1	GTTATGTTGAGGATGGATAT (35)	TGGATTTGCCCCTAT	……	CAGGGGGCCAGTCTA	GATATGATCAACGTAATACC (35)	-
SPC35^{R} MG-S2	TCGGCGACGCTCGATGAGAT (60)	TGGATTTGCCCCTAT	……	CAGGGGGCCAGTCTA	GAGATGAAGCAATACACCGT (45)	-
IS*2*A[c]	ACGAATAAACGCATAATTAC (30)	TAGACTGGCCCCCTG	……	ATAGGGGCAAATCCA	ATTACCTATCAGGCAGTTTG (40)	+
IS*2*D	ACATCATGGGACAAGAACCC (50)	TGGATTTGCCCCTAT	……	CAGGGGGCCAGTCTA	AACCCCAGAACTTACTTATG (40)	-
IS*2*E[d]	CACTGGATGACGAACTGGCC (60)	truncated IS*2*	……	ATAGGGGCAAATCCA	CAGGTGAACTACGCTCCTCT (55)	+
IS*2*F	AACAAATCCGCATTCGTGGC (50)	TGGATTTGCCCCTAT	……	CAGGGGGCCAGTCTA	GTGGCGAAGCATCCTCCCGT (65)	-
IS*2*H	AGTAAATTCCAATTGTTTAT (20)	TGGATTTGCCCCTAT	……	CAGGGGGCCAGTCTA	AACTTGCTCTTTTCTTCTGG (40)	-
IS*2*I	TATTGTTCATTATCCGTTAT (25)	TAGACTGGCCCCCTG	……	ATAGGGGCAAATCCA	GTTATCATCATTGGTTGTGC (40)	+
IS*2*K	AGGGATTTGATGGTCCCTTG (50)	TAGACTGGCCCCCTG	……	ATAGGGGCAAATCCA	CCTTGTGCTGATATGAATAC (40)	+

[a] The 5-bp flanking sequence duplications are underlined. Note that IS*2*E and IS*2*H do not have these duplication sequences.

[b] +, IS*2* inserted in the same orientation as the direction of *btuB* transcription; -, IS*2* inserted in the opposite orientation as the direction of *btuB* transcription.

[c] Sequences for IS*2* elements have been retrieved from GenBank accession no. U00096.

[d] IS*2*E is a truncated element that does not contain a *insC* (*orfA*) gene.

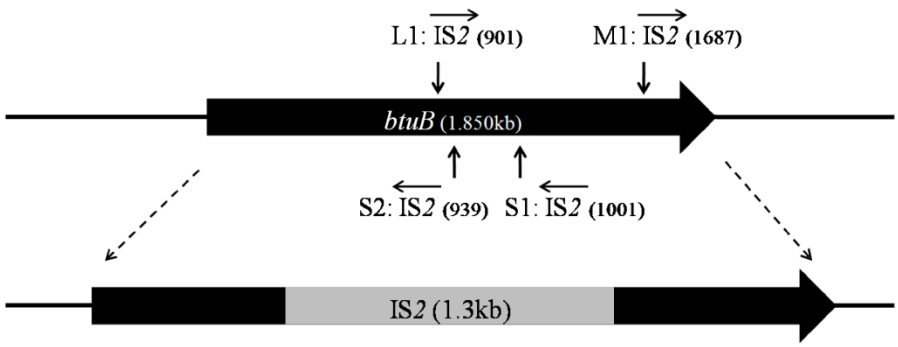

Fig. II-8. Schematic representation of IS*2* insertion sites in the *btuB* gene of SPC35-resistant *E. coli* mutants. Vertical arrow indicates insertion of IS*2*, and corresponding insertion sites are shown in parentheses. Horizontal arrow indicates orientation of IS*2*.

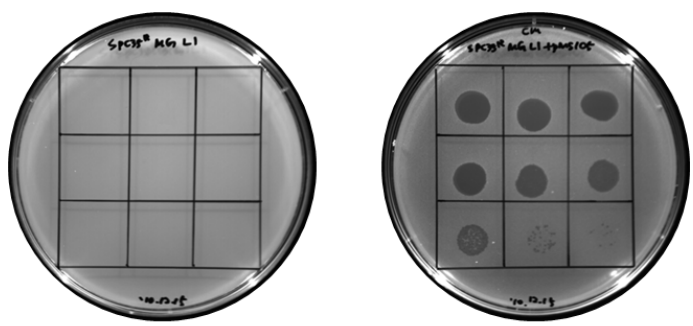

Fig. II-9. Retoration of SPC35-susceptibility by *btuB* complementation in SPC35-resistant *E. coli* mutant. Serial dilutes (10-fold) of SPC35 stock were spotted on lawns of SPC35^{R} MG-L1 and its derivative expressing an *E. coli*-derived *btuB* gene from pMS106.

SPC35^{R}MG-L1, with plasmid pMS106 containing an intact *btuB* gene resulted in the formation of clear plaques of SPC35 in spotting assay (Fig. II-9). This result strongly suggested that IS*2* insertion in *btuB* gene provided the SPC35 resistance in *E. coli*. The PCR amplified *btuB* fragment of SPC35^{R}MG-M2 was the same size as that of WT *E. coli* MG1655; however, one nucleotide (G) was deleted among the five Gs between 325 and 329 nucleotides from the start codon.

In contrast, PCR analysis and sequencing of the *btuB* gene of all five SPC35-resistant *S*. Typhimurium isolates revealed no mutations in their *btuB* gene.

II-4. Discussion

Using *S.* Typhimurium SL1344 as the host bacteria, I isolated a novel bacteriophage SPC35, which can infect *Salmonella* species and *E. coli*. Morphological analysis by TEM revealed that SPC35 has an icosahedral head and non-contractile tail, indicating that it belongs to the family *Siphoviridae* (Fig. II-1). Sequencing and genome analysis revealed that SPC35 genome was highly homologous to that of T5 group phages T5, BF23, H8, and EPS7 (Hong *et al.*, 2008; Rabsch *et al.*, 2007; Wang *et al.*, 2005).

Attachment to the host cell is the initial step of phage infection. Various components of the host bacteria such as flagella, fimbriae, lipopolysaccharides (LPS), and outer membrane proteins are used as bacteriophage receptors (Skurnik and Strauch, 2006). Phage T5 uses the ferrichrome outer membrane transporter FhuA as a receptor (Rabsch *et al.*, 2007). Phage BF23 and EPS7 utilize BtuB, the outer membrane protein involved in vitamin B_{12} uptake (Hong *et al.*, 2008), and H8, binds to the host ferric enterobactin receptor FepA (Rabsch *et al.*, 2007). In the present study, mutant and genome analysis revealed that BtuB is the host receptor for SPC35. Although the SPC35 genome was highly homologous to the T5 genome (Fig. II-3), the putative receptor binding protein of SPC35, which interacts with its corresponding host receptor, was more similar to that of BF23 (amino acid

homology: BF23, 84% identity and 91% positive; T5, 29% identity and 43% positive).

Although the SPC35 genome does not appear to contain genes involved in lysogen formation (Table II-2), several strains of bacteria, including *S. enterica* isolate 3605, *E. coli* FS575, *E. coli* W3110 and *Salmonella* colonies which obtained after the SPC35 challenging, produced turbid plaques. These results may be due to the inefficient lysis of the host bacteria or unknown phage resistance-related factor(s).

The bacterial challenge assay with *S.* Typhimurium and *E. coli* showed that host bacteria could not be completely eliminated at any MOI tested (Fig. II-5). This result can be explained by the proportion of phage resistant bacteria in initial inoculated population or, by the rapid appearance of resistant phenotype. Carey-Smith et al. and Fischer et al. proposed that the growth retardation in the presence of bacteriophage occurs when only a subpopulation of the host bacteria is susceptible to phage infection (Carey-Smith *et al.*, 2006; Fischer *et al.*, 2004). Similar explanation was also suggested previously that the only 15% of *E. coli* O157:H7 Mu^{L} were resistant to phage PP01 (Fischer *et al.*, 2004). In contrast, Kocharunchitt at al. suggested that incomplete lysis of phage-treated bacteria was due to physiologic and/or genetic modification of host cells during phage infection (Kocharunchitt *et al.*, 2009). In the present study, I found that the physiologic

and/or genetic modification of host cells during phage infection, but was differed between genera of host bacteria. In most of the SPC35-resistant *E. coli* isolates, an IS*2* insertion disrupted the *btuB* gene, whereas the SPC35-resistant *S.* Typhimurium isolates had intact *btuB* genes and only showed the transient phage resistance.

Insertion sequences are transposable elements (typically 700–2000 bp) present in many bacterial chromosomes and plasmids (Deonier, 1996). IS*2*, which naturally occurs in *E. coli* K-12, is a 1327-bp insertion sequence (Ghosal *et al.*, 1979; Ronecker and Rak, 1987) of the IS*3* family, which consists of IS*3*, IS*150*, IS*911*, and other elements (Mahillon and Chandler, 1998). IS*3* family members contain only two open reading frames, *orfA* and *orfB*, from which the fusion protein transposase, OrfAB, is generated by a -1 translational frameshift; its transposition is mediated by this transposase via circular and linear intermediates (Haas and Rak, 2002; Lewis *et al.*, 2001). Mutations by IS*2* insertion have been reported in both regulatory regions and coding regions of target genes; for example, the promoter regions of the *galOPETK* and *lac* operons and the coding sequences of *galK*, *relA*, and *hemB* have been mutated by IS*2* (Lewis *et al.*, 1994a). In the present study, all of the IS*2* elements in SPC35-reistant *E. coli* mutants were inserted into the *btuB* coding region (Fig. II-8). In these mutants, the IS*2* was flanked by 5-bp sequence duplications, which normally formed during IS*2* integration into its target gene

(Ghosal *et al.*, 1979). IS*2* showed no apparent preference for G+C percent in target sequences (Table II-3).

Only a few studies have reported phage-resistant mutants with IS insertions in their receptor genes. The *tonB* gene of *E. coli*, which encodes for a protein that assists in phage T1 infection as well as the colicin B action, was spontaneously inactivated by IS*1*, IS*2*, and IS*10* in the presence of colicin B (Kitamura *et al.*, 1995). The *Vibrio cholerae* phage K139 uses the O1 antigen of LPS as host receptor for infection, and K139-resistant *V. cholerae* mutants were reported to have IS*1004* inserted into the O-antigen biosynthesis gene cluster (*manB*, involved in the biosynthesis of perosamine, and *wbeW*, which encodes a glycosyltransferase) (Nesper *et al.*, 2000).

Salmonella Typhimurium LT2 contains only one type of IS element, IS*200*, whereas *E. coli* K-12 contains eight to 12 different IS elements, including IS*2*. Moreover, IS*200* transposes less frequently than *E. coli* K-12 IS elements (Deonier, 1996). Thus, non-heritable phase-variable physiologic changes stimulated by phage infection may be a more common reason of phage resistance than IS*200* insertion in *S.* Typhimurium.

IS elements are widespread; therefore, spontaneous phage-resistant mutants due to IS element insertion into genes required for phage infection might be a problem. However, the low transposition frequency of IS elements suggests that this problem

could be overcome with phage cocktails consisting of various virulent phages that use different host receptors. Simultaneous IS elements insertions into every gene encoding the host receptor which targeted by the phage cocktail is unlikely. Thus, identification of the phage receptor is prerequisite for the production of better phage cocktails.

The transience of SPC35-resistance in *S.* Typhimurium strains was not due to pseudolysogeny, as described previously (Ackermann and DuBow, 1987), because I did not observe spontaneous production of phages from the SPC35-resistant *S.* Typhimurium isolates. PCR analysis also revealed that these isolates did not contain the SPC35 genome (data not shown). Differential resistance is not specific to SPC35; *E. coli* and *Salmonella* also showed differential resistance to several other phages isolated previously (data not shown). Similar transient phage resistance observed with *S.* Typhimurium SL1344 was also reported in *E. coli* O157:H7 and other *Salmonella* spp. (Kocharunchitt *et al.*, 2009; Sheng *et al.*, 2006). The underlying mechanism(s) for this phenomenon is not known clear; thus, the aim of my next study is elucidation of this mechanism and, the results will be described in Chapter III.

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Chapter III.

Spontaneous and Transient Defense against Bacteriophage by Phase-Variable Glucosylation of O-Antigen in *Salmonella enterica* serovar Typhimurium

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III-1. Introduction

In their environment, bacteria continuously encounter bacteriophages. Although bacteriophages are estimated to outnumber bacteria 10 to 1 (Brüssow and Hendrix, 2002), bacteria are able to overcome phage's invasions by various evasive mechanisms. These mechanisms include prevention of phage adsorption, prevention of phage DNA entry, restriction of penetrated phage DNA (the restriction/modification [RM] system or the clustered regularly interspaced short palindromic repeats [CRISPR] system) and activation of the phage abortive infection (Abi) system (Fineran *et al.*, 2009; Hyman and Abedon, 2010; Labrie *et al.*, 2010; Marraffini and Sontheimer, 2010; Sorek and Stern, 2011). Since phage adsorption to the host bacteria is essential for successful phage infection, bacteria have frequently developed phage resistance mechanisms that target this process. Various bacterial cell surface appendages, such as lipopolysaccharides (LPS), teichoic acids, flagella, pili and some outer membrane proteins, serve as host-specific phage receptors, that are often blocked by masking proteins (Nordstrom and Forsgren, 1974; Riede and Eschbach, 1986), or modified by the enzymes of serotype-converting phages (Allison and Verma, 2000; Perry *et al.*, 2009; Vander Byl and Kropinski, 2000) to avoid phage adsorption. In some cases, the receptors are even not synthesized by mutations (Liu *et al.*, 2002; Nesper *et al.*, 2000; Scott

et al., 2007; Sorensen *et al.*, 2011; Zaleski *et al.*, 2005). Alternatively, some bacteria shield their host receptors through the production of extracellular matrices that act as physical barriers (Scholl *et al.*, 2005). These strategies protect bacteria from phage infections; however, such defenses are potentially costly to the bacteria because these host receptors normally play critical roles in bacterial metabolism and immune system evasion as chemical receptors, membrane porins, adhesins, etc. (Hyman and Abedon, 2010).

I previously identified one example of receptor-loss strategies in the *Escherichia coli*-phage SPC35 system, where the insertion sequence 2 (IS*2*) disrupt the receptor encoding *btuB* gene (Kim and Ryu, 2011). This virulent T5-like phage can infect both *E. coli* and *S.* Typhimurium, utilising the vitamin B_{12}/cobalamin outer membrane transporter BtuB as its host receptor. Although both bacteria have developed SPC35-resistance, the features of resistance in each host are quite different. *E. coli* mutants have maintained SPC35-resistance across multiple generations through the *btuB* mutation described above. In contrast, the rapidly developed SPC35-resistance in *S.* Typhimurium is quickly lost in the absence of SPC35, suggesting the possibility of phase variable physiological change(s) in this species. Similarly, transient phage-resistance has been observed in a number of other Gram-negative bacteria-phage systems (Carey-Smith *et al.*, 2006; Fischer *et al.*, 2004; Kocharunchitt *et al.*, 2009; Pasharawipas *et al.*, 2008), although the

biological mechanisms underlying this process have not yet been elucidated.

Phase variation is a gene regulation process that induces the heterogenic and dynamic expression of specific genes within a clonal population (Henderson *et al.*, 1999; van der Woude, 2006, 2011). The process is usually heritable but reversible; thus, the 'ON' and 'OFF' phenotypes are interchangeable through the generations. Generally, the switch from ON to OFF or vice versa randomly occurs with a relatively high frequency ranging from 10^{-4} to 10^{-1} per generation (van der Woude, 2006). It has been proposed that phase variation facilitates bacterial adaption to more than one specific environment, thereby ensuring their survival within a rapidly changing environment (Henderson *et al.*, 1999). The facilitating of immune evasion of pathogens in a host has also been suggested as the particular roles of phase variation. This phenomenon was first described as the distinguishing behavior of *Salmonella* in agglutination (Andrews, 1922). Since then, phase variation has been observed in many types of bacteria such as *E. coli*, *Salmonella enterica* ssp., *Haemophilus influenzae, Neisseria* spp. and *Bordetella* spp. with various underlying molecular mechanisms (site-specific recombination, slipped-strand mispairing and differential DNA methylation) [reviewed in Henderson *et al.* (1999) and van der Woude (2011)]. The site-specific inversion of a promoter-containing DNA segment in *S.* Typhimurium is a well-known example of phase variation, where the inversion results in the alternate gene expression of either

FliC- and FljB-flagellin (Zieg *et al.*, 1977). Fimbrial phase variations have also been observed in *S.* Typhimurium, including the epigenetically controlled expression of plasmid-encoded fimbriae (Pef) through Lrp- and Dam-dependent phase variation (Nicholson and Low, 2000), and the variable expression of long polar fimbriae (Lpf), which occurs through an undetermined mechanism (Norris *et al.*, 1998). Recently, a new epigenetic system has been identified in *S. enterica* spp., where OxyR- and Dam-dependent phase variation controls the expression of LPS-modifying *gtr* operon (Broadbent *et al.*, 2010).

Here, I have identified an indispensable role for *S.* Typhimurium O-antigens in SPC35 adsorption. The results of this study suggest that although the BtuB is a host receptor for SPC35 infection, the temporal development of SPC35-resistance in *S.* Typhimurium occurs through the phase variable glucosylation of the O-antigen.

III-2. Materials and Methods

Bacterial strains, bacteriophages, plasmids and growth conditions. The bacterial and phage strains and plasmids used in this study are listed in Table III-1. Prophage-cured *Salmonella enterica* serovar Typhimurium strain LT2 [referred to LT2(c)] was provided by the Cancer Research Center (Colombia, MO) (Erickson *et al.*, 2009). All *S.* Typhimurium strains with a specific gene(s) deletion were derived from LT2(c) according to previously described methods (Datsenko and Wanner, 2000). Bacterial cells were grown aerobically in LB broth or on LB agar plates [1.5% (wt/vol) agar] at 37°C, unless mentioned otherwise. Antibiotics or chemicals were supplemented to the media at the following concentrations: ampicillin, 50 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 100 μg ml^{-1}. The bacteriophages were propagated with a proper host bacteria, and concentrated by precipitation with polyethylene glycol 6000 according to previously described methods (Kim and Ryu, 2011). Concentrated phage stocks were stored at 4°C until further use.

Table III-1. Bacterial strains, bacteriophages and plasmids used in Chapter III.

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
Salmonella enterica serovar Typhimurium		
LT2(c)	Prophage cured strain LT2; wild-type; host for phage SPC35, P22, SFP10, SPN10H, SPN14, SPN9C, SPN17T and SPC41	(Erickson *et al.*, 2009)
SPC35^{R}LT2(c)	SPC35-resistant LT2(c); transiently exhibited SPC35-resistance	This study
SPC35^{RS}LT2(c)	revertant of SPC35^{R}LT2(c); sensitive to SPC35	This study
SR5015	Δ*rfaL*	This study
SR5016	Δ*rfbP*	This study
SR5017	SR5016 with pUHE21-2 *lacI*^{q}	This study
SR5018	SR5016 with p*rfbP*; *rfbP* overexpression strain	This study
SR5019	Δ*btuB*	This study

SR5021	Δ*btuB* Δ*rfbP*	This study
SR5011	Δ*rfe*	This study
SR5007	Δ*wcaD*	This study
SR5025	Δ*oafA*	This study
SR5026	SR5025 with pUHE21-2 *lacI*^{q}	This study
SR5027	SR5025 with p*oafA*; *oafA* overexpression strain	This study
SR5003	Δ^{LT2}*gtrABC1*	This study
SR5004	SR5003 with pUHE21-2 *lacI*^{q}	This study
SR5005	SR5003 with p^{LT2}*gtrABC1*; ^{LT2}*gtrABC1* overexpression strain	This study
SR5006	SR5003 with p^{P22}*gtrABC*; ^{P22}*gtrABC* overexpression strain	This study
SR5024	^{LT2}*gtrABC1*::*lacZ*; Km^{R}	This study
SL4123	*metA22 metE551 galE496 rpsL120 xyl-404* Δ[Fels2] H1-b H2-*e,n,x nml hsdL6 hsdSA29 ilv-452*	(Matic *et al.*, 1994)

SR5129	Δ^{LT2}*gtrABC1 galE*::Km^R; Km^R	This study
SR5132	Δ^{LT2}*gtrABC1 galE*::Km^R (pMS1218); Km^R Cm^R	This study
SR5133	Δ^{LT2}*gtrABC1 galE496*	This study
SR5135	SR5133 with p^{LT2}*gtrABC1*	This study
Escherichia coli		
K-12 MG1655	Host for phage SPC35	Laboratory collection
S17-1 lambda *pir*	*recA thi hsdRM*$^+$ RP4::2-Tc::Mu::Km Tn7, lambda *pir*; Tp^R Sm^R	(Simon *et al.*, 1983)

Bacteriophages

SPC35	T5-like coliphage; infect both *S.* Typhimurium and *E. coli*; BtuB-specific	(Kim and Ryu, 2011)
P22	General transduction phage; infect *S.* Typhimurium; serotype converting; LPS-specific	Laboratory collection
SFP10	Infect both *S.* Typhimurium and *E. coli* O157:H7; LPS-specific	(Park *et al.*, 2012)
SPN10H	Infect *S.* Typhimurium; BtuB-specific	Laboratory collection

SPN14	Infect *S.* Typhimurium; BtuB-specific	Laboratory collection
SPN9C	Infect *S.* Typhimurium; BtuB-specific	Laboratory collection
SPN17T	Infect *S.* Typhimurium; BtuB-specific	Laboratory collection
SPC41	Infect *S.* Typhimurium; flagella-specific	Laboratory collection

Plasmids

pKD46	P_{BAD}-*gam-beta-exo oriR101 repA101*^{ts}; Ap^R	(Datsenko and Wanner, 2000)
pKD13	FRT Km^R FRT PS1 PS4 *oriR6Kγ*; Ap^R	(Datsenko and Wanner, 2000)
pCP20	*cI*857 λP_R*flp oripSC101*^{ts}; Ap^R Cm^R	(Datsenko and Wanner, 2000)
pCE70	FRT *tnpR lacZY*$^+$ *oriR6Kγ*; Km^R	(Merighi *et al.*, 2005)
pUHE21-2 *lacI*^{q}	rep_{pMB1} *lacI*^{q}; Ap^R	(Soncini *et al.*, 1995)
p*rfbP*	pUHE21-2 *lacI*^{q}-*rfbP*; Ap^R	This study
p*oafA*	pUHE21-2 *lacI*^{q}-*oafA*; Ap^R	This study

p^{LT2}*gtrABC1*	pUHE21-2 *lacI*^{q}-^{LT2}*gtrABC1*; Ap^{R}	This study
p^{P22}*gtrABC*	pUHE21-2 *lacI*^{q}-^{P22}*gtrABC*; Ap^{R}	This study
pDS132	*R6K ori mobRP4 cat sacB*; Cm^{R}	(Philippe *et al*., 2004)
pDS*galE*	pDS132-*galE*	This study

[a] Km^{R}, kanamycin resistant; Ap^{R}, ampicillin resistant; Cm^{R}, chloramphenicol resistant; Tp^{R}, trimethoprim resistant; Sm^{R}, streptomycin resistant.

Construction of *Salmonella* strains. The methods of lambda Red recombineering (Datsenko and Wanner, 2000) were used to introduce specific gene deletions into the *Salmonella* mutant strains. For the construction of SR5015, a derivative of LT2(c) with a deletion of *rfaL*, the Km^R cassette from plasmid pKD13 was amplified via PCR with oligonucleotides rfaL-Red-F and rfaL-Red-R. The LT2(c) strain harboring the plasmid pKD46 was transformed with the resulting PCR product then plated on a kanamycin plate for the selection of *rfaL*::Km^R transformants. The Km^R cassette was removed from the transformants by introducing plasmid pCP20 (Datsenko and Wanner, 2000). Similar methods were adapted to construct other chromosomal gene deletion *Salmonella* mutants using appropriate oligonucleotides for each deletion. SR5021, a *btuB rfbP* double deletion mutant, was constructed as follows. The Km^R cassette from plasmid pKD13 was amplified using oligonucleotides rfbP-Red-F and rfbP-Red-R, and transformed into the SR5019, a Δ*btuB* mutant, harboring plasmid pKD46. The plasmid pCP20 was introduced into the selected transformants in order to remove the Km^R cassette and generate SR5021.

SR5024 carries a *lacZ* fusion to the ^{LT2}*gtrABC1* cluster and was constructed as previously described (Ellermeier *et al.*, 2002). The Km^R cassette from plasmid pKD13 was amplified using oligonucleotides LT2 gtrC-lac Red-F and LT2 gtrC-lac

Red-R. The LT2(c) strain harboring plasmid pKD46 was transformed with the resulting PCR product, and the Km^R cassette was excised with the introduction of plasmid pCP20. Using plasmid pCE70 (Merighi *et al.*, 2005), the *lacZY* genes were integrated at the FRT site to generate a transcriptional fusion of *lacZ* and ^{LT2}*gtrABC1*.

To generate the Δ^{LT2}*gtrABC1galE496* mutant (SR5133), the *galE496* mutation was moved from *S.* Typhimurium strain LT2 SL4213 (Matic *et al.*, 1994) to Δ^{LT2}*gtrABC1* mutant (SR5003) by a double homologous recombination-based method as previously described (Kim *et al.*, 2011) with some modifications. The *galE* gene contains *galE496* point mutation from strain SL4213 was PCR amplified using oligonucleotides LT2-galE-CF2 and LT2-galE-CR2, and ligated with pGEM®-T easy vector (Promega, Madison, WI). The sequence confirmed insert was sub-cloned into the SphI-SacI digested suicide conjugal vector pDS132 (Philippe *et al.*, 2004) to construct a plasmid pDS*galE*. To generate the Δ^{LT2}*gtrABC1galE496* mutant (SR5133) by double homologous recombination, *E. coli* S17-1 λ *pir* (Simon *et al.*, 1983) harbouring pDS*galE* was used as a conjugal donor to recipient *S.* Typhimurium Δ^{LT2}*gtrABC1 galE::*Km^R mutant (SR5129) which constructed by the lambda Red recombineering method with oligonucleotides LT2-galE-Red-F1 and LT2-galE-Red-R1 as described in the manuscript. The stationary phase donor and recipient strains grown in LB broth

were washed three times with one-milliliter of 10 mM $MgSO_4$, mixed thoroughly with 100-μl of 10 mM $MgSO_4$, and spotted on LB agar plate. After incubation at 37°C for 16 hr, the grown cells were recovered with a sterile loop and then resuspended in 1 ml of LB broth. Portions of the cell suspension (100 μl) were plated on LB agar plate supplemented with chloramphenicol and kanamycin, and incubated overnight at 37°C. The insertion of p pDS*galE* into the *galE* region of transconjugants' chromosome by a first homologous recombination was PCR confirmed, and the positive clone (SR5132) was subjected to a second homologous recombination which forced by a sucrose challenging. Overnight grown SR5132 strain was inoculated in 1:1000 ratio into 5 ml LB broth supplemented with 20% sucrose, and incubated at 37°C for 10 hr. The culture was serially diluted (10-fold) in LB broth, and 100-μl of each dilutes were plated on LB agar plates supplemented with 5% sucrose and without NaCl. After overnight incubation at 37°C, the counter-selected clones were replicated on LB Cm, LB Km, and LB agar plates. The Cm^S Km^S clone was selected, and the *galE496* mutation was confirmed by PCR and consequent nucleotides sequencing. The oligonucleotides used for construction of these *Salmonella* strains are listed in Table III-2.

Table III-2. The oligonucleotides used in Chapter III.

Oligonucleotides	Sequences (5'→ 3')[a]	Purpose
rfaL-Red-F	CTG GTT TTT CTT TTT GTT GCC ACG TAT TTT CTG GAT GGT ATG TAG GCT GGA GCT GCT TCG	SR5015 construction
rfaL-Red-R	TGG ATA ATC GAC AAC GCG TTT ATT ATA AAC ACC ATC ATA CAT TCC GGG GAT CCG TCG ACC	SR5015 construction
rfbP-Red-F	ATG GAT AAT ATT GAT AAT AAG TAT AAT CCA CAG CTA TGT ATG TAG GCT GGA GCT GCT TCG	SR5016 construction
rfbP-Red-R	TTA ATA CGC ACC ATC TCG CCG CAA AAC AAC TTT CGC TGT TAT TCC GGG GAT CCG TCG ACC	SR5016 construction
LT-rfbP-CF1	TAA TGC ACC TGA ATT CCG CTG CTA TG	p*rfbP* construction
LT-rfbP-CR1	ATT AAG GTA GCG ATG GAT CCG TTC AG	p*rfbP* construction
btuB-Red-F	ATG ATT AAA AAA GCT ACG CTG CTG ACG GCG TTC TCC GTC ATG TAG GCT GGA GCT GCT TCG	SR5019 construction
btuB-Red-R	TAA TGG CGT ATC GGT AAT CGC ATT ACG CGC ATC AAC GTA AAT TCC GGG GAT CCG TCG ACC	SR5019 construction
rfe-Red-F	GTG AAG TTA CTC ACC GCT TTA TCT GAG CTA ATC AGT ATT TTG TAG GCT GGA GCT GCT TCG	SR5011 construction
rfe-Red-R	ACG GTA AAG CCA ATC AGT GTA CTA CCG GCA TCG CCC ATA AAT TCC GGG GAT CCG TCG ACC	SR5011 construction
wcaD-Red-F	CTG CTG CCG CTG ATC TAC CTA CTG GTC AAT GTC AAG ATT GTG TAG GCT GGA GCT GCT TCG	SR5007 construction

wcaD-Red-R	ATG AAA TTT TTA ACG CCT TGC GCA AAA TGA ACG GAC AGA CAT TCC GGG GAT CCG TCG ACC	SR5007 construction
LT-oafA-Red-F	ATG ATC TAC AAG AAA TTC AGA CTC GAT ATA AAT GGG CTA ATG TAG GCT GGA GCT GCT TCG	SR5025 construction
LT-oafA-Red-F	TTA TTT TGA AAT CTG CTT TTT CAC TTC CTC AAT AAA CCA CAT TCC GGG GAT CCG TCG ACC	SR5025 construction
LT-oafA-CF1	ATC ATC CAT TAT CTG AAT TCC GTC	p*oafA* construction
LT-oafA-CR2	AAA ATA TAG GAT CCC CCA TCA GAA	p*oafA* construction
rfbI-Red-F	ATG TTG AAG TTA TTC GCT AAG TAC ACA TCG ATC GGT GTT CTG TAG GCT GGA GCT GCT TCG	SR5003 construction
gtrC-Red-R	TTA TTT AAT TAT TTC CGT AAT ATT CTC ATT TGT CCT CGC CAT TCC GGG GAT CCG TCG ACC	SR5003 construction
LT-gtr-CF1	TCG ATC AAA GGA ATT CGT AGT GCT	p^{LT2}*gtrABC1* construction
LT-gtr-CR1	TTG TGA CAT AGG ATC CGT AGG AAT	p^{LT2}*gtrABC1* construction
P22-gtr-CF1	GAT CGC TTG AAT TCA TCA AAA CAA	p^{P22}*gtrABC* construction
P22-gtr-CR1	TTA GAT TAA CGT CGA CAA CAC TTT	p^{P22}*gtrABC* construction
LT2 gtrC-lac Red-F	GGC GAG GAC AAA TGA GAA TAT TAC GGA AAT AAT TAA ATA ATG TAG GCT GGA GCT GCT TCG	SR5024 construction
LT2 gtrC-lac Red-R	CCG CCG CCC GTT ACC CAT TGG TGG CGG GGA ACA TTA ATT AAT TCC GGG GAT CCG TCG ACC	SR5024 construction

btuB-CF-2	TTG TAG GGC ATG CTC AGT GGA TGT	*btuB* sequencing
btuB-SF1	ACC CAG GCA CAG AAT TAA CC	*btuB* sequencing
btuB-CR-2	ATA CAA GCT TGG TGG GAC GTG GTT	*btuB* sequencing
LT2-galE-Red-F1	TAA AAT CAC CAG TGT GTA AAC GAT TCC ACT AAT TTA TTA CTG TAG GCT GGA GCT GCT TCG	SR5129 construction
LT2-galE-Red-R1	CGG ATG ATC GAT GGG ATT AAA TGG GGT CAT AAC AAC GTC CAT TCC GGG GAT CCG TCG ACC	SR5129 construction
LT2-galE-CF2	CCT ACC TAC GAT GCA ACC AAT C	pDS*galE* construction
LT2-galE-CR2	CAG TTC TCG CTG GAC ATA ATC C	pDS*galE* construction

[a] Restriction enzyme sites are underlined.

Plasmid construction. Plasmid p*rfbP*, which expresses the *rfbP* gene under the control of the *lac* promoter, was constructed as follows. The *rfbP* gene from the LT2(c) strain was amplified using oligonucleotides LT-rfbP-CF1 and LT-rfbP-CR1. The resulting PCR product was purified and digested with the restriction enzymes EcoRI and BamHI prior to cloning between the EcoRI and BamHI sites of the pUHE21-2 *lacI*^{q} plasmid vector (Soncini *et al.*, 1995). All other plasmids were constructed using similar experimental procedures and the appropriate oligonucleotides; SalI was used instead of BamHI in the construction of p^{P22}*gtrABC*. The oligonucleotides used in the plasmid construction are listed in Table III-2.

Phage spotting assay. The mixture of 100 µl of overnight bacterial culture and 5 ml LB soft agar [0.4% (wt/vol) agar] was poured onto a prepared LB plate (supplemented with appropriate antibiotics, if required) to prepare a bacterial lawn. Isopropyl-β-D-thiogalactopyranoside (IPTG) was additionally added to both the bacterial seed culture and the mixtures to final concentrations of 100, 200, 500 and 1000 µM for the overexpression of specific genes. After 30 minutes of agar solidification, 10 µl of serially diluted phage lysates [10-fold diluted in sodium chloride-magnesium sulphate buffer (SM buffer; 100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl adjusted to pH7.5)] were spotted onto the lawn, and the plate was

allowed to dry for 30 minutes. The plate was then incubated at 37°C for 8 hours, after which the produced plaques or lysis zones were inspected. Plate images were captured with the Red™ Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA).

Bacterial challenge assay. LB broth (50 ml) was inoculated with 1% bacterial overnight culture (final concentration), and incubated at 37°C with (at 220 rpm) or without shaking, until the culture reached a density of OD_{600} = ~0.6. The incubation was continued after the addition of 100 µl SPC35 stock diluted to a multiplicity of infection (MOI) of 0.1, except where otherwise indicated. The OD_{600} was measured periodically to monitor bacterial growth. SM buffer was used in the place of diluted SPC35 dilute as a negative control.

Isolation of SPC35-resistant *S*. Typhimurium mutants. To isolate SPC35-resistant mutants, bacteria from a 24 hr-SPC35-challenged culture were collected using sterile loops and sequentially streaked onto fresh LB plates at least 3 times. Alternatively, the high-titer overlay assay was carried out as previously described (Kim and Ryu, 2011). Briefly, 100 µl of serially diluted *Salmonella* culture was inoculated in 5 ml LB soft agar [0.4% (wt/vol) agar] and mixed with 100 µl SPC35 lysate at an MOI of >10, prior to pouring onto an LB plate. After 24 hr incubation

at 37°C, the SPC35-resistant colonies were picked and repeatedly streaked onto LB plates at least 3 times. The SPC35-resistance of the daughter clones was verified at each streaking using phage spotting assay, and this verification was always conducted in parallel with any experiments that used the resistant mutant.

Phage adsorption assay. The phage adsorption assay was performed as previously described (Heller and Braun, 1979) with some modifications. Briefly, *S.* Typhimurium strain cultures were harvested at OD_{600} = 2.3–2.5, washed with PBS twice, and re-suspended in M9 minimal medium to an OD_{600} of 2.5 (8–10 x 10^8 CFU ml^{-1}). Pre-warmed (37°C) phage SPC35 was added at an MOI of 0.1, and the adsorption was performed for 15 min at 37°C. Samples of 50 µl each were collected at designated time points and diluted in 5 ml ice-cold PBS to halt phage adsorption. The bacteria were removed by centrifugation (10,000 x g, 4°C for 10 min) and filtration (0.22-µm pore size, Millipore), after which unadsorbed phages in the filtrates were counted with the double-agar overlay assay (Kim and Ryu, 2011) using *S.* Typhimurium LT2(c) as an indicator strain. An adsorption constant (k) was calculated according to the following formula: $k = - \ln (P_t/P_0)/Nt$, 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). The control group (SPC35 only in M9 medium) was assessed in parallel, and its phage titer was considered as P_0.

Phage adsorption to LPS. The adsorption of phage particles to LPS was examined by modified methods of previous report (Heller and Braun, 1979). Briefly, dilutions of phage SPC35 (~ 8 x 10^6 PFU ml^{-1}) were mixed with various concentrations of *S*. Typhimurium LPS (Sigma, cat No. L6511) or *P. aeruginosa* LPS (Sigma, cat No. L9143), that dissolved in distilled and deionised water (ddH_2O). After incubation at 37°C for 15 min, half of the mixture was centrifuged (16,000 x g, 4°C for 2 min) and the resulting supernatant was diluted 50-fold in ice-cold PBS. The other half was directly diluted 50-fold without centrifugation for use as the 100% control. The diluted samples were incubated at 32°C for 10 minutes to dissociate the phages from the LPS, and the free phage particles were counted by the double-agar overlay assay. A parallel assay was performed with ddH_2O instead of LPS and used as a negative control.

LPS extraction and analysis. LPS was extracted from overnight bacterial cultures using hot phenol-water micro-extraction methods (Wang *et al.*, 2010b). One-milliliter of bacterial cultures (ca. 2 x 10^9 CFU ml^{-1}) was harvested, washed once with 1 ml DPBS (Dulbecco's phosphate buffered saline; PBS containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$), and re-suspended in 300 µl ddH_2O. An equal volume of pre-heated (68°C) phenol solution was added prior to incubation at 68°C

with vigorous vortex mixing every 5 minutes. The samples were chilled on ice for 5 minutes, and the aqueous phases were separated by centrifugation at 10,000 x g, 4°C for 5 min. The LPS was extracted again from the phenol phase with another 300 µl of ddH_2O. After the addition of sodium acetate to the pooled aqueous phases at a final concentration of 0.5 M, 10 volumes of 95% ethanol was added, and the mixture was incubated overnight at -20°C. The crude LPS were sedimented by centrifugation at 16,000 x g, 4°C for 5 minutes, re-suspended in 100 µl ddH_2O, and precipitated again with 95% ethanol. Finally, the precipitated LPS was re-dissolved in 50 µl ddH_2O and stored at -20°C.

The extracted LPS were analysed by deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) on a 15% acrylamide gels (Reuhs *et al.*, 1998). Briefly, the resolving gel (15%) was prepared with 5 ml of monomer stock solution [30% (wt/vol) acrylamide, 0.8% (wt/vol) *N, N'*-methylenebisacrylamide], 2 ml of resolving gel buffer solution (1.875 M Tris base, adjust to pH 8.8), 3 ml of ddH_2O, 17.5 µl of 10% ammonium persulfate and 8.75 µl of *N, N, N', N'*-tertramethylethylenediamine (TEMED). The stacking gel (4%) consisting 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 ddH_2O, 12.5 µl of 10% ammonium persulfate and 6.25 µl of TEMED was laid onto the solidified resolving gel. 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. Four-microliters of extracted LPS was mixed with equal volumes of sample buffer (containing 2 ml of stacking gel buffer solution, 1 ml of glycerol and 2.5 mg of bromophenol blue, brought up to 10 ml with ddH_2O), and the mixtures were loaded onto the gels prior to running each gel for ~60 min at 15 mA. The gels were fluorescently stained using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes, cat No. P20495; Eugene, OR, USA) according to the manufacturer's instructions. The samples were visualised under the 300 nm UV by the Red™ Imaging System.

β-Galactosidase assay. β-Galactosidase assays were performed in triplicate according to the methods of Miller (Miller, 1972). Briefly, *Salmonella* cultures in the exponential growth phase (OD_{600} = 2.5) were harvested and re-suspended in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 2 mM $MgSO_4$ and 40 mM β-mercaptoethanol, adjust to pH 7.0). The cell suspensions were diluted 1:5 in ice-cold Z-buffer, and the OD_{600} was measured. Aliquots (0.2 ml) of the cell suspensions were added to Pyrex tubes containing 20 µl of 0.1% SDS, 40 µl of chloroform and 0.8 ml of Z-buffer. After vortex mixing for 10 sec, the samples were incubated for 10 min prior to the start of reaction by adding 0.2 ml of 2-nitorophenyl β-D-galactopyranoside (ONPG; 4 mg ml^{-1}). The reaction was

continued for 10 min and subsequently terminated with the addition of 0.5 ml of 1 M Na_2CO_3. The samples were centrifuged at 16,000 x g for 1 min to sediment all cell debris, and the optical density of supernatants was measured at 420 nm or 550 nm. The β-galactosidase activity was calculated as previously described and expressed in Miller units (Miller, 1972).

III-3. Results

Transient SPC35-resistance associated with *S*. Typhimurium O-antigens. The contrasting features of SPC35-resistance in *S*. Typhimurium and *E. coli* (rapid emergence and non-heritable resistance in *S*. Typhimurium vs. relatively slow emergence and heritable resistance in *E. coli*) detailed in my previous study (Kim and Ryu, 2011) suggested that physiological change(s) might be associated with SPC35-resistance in *S*. Typhimurium. The LPS of *Salmonella* are more complex compared to those in *E. coli* (Lerouge and Vanderleyden, 2002), and LPS is known to play a supporting role in phage T5 infection (Heller and Braun, 1979, 1982). Therefore, I tested whether LPS is responsible for the differences in the SPC35-resistance between *S*. Typhimurium and *E. coli*.

Firstly, two *S*. Typhimurium mutants with mutations in genes involved in LPS biosynthesis were tested: *rfaL,* which encodes a lipid A-surface polysaccharide ligase and *rfbP,* which encodes an undecaprenyl-phosphate galactosephosphotransferase. The former enzyme mediates the ligation of the completely synthesised O-antigen chain to the lipid A-core in the final step of LPS biosynthesis, while the latter transfers the galactose phosphate from UDP-galactose to the undecaprenyl phosphate to initiate O-antigen biosynthesis (Wang *et al.*, 2010a). Although the specific roles of these enzymes in LPS biosynthesis are quite

different, the phenotype resulting from their mutation was the same; O-antigens were absent in both Δ*rfaL* and Δ*rfbP* mutants (Kong *et al.*, 2011). SPC35 made clearer plaques on these mutant strains than those on wild-type (WT) strain *S.* Typhimurium LT2(c) (Fig. III-1), while the EOP (efficiency of plating) of each mutant was not significantly different from that of the WT (data not shown). Complementation with the Δ*rfbP* mutant (Δ*rfbP*+p*rfbP*) restored the plaque turbidity to that of the WT (Fig. III-1), indicating that the O-antigens of LPS might affect the SPC35 infection of *S.* Typhimurium.

Based on these results and the result from my previous study describing the heritable SPC35-resistance in O-antigen-lacking *E. coli* strain, I hypothesized that the elimination of O-antigens from *Salmonella* might confer a similar phenotype to *E. coli* against SPC35 infection. To confirm the possibility that *Salmonella* O-antigens contribute to the development of transient SPC35-resistance, I analysed SPC35-resistant mutants obtained from a high-titer overlay assay with the Δ*rfaL* strain. Unlike in the assay with WT *Salmonella*, I could collect a few colonies from within the confluent lysis zone of the Δ*rfaL* strain (Fig. III-2A), and they stably retained the phage-resistance through subsequent generations. The nucleotide sequencing of the *btuB* receptor gene revealed various random mutations that resulted in the truncation and inactivation of BtuB protein (Fig. III-2B), as observed previously in the SPC35-resistant *E. coli* mutants (Kim and Ryu, 2011).

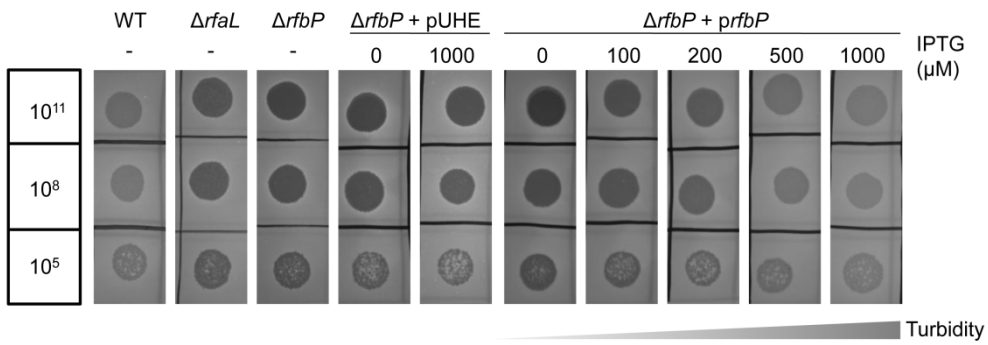


Fig. III-1. SPC35 spotting assay on WT *S*. Typhimurium and various LPS mutants. The absence of *S*. Typhimurium O-antigens reduces SPC35 plaque turbidity with no significant changes in the EOP (efficiency of plating). Ten-fold serial dilutions of SPC35 stock (about ~10^{12} PFU ml^{-1}) were spotted on lawns of the following bacterial strains: wild type (WT; LT2(c)); Δ*rfaL* (SR5015); Δ*rfbP* (SR5016); Δ*rfbP* harbouring the empty vector (Δ*rfbP*+pUHE; SR5017); and Δ*rfbP* harbouring the *rfbP* gene under IPTG control (Δ*rfbP*+p*rfbP*; SR5018). IPTG was added to the lawns at the indicated concentrations to induce *rfbP* gene expression. Note that the values in the boxes on the left correspond to the titer of phages spotted (PFU ml^{-1}).

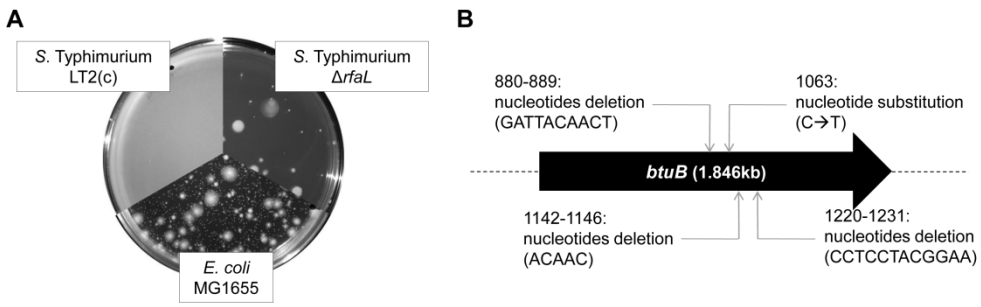


Fig. III-2. The LPS-lacking Δ*rfaL* strain generates SPC35-resistant mutants that contain several heritable mutations within the *btuB* gene.

A. High-titer overlay assay of *S.* Typhimurium LT2(c), Δ*rfaL* and *E. coli* MG1655. A number of SPC35-resistant colonies of various sizes were generated with Δ*rfaL* and *E. coli* MG1655, but a bacterial lawn was formed with LT2(c).

B. Schematic representation of the *btuB* mutations in SPC35-resistant Δ*rfaL* strain. The numbers correspond to mutation sites, and the mutated nucleotides are shown in parentheses.

In contrast, resistant mutants derived from the WT [termed SPC35^{R}LT2(c)] easily regained their SPC35-susceptibility; even following two sequential isolations through streaking on fresh LB plates were sufficient for the susceptibility regaining. No mutations were identified in the *btuB* gene in either SPC35^{R}LT2(c) or the strain with regained susceptibility [termed SPC35^{RS}LT2(c)]. Therefore, O-antigens enable *Salmonella* to avoid the costly *btuB* mutations against SPC35 infection, strongly suggesting a role for O-antigens in the development of transient SPC35-resistance in *S*. Typhimurium.

***Salmonella* O-antigens play an assisting role in phage adsorption.** To compare the SPC35 infection kinetics between WT *Salmonella* and O-antigen-lacking *Salmonella* mutants, which exhibited heritable phage-resistance similar to *E. coli*, I conducted a bacterial challenge assay with these strains. Unexpectedly, although SPC35 made clearer plaques on the Δ*rfaL* and Δ*rfbP* mutant strains than on the WT strain, SPC35 did not inhibit the growth of mutants in shaking culture conditions at an MOI of either 0.1 or 1 (Fig. III-3). Counting of viable cells revealed that SPC35 could not infect and kill these mutants, while SPC35 significantly reduced the number of viable WT cells (data not shown). However, considering that SPC35 can infect the *Salmonella* mutants (Fig. III-3), and consequently propagated (data not shown) under static culture conditions, I

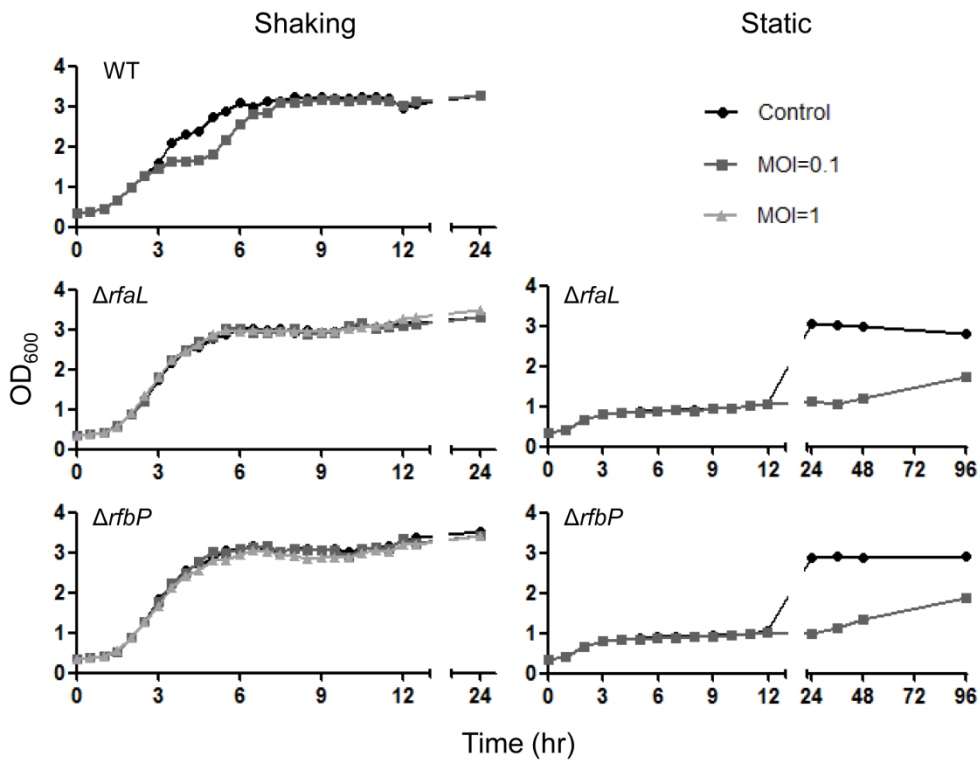


Fig. III-3. *S*. Typhimurium O-antigens play a critical role in efficient SPC35 infection under shaking culture conditions. The growth (OD_{600}) of WT (LT2(c)), Δ*rfaL* (SR5015) or Δ*rfbP* (SR5016) bacterial strains cultured with (shaking) or without (static) regular agitations was measured after the addition of SPC35 (MOI of 0.1 or 1) or SM buffer (control).

hypothesized that, although BtuB is a receptor of SPC35, the O-antigen might play an essential role in SPC35 infection of *S.* Typhimurium under shaking conditions.

To test this hypothesis, I conducted a phage adsorption assay using various mutant strains. The result of this assay revealed that the SPC35 adsorption constant (*k*) of Δ*rfbP* was approximately 3.7-fold higher than that of Δ*btuB* but 1.7-fold lower than that of WT (Fig. III-4A). The adsorption efficiency of SPC35 was further reduced in the Δ*btuB* Δ*rfbP* double mutant; no SPC35 particles were able to adsorb to this mutant. Similarly, no phage particles were able to adsorb to the double mutant Δ*btuB* Δ*rfaL* (data not shown). Moreover, it was noteworthy that strains with O-antigens displayed meaningful levels of phage adsorption within the first minute, with 8-fold higher adsorption constant for WT than for Δ*btuB* (Fig. III-5). These results imply that O-antigens in *S.* Typhimurium might facilitate the binding of SPC35 to BtuB during the initial phage adsorption step. Additionally, I performed phage adsorption assays using the LPS extracted from *S.* Typhimurium or *Pseudomonas aeruginosa* to determine whether *Salmonella* LPS specifically interact with SPC35. The result revealed that only *S.* Typhimurium LPS could absorb SPC35 in a concentration-dependent manner (Fig. III-4B). Approximately 30% of SPC35 particles were adsorbed to 0.5 mg ml^{-1} *S.* Typhimurium LPS within a 15 minute period at 37°C. To assess the possibility that other outer membrane polysaccharide moieties were involved in the SPC35 interaction, I constructed an

enterobacterial common antigen (ECA)-lacking mutant (Δ*rfe*) and a capsular polysaccharide (CPS)-lacking mutant (Δ*wcaD*). However, these mutations did not affect the SPC35 infection of *S*. Typhimurium (Fig. III-6).

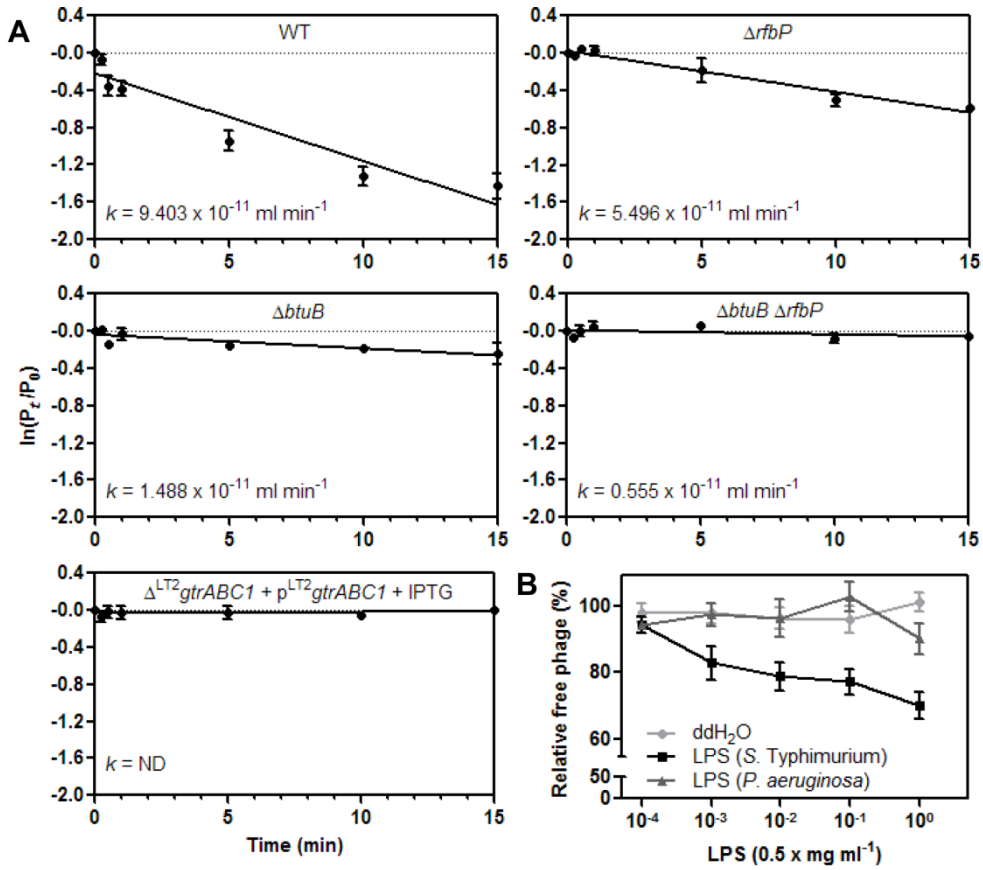


Fig. III-4. *S*. Typhimurium O-antigens assist SPC35 adsorption.

A. SPC35 adsorption kinetics of the *S*. Typhimurium strains. SPC35 adsorption assays were performed with the following bacterial strains: WT (LT2(c)); Δ*btuB* (SR5019); Δ*rfbP* (SR5016); Δ*btuB* Δ*rfbP* (SR5021); and Δ^{LT2}*gtrABC1* harboring the ^{LT2}*gtrABC1* cluster under IPTG control (Δ^{LT2}*gtrABC1*+p^{LT2}*gtrABC1*; SR5005; "+ IPTG" represents the induction with 1000 μM IPTG). Linear regression lines were drawn from the data points and correspond to the natural logarithm of the ratio of end to initial phage titer (ln [P_t/P_0]). The adsorption constant (*k*) is shown at the bottom left of each graph. ND, not determined.

B. SPC35 specifically interacts with the *S*. Typhimurium LPS. The SPC35 adsorption ability of *S*. Typhimurium LPS or *P. aeruginosa* LPS at 37°C for 15 min was assessed by counting unbound phage numbers from the mixture of SPC35-LPS. The means with SEM for three independent experiments are shown.

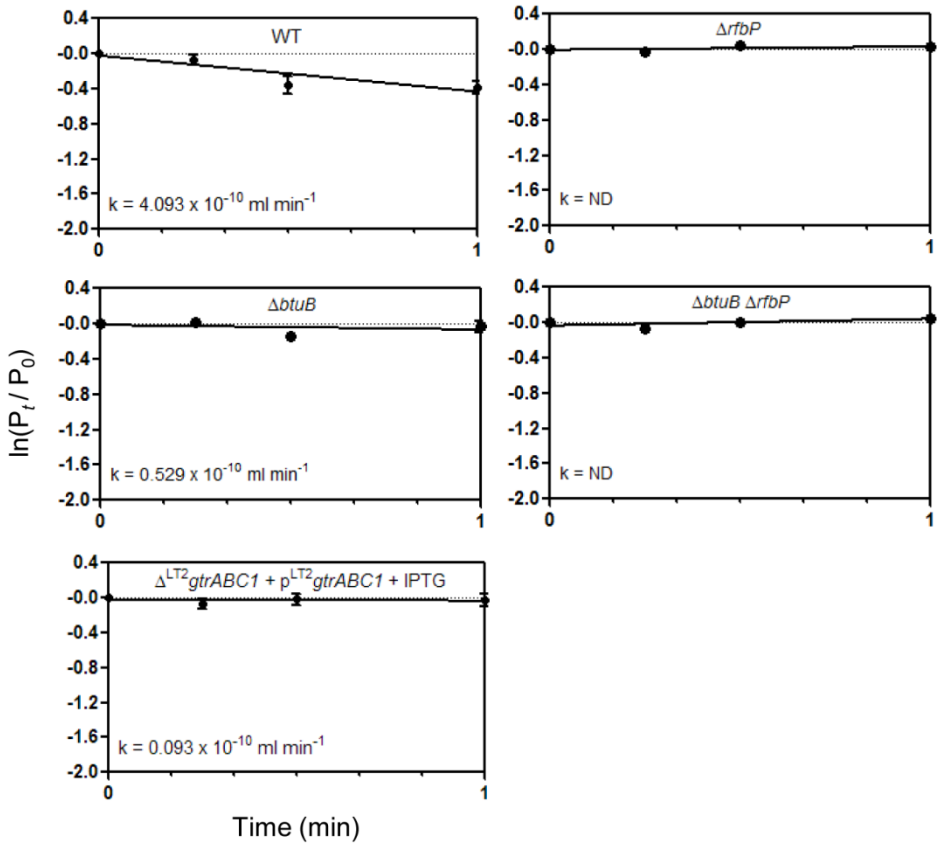


Fig. III-5. SPC35 adsorption kinetics of *S*. Typhimurium strains during the first minute of adsorption. Initial SPC35 adsorption was only exhibited by O-antigen-possessing strains: WT (LT2(c)), Δ*btuB* and the ^{LT2}*gtrABC1* overexpression strain with 1000 µM IPTG induction (Δ^{LT2}*gtrABC1* + p^{LT2}*gtrABC1*; SR5005). The adsorption constants (*k*) of other strains could not be determined (ND).

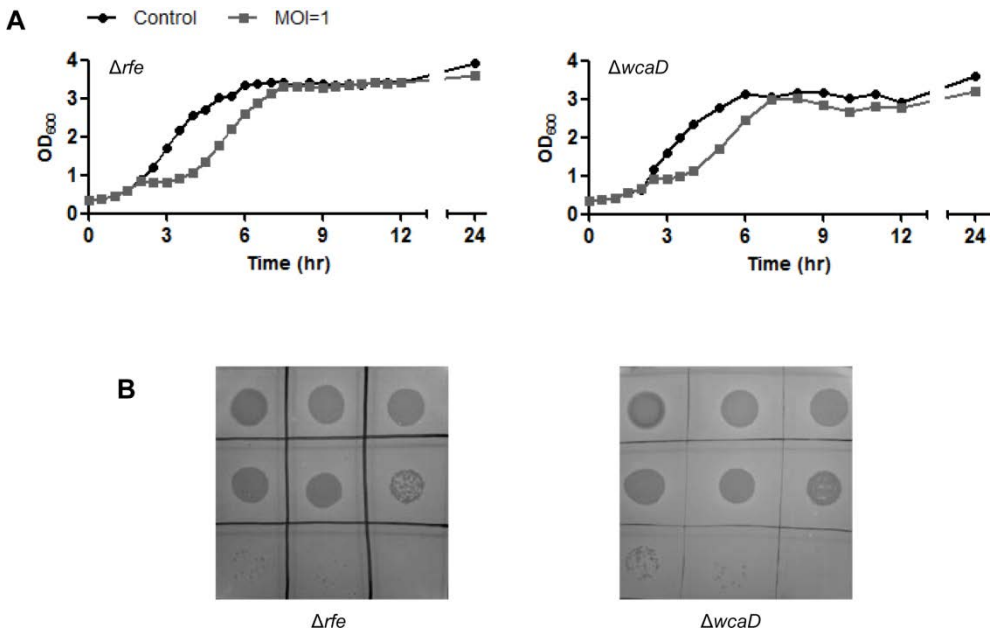


Fig. III-6. Enterobacterial common antigens (ECA) or capsular polysaccharides (CPS) were not involved in SPC35 infection.

A. In the bacterial challenge assay with Δ*rfe* (SR5011; ECA-lacking) or Δ*wcaD* (SR5007; CPS-lacking), no differences in growth inhibition compared with the wild type were observed.

B. SPC35 spotting assay of Δ*rfe* or Δ*wcaD*. Plaque turbidity was similar to wild-type levels.

α-1,4-glucosylation of O-antigens confers SPC35-resistance to *S.* Typhimurium by preventing phage adsorption. The proposed role for the *S.* Typhimurium O-antigen in SPC35 infection indicates that changes in the O-antigen might affect SPC35 infectivity; thus, two different modifications of the *S.* Typhimurium O-antigen, through the overexpression of either the *oafA* gene or the ^{LT2}*gtrABC1*gene cluster, were tested for SPC35 infection. In *S.* Typhimurium, *oafA* is responsible for O5-antigen formation through the acetylation of the 2-hydroxyl group of the abequose residues of O4-antigens. Similarly, ^{LT2}*gtrABC1* is involved in the biosynthesis of O12-2-antigens through the addition of a glucose residue to the C-4 position of each galactose in the O12-antigen repeats (Fig. III-7) (Bogomolnaya *et al.*, 2008; Nikaido *et al.*, 1971; Nikaido and Nikaido, 1971; Slauch *et al.*, 1996). No changes in plaque morphology or EOP were observed between the strains with *oafA* deletion or *oafA* overexpression and the WT strain (Fig. III-8A). However, SPC35 produced slightly clearer plaques on the Δ^{LT2}*gtrABC1* mutant than it did on WT (Fig. III-8B). The effect of O-antigen α-1,4-glucosylation on SPC35 infection was further analysed by differentially expressing ^{LT2}*gtrABC1* under the control of the *lac* promoter. In the SPC35 spotting assay for this strain, SPC35 could not form any distinguishable single plaques even when lower titer-phages were spotted; hence, I could not determine the EOP. The bacterial lysis zone was gradually disappeared with decreasing phage titers

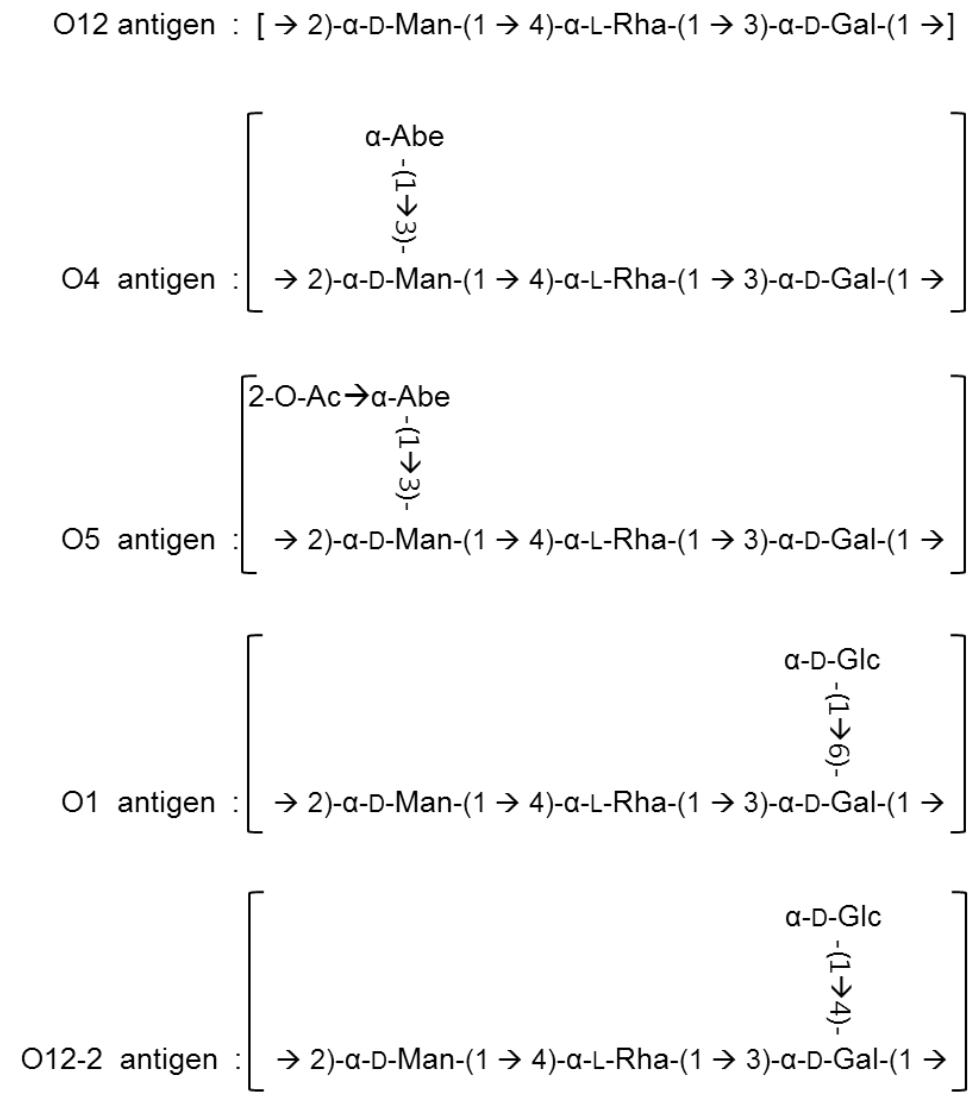


Fig. III-7. The various structures of the O-antigen repeat unit in *S.* Typhimurium. The basic O-antigen repeat unit of *S.* Typhimurium consists of a trisaccharide (mannose-rhamnose-galactose) backbone, known as the O12-antigen. An abequose addition to the mannose residue in the backbone unit results in the formation of O4-antigen, and the acetylation of the 2-hydroxyl group of this abequose residue generates the O5-antigen. Glucose side-branching via an α-1→6 linkage to the galactose residue in backbone unit is responsible for the O1-antigen, while the same modification but via an α-1→4 linkage creates the O12-2-antigen. D-Man, D-mannose; L-Rha, L-rhamnose; D-Gal, D-galactose; Abe, abequose; D-Glc, D-glucose; O-Ac, O-acetyl group.

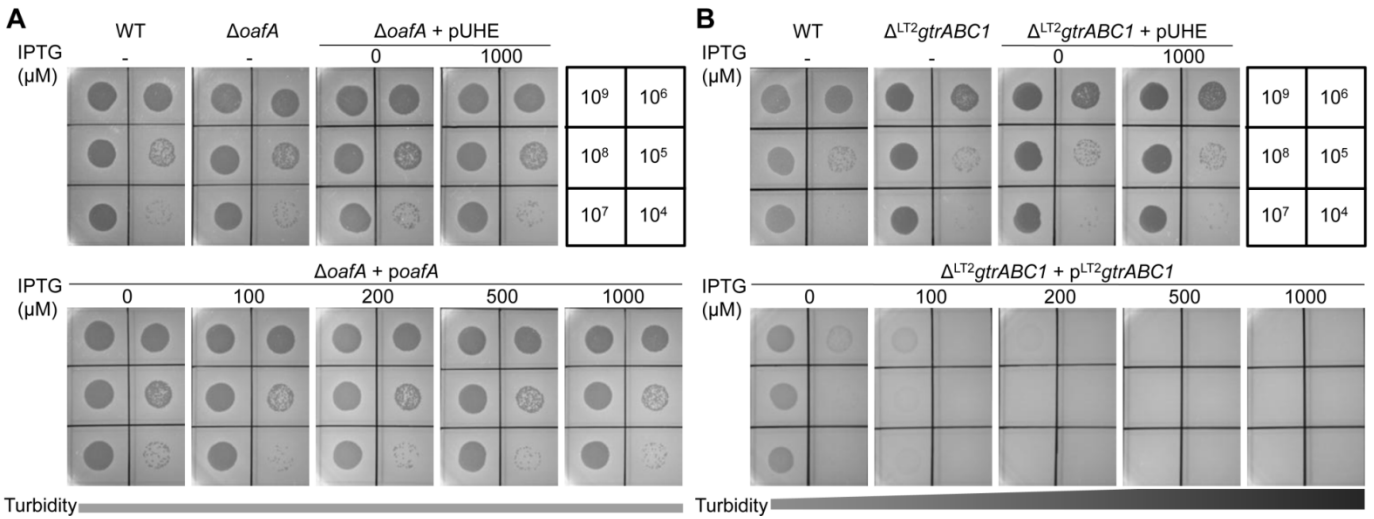


Fig. III-8. α-1,4-glucosylation, not acetylation, of O-antigen conferred SPC35-resistance to *S*. Typhimurium. SPC35 were serially diluted (10-fold dilutions from ~10^{10} PFU ml^{-1} in SM buffer) and spotted onto lawns of (A) WT (LT2(c)); Δ*oafA* (SR5025); Δ*oafA* harbouring the empty vector (Δ*oafA*+pUHE; SR5026); and Δ*oafA* harbouring the *oafA* gene under IPTG control (Δ*oafA*+p*oafA*; SR5027); or (B) WT (LT2(c)); Δ^{LT2}*gtrABC1* (SR5003); Δ^{LT2}*gtrABC1* harbouring the empty vector (Δ^{LT2}*gtrABC1*+pUHE; SR5004); and Δ^{LT2}*gtrABC1* harbuoring the ^{LT2}*gtrABC1* cluster under IPTG control (Δ^{LT2}*gtrABC1*+p^{LT2}*gtrABC1*; SR5005). Expression of the *oafA* gene and the ^{LT2}*gtrABC1* cluster from SR5027 and SR5005, respectively, were induced by IPTG addition at the designated concentrations. Note that the values in right upper boxes correspond to the titer of phages spotted (PFU ml^{-1}).

A. The deletion or overexpression of the *oafA* gene, which is responsible for O-antigen acetylation, did not affect SPC35 infectivity.

B. O-antigen α-1,4-glucosylation by the ^{LT2}*gtrABC1* cluster confers SPC35-resistance to *S*. Typhimurium.

(Fig. III-8B), indicating the poor plating efficiency of the strain resulting from the ^{LT2}*gtrABC1* cluster expression. The turbidity of the lysis zone at high titers was dramatically increased as more ^{LT2}*gtrABC1* was expressed, and eventually, SPC35 did not form any lysis zones when the overexpression of ^{LT2}*gtrABC1* was induced with more than 500 μM of IPTG. The adsorption assay also indicated that SPC35 could not bind to *S*. Typhimurium when the ^{LT2}*gtrABC1* is overexpressed (Fig. III-4A). These results strongly suggest that the α-1,4-glucosylation of *S*. Typhimurium O-antigens suppresses its adsorption-assisting function and consequently prevents SPC35 infection.

Some serotype-converting bacteriophages such as phage P22 also contain *gtrA*, *gtrB* and *gtr(type)* gene clusters that resemble the ^{LT2}*gtrABC1* (Bogomolnaya *et al.*, 2008; Vander Byl and Kropinski, 2000). However, the position for glucose addition is different for each glucosylation: the P22 encoded-*gtrA*, *gtrB* and *gtr(type)* genes (hereafter refer to ^{P22}*gtrABC*) glucosylate the O12-antigen at the C-6 position of galactose to generate the O1-antigen (Vander Byl and Kropinski, 2000), while the ^{LT2}*gtrABC1* genes glucosylate the O12-antigen at the C-4 position of galactose to generate the O12-2 variant (Fig. III-7) (Bogomolnaya *et al.*, 2008; Nikaido *et al.*, 1971; Nikaido and Nikaido, 1971). Although P22 did not infect ^{P22}*gtrABC* gene cluster-overexpressing *S*. Typhimurium, the overexpression of this gene cluster did not affect SPC35 infection (Fig. III-9). Therefore, neither the *oafA* gene-mediated

O-antigen acetylation nor the ^{P22}*gtrABC* cluster-mediated α-1,6-glucosylation affected SPC35-resistance, whereas the α-1,4-glucosylation of the O-antigen through the ^{LT2}*gtrABC1* cluster conferred SPC35-resistance to *S.* Typhimurium. These results demonstrate that the position of glucose added is a key determinant of O-antigen glucosylation-mediated SPC35-resistance and that particularly addition at the C-4 position of galactose is responsible for the destruction of an SPC35-interacting moiety. Interestingly, other phages employing BtuB or LPS as a receptor could not infect the α-1,4-glucosylated strain but could infect the α-1,6-glucosylated strain (Fig. III -9).

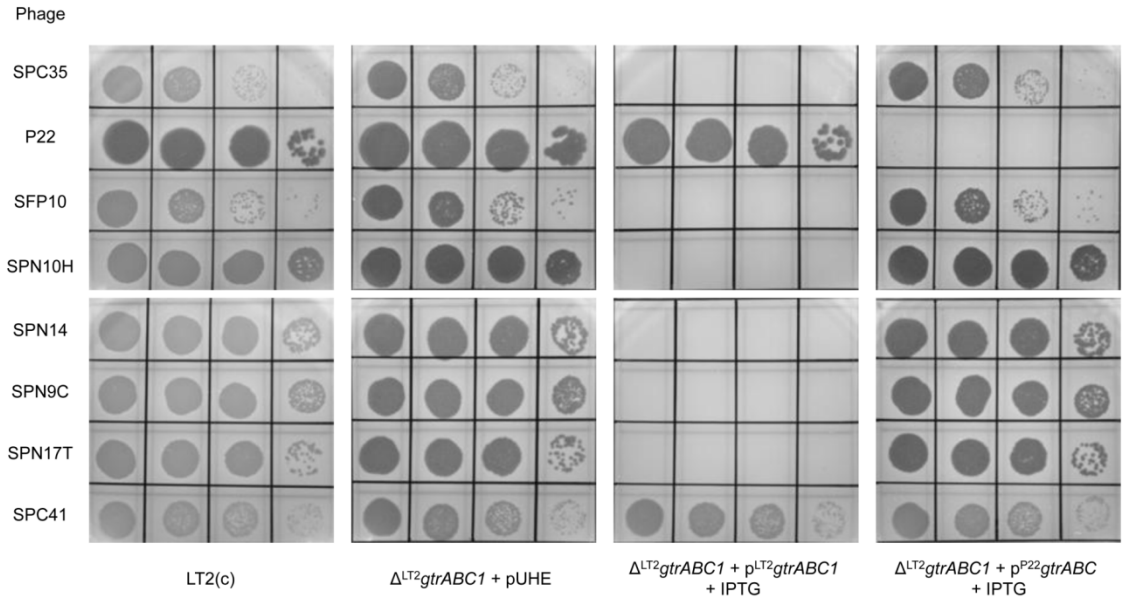


Fig. III-9. O-antigen α-1,6-glucosylation by the ^{P22}*gtrABC* cluster confers P22-resistance, but not SPC35-resistance, to *S*. Typhimurium. Ten-fold serial dilutions of phage stocks (SPC35, P22, SFP10, SPN10H, SPN14, SPN9C, SPN17T and SPC41) were spotted onto lawns of the following bacterial strains: WT (LT2(c)); Δ^{LT2}*gtrABC1* harboring the empty vector (Δ^{LT2}*gtrABC1*+pUHE; SR5004); Δ^{LT2}*gtrABC1* harboring the ^{LT2}*gtrABC1* cluster under IPTG control (Δ^{LT2}*gtrABC1*+p^{LT2}*gtrABC1*; SR5005); and Δ^{LT2}*gtrABC1* harboring the ^{P22}*gtrABC* cluster under IPTG control (Δ^{LT2}*gtrABC1*+p^{P22}*gtrABC*; SR5006). "+ IPTG" represents the induction with 1000 μM IPTG. Note that the titer of each phage is different.

Phase variable α-1,4-glucosylation of O-antigens is responsible for transient SPC35-resistance in *S.* Typhimurium. The slower migration of the O-antigen repeats after the addition of glucose residues at the C-4 position of each galactose can be observed using deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) (Bogomolnaya *et al.*, 2008). The analysis of the glucosylation pattern of the O-antigen repeats by DOC-PAGE revealed that both the SPC35-resistant mutant (SPC35^{R}LT2(c)) and the ^{LT2}*gtrABC1* overexpression strain had slower migrating O-antigen repeats than either the WT or Δ^{LT2}*gtrABC1*; however, the revertant (SPC35^{RS}LT2(c)) contained normal O-antigen repeats (Fig. III-10A). The similarly-migrating O-antigen repeats of the SPC35^{R}LT2(c) and ^{LT2}*gtrABC1* overexpression strain indicate that similar pattern of glucosylation had occurred in both strains.

A Dam methylation-mediated phase variation controls the expression of the ^{LT2}*gtrABC1* cluster (Broadbent *et al.*, 2010); therefore I postulated that transient phage-resistance might be associated with the phase variable regulation of the ^{LT2}*gtrABC1* promoter. To examine my hypothesis, I constructed the ^{LT2}*gtrABC1-lacZ* fusion strain and monitored the expression of ^{LT2}*gtrABC1* with or without the phage challenge. The *lacZ* gene was inserted after the stop codon of the *gtrC* gene, thereby the β-galactosidase encoded by *lacZ* gene was polycistronically expressed with the GtrABC1. Without the SPC35 challenge, both ON (blue) and OFF (white) phase colonies appeared on the LBX (LB supplemented with 100 μg ml^{-1} X-gal)

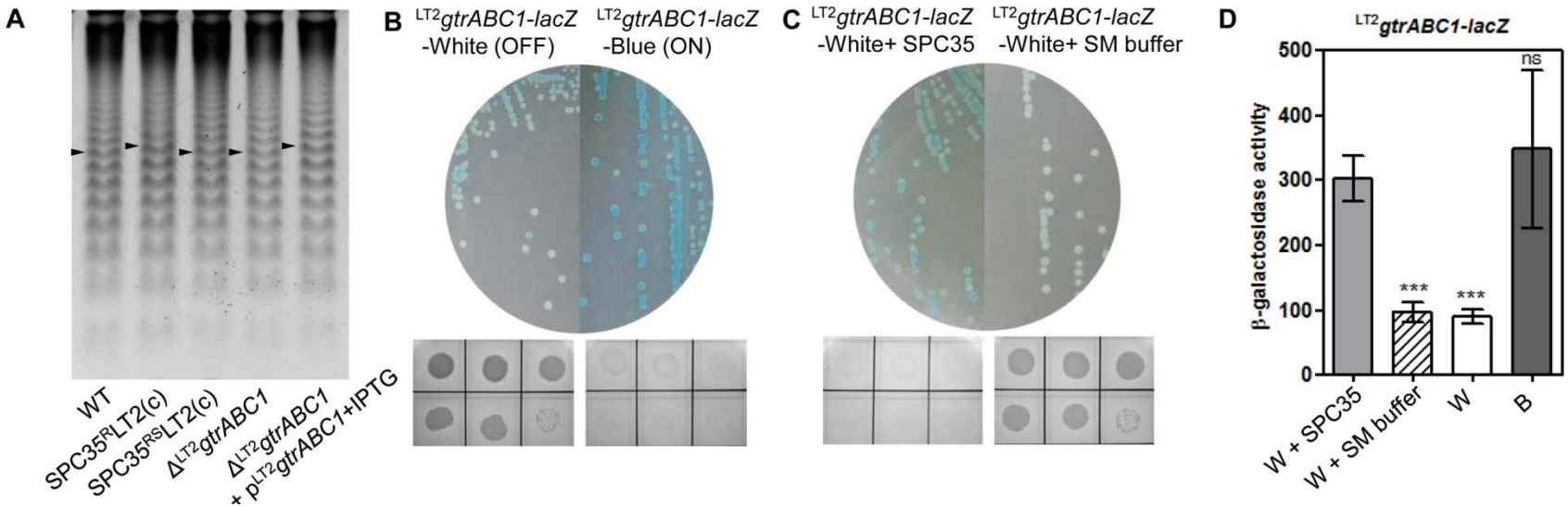

A
WT
SPC35RLT2(c)
SPC35RSLT2(c)
ΔLT2gtrABC1
ΔLT2gtrABC1 + pLT2gtrABC1+IPTG
B
LT2gtrABC1-lacZ -White (OFF)
LT2gtrABC1-lacZ -Blue (ON)
C
LT2gtrABC1-lacZ -White+ SPC35
LT2gtrABC1-lacZ -White+ SM buffer
D
LT2gtrABC1-lacZ
β-galactosidase activity
500
400
300
200
100
0
***
***
ns
W + SPC35
W + SM buffer
W
B

Fig. III-10. The phase variably expressed ^{LT2}*gtrABC1* cluster is responsible for transient SPC35-resistance in *S*. Typhimurium.

A. Both the SPC35-resistant mutant and the ^{LT2}*gtrABC1* overexpression strain show a slower migration of O-antigen repeats. LPS of LT2(c), SPC35-resistant mutant (SPC35^{R}LT2(c)), the revertant (SPC35^{RS}LT2(c)), Δ^{LT2}*gtrABC1* and the ^{LT2}*gtrABC1* overexpression strain (Δ^{LT2}*gtrABC1*+p^{LT2}*gtrABC1*; "+ IPTG" represents the induction with 1000 μM IPTG) were extracted and analysed by DOC-PAGE. The arrowheads indicate the position differences of each O-antigen band.

B. SPC35-rsistance is dependent on the phase variable expression of the ^{LT2}*gtrABC1* cluster. The presence of white (OFF phase) and blue (ON phase) colonies of the ^{LT2}*gtrABC1-lacZ* fusion strain (SR5024) on LBX plates corresponds with phase variation. The SPC35-susceptibility of each clone was assessed using spotting assays.

C. SPC35-challenging selects the resistant blue clones. The SPC35-challenged white ^{LT2}*gtrABC1-lacZ* strain was streaked onto LBX and subjected to the SPC35 spotting assay. The white ^{LT2}*gtrABC1-lacZ* strain was challenged with SM buffer for use as a control group.

D. SPC35-resistant mutants actively express the ^{LT2}*gtrABC1* cluster. Transcription levels of the ^{LT2}*gtrABC1* cluster from the SPC35-challenged white ^{LT2}*gtrABC1-lacZ* strain (W+SPC35), the control group (W+SM buffer), the white ^{LT2}*gtrABC1-lacZ* strain and the blue ^{LT2}*gtrABC1-lacZ* strain were assessed using a β-galactosidase assay. The values shown represent the means with SD of three independent experiments, which were analysed using Student's *t* test. ***, $P < 0.001$; ns, not significant.

plate (Fig. III-10B) in accordance with the phase variable regulation of the ^{LT2}*gtrABC1* promoter (Broadbent *et al.*, 2010). An SPC35 spotting assay with cultures from the ON (blue) or OFF (white) colonies revealed that SPC35 formed clear plaques in the absence of ^{LT2}*gtrABC1* expression, whereas turbid plaques when ^{LT2}*gtrABC1* was expressed (Fig. III-10B). When the OFF (white) phase fusion strain was streaked onto an LBX plate after being challenged with SPC35, the majority of the daughter colonies were ON (blue) phase, whereas when the fusion strain was challenged with SM buffer, the majority of the daughter colonies were remained as OFF (white) phase (Fig. III-10C), indicating that SPC35 infection selects ^{LT2}*gtrABC1*-expressing and SPC35-resistant populations. To quantitatively compare the expression of the ^{LT2}*gtrABC1*cluster between the SPC35-challenged and the control groups, a β-galactosidase assay was conducted. The results demonstrated that the SPC35-challenged ^{LT2}*gtrABC1-lacZ* strains had significantly higher activity than the SM buffer-challenged control group or the parental OFF (white) phase clones; the activity of these strains was comparable with that of the ON (blue) phase colonies that vigorously expressed the ^{LT2}*gtrABC1* cluster (Fig. III-10D).

When the ON (blue) phase colonies of the SPC35-challenged strain were sequentially streaked onto fresh LBX plates, the daughter colonies showed a mixture of ON (blue) and OFF (white) phases (Fig. III-11), and the OFF (white)

phase colonies exhibited SPC35-susceptibility again. Hence, an unintended selection of these OFF phase clones in the sequential streaking of SPC35-resistant mutants explains the transience of SPC35-resistance in *S.* Typhimurium.

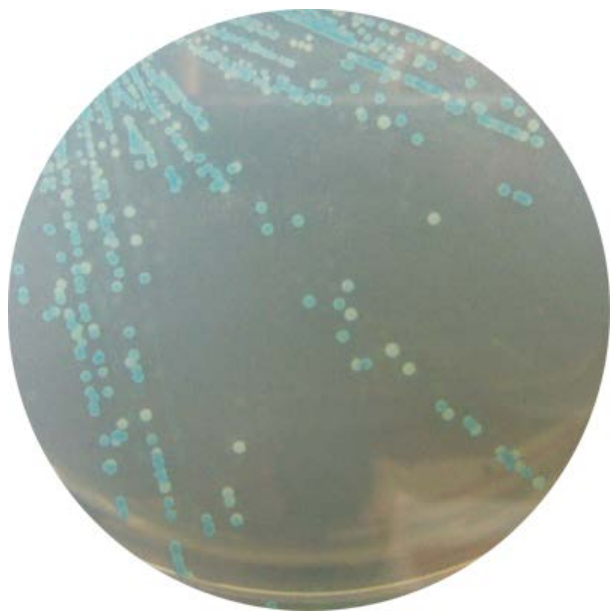


Fig. III-11. The ^{LT2}*gtrABC1* cluster is phase variable. The SPC35-challenged ^{LT2}*gtrABC1-lacZ* fusion strain (SR5024) produced ON (blue) phase colonies with a few OFF (white) phase colonies on LBX plates, indicating that SPC35 infection select the ^{LT2}*gtrABC1* ON phase clones.

III-4. Discussion

The recognition of specific host receptors is essential for successful bacteriophage infections. Most of the receptors, such as the porin OmpF for phage T2, the selective transport protein LamB for phage λ and flagellar proteins for phage χ, are bacterial components that are presented on the cell surface (Hantke, 1978; Lindberg, 1973; Randall-Hazelbauer and Schwartz, 1973; Samuel *et al.*, 1999). LPS is one of the distinctive surface appendages frequently used by Gram-negative bacteria-specific phages: phage P22 specifically binds to the O-antigens of *Salmonella* LPS, and phage ΦX174 specifically binds to the *Salmonella* core antigens that are not protected by O-antigens (Lindberg, 1973; Rakhuba *et al.*, 2010). The other surface appendages such as pili, capsular antigens and the teichoic acids also serve as phage host receptors.

In some cases, however, two different bacterial receptors are required for the two-step phage attachment process (Lindberg, 1973; Rakhuba *et al.*, 2010). In the initial phage adsorption step, phage particles collide with host bacterial cells through Brownian motion, and reversibly, but specifically, adsorb to the primary host receptor. In the second step, an irreversible binding between the virus and its secondary receptor occurs, triggering phage DNA ejection. An example of this process was recently reported for phages ΦCb13 and ΦCbK, which reversibly

interact with the flagellum of *Caulobacter crescentus* through a phage head filament and irreversibly bind to the pili portal, which is the actual host receptor (Guerrero-Ferreira *et al.*, 2011). Other example of dual step adsorption occurs in coliphage T5 infection, where the initial interaction of the L-shaped tail fibers of T5 with the polymannose O-antigens of *E. coli* F is followed by the irreversible binding of the T5 *oad* gene product (pb5) to the *E. coli* ferrichrome-iron uptake protein FhuA (Heller and Braun, 1979, 1982).

The present study identified two steps of phage SPC35 infection mediated by the *S.* Typhimurium O-antigens and the BtuB protein. Interestingly, the O-antigens play an influential role only in the initial binding of phage to the host bacteria but do not in the actual phage genome injection, given that phage SPC35 could not infect Δ*rfaL* or Δ*rfbP* mutants under shaking culture conditions but successfully infect these mutant under static conditions, such as on the surface of an agar plate or in the static liquid culture (Fig. III-1 and III-3). As the adsorption-assisting apparatus, *S.* Typhimurium O-antigens initially recruit phages to the cell surface from the surrounding environment (Fig. III-12A), and then facilitate the irreversible binding of SPC35 to its host receptor BtuB. As with T5 infection, viral tail fibers might be involved in the specific interactions between SPC35 and the *Salmonella* O-antigens (Fig. III-4B) because the L-shaped tail fibers at the distal end of SPC35 tail were observed using TEM (Kim and Ryu, 2011).

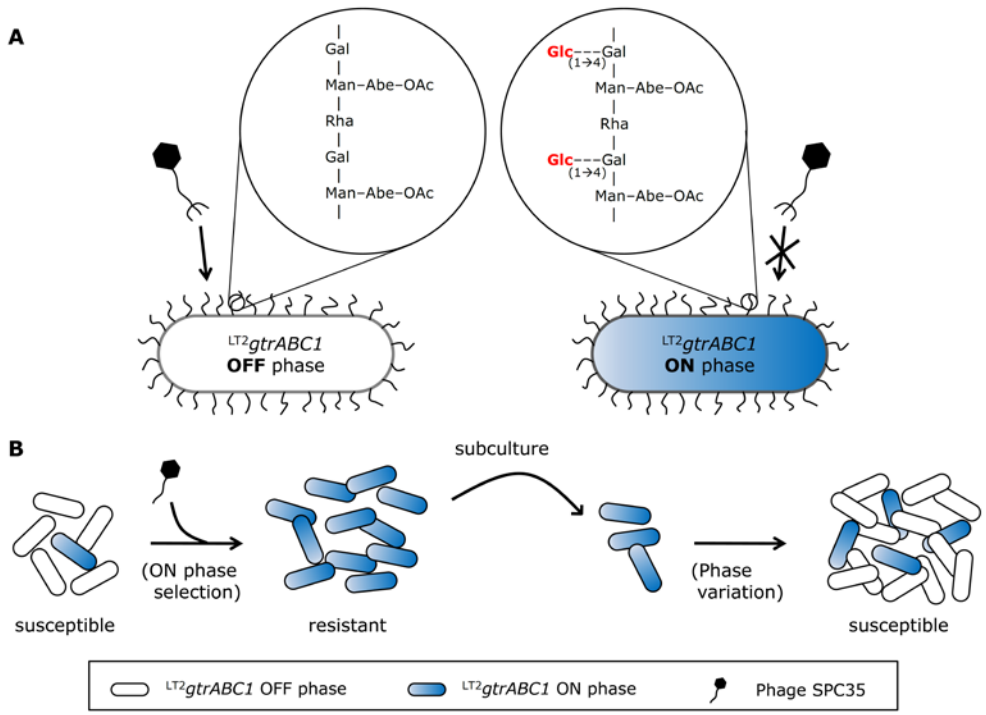


Fig. III-12. Model for the development of transient resistance against SPC35 through phase variable O-antigen glucosylation.

A. Schematic representation of the initial *Salmonella*-SPC35 interaction via the O-antigen. The non-glucosylated O-antigen can assist the initial adsorption of SPC35 to *Salmonella* even though SPC35 uses BtuB as a primary host receptor. In contrast, the α-1,4-glucosylated O-antigen produced by ^{LT2}*gtrABC1* expression prevent the initial adsorption of SPC35 to *Salmonella*. Gal, D-galactose; Man, D-mannose; Abe, abequose; Rha, L-rhamnose; Glc, D-glucose; OAc, O-acetyl group.

B. Hypothetical model for the spontaneous and transient SPC35-resistance in *S.* Typhimurium. Phage SPC35 infects and kills the majority of ^{LT2}*gtrABC1* OFF phase clones in the initial culture, but a small number of the phage-resistant ON phase clones in the population survived. After subculturing in a fresh media in the absence of SPC35, a majority of those ON phase clones spontaneously become phage-susceptible OFF phase through the phase variable switching of ^{LT2}*gtrABC1* expression. The size of bacteria and bacteriophage are not on scale.

The α-1,4-glucosylation of O-antigens by overexpression of the ^{LT2}*gtrABC1* cluster reduced SPC35 adsorption to *Salmonella* (Fig. III-4A), and resulted in the resistance against SPC35 (Fig. III-8B). The possibility of adverse effects from an increased number of inner membrane proteins (GtrA, GtrB and GtrC) on the expression of BtuB can be ruled out because SPC35 normally produces plaques on the ^{P22}*gtrABC* overexpression strain (Fig. III-9). Moreover, the α-1,4-glucosylation of O-antigens conferred resistance not only to BtuB-specific phages, including SPC35, but also to other LPS-specific phages (Fig. III-7), indicating that this type of phage-resistance mechanism might be widespread among Gram-negative bacteria.

Paradoxically, the strain expressing α-1,4-glucosylated O-antigen was resistant (Fig. III-8B) while the strain without O-antigen was sensitive (Fig. III-1) to SPC35 infection in static culture although the phage adsorption–assisting function was lost in both strains (Fig. III-4A). This discrepancy might be due to the different accessibilities of BtuB receptor on bacterial cell surface to the phage. By contrast to the O-antigen glucosylated strain, the fully exposed BtuB on the O-antigen-lacking strain surface may allow direct irreversible binding of phage to the receptor without the O-antigen-mediated adsorption-assisting. However, the interaction between BtuB and SPC35 in the absence of O-antigen seems fragile that it was not possible in the shaking culture condition (Fig. III-3). The accessibility of BtuB to

SPC35 was also tested with the *galE496* mutant, which produces significantly reduced amount of O-antigen (less than 1% wild-type) (Palva and Makela, 1980; Yun *et al.*, 2009). Similar to the Δ*rfbP* mutant, the Δ^{LT2}*gtrABC1 galE496* mutant also produced the clear SPC35 plaques (Fig. III-13A). In addition, the SPC35 could make plaques on the Δ^{LT2}*gtrABC1 galE496* mutant overexpressing the glucosylated O-antigen by complementation with p^{LT2}*gtrABC1* (Fig. III-13A), suggesting that the exposure level of BtuB on the cell surface can be determinant for SPC35-susceptibility in the static culture condition, regardless of the glucosylation state of O-antigen. In a liquid condition, however, the α-1,4-glucosylation of O-antigen dramatically affected the phage adsorption ability; the SPC35 adsorption constant was approximately 8.5-fold lowered by the α-1,4-glucosylation of O-antigen on the *galE496* background (Fig. III-13B). Taken together, these results strongly demonstrated that the loss of adsorption assisting function of O-antigen by α-1,4-glucosylation would be mainly responsible for the SPC35-resistance both in shaking and static culture conditions, although the full exposure of BtuB in the O-antigen-lacking strain can overcome the loss of adsorption assisting function of O-antigen in the static condition, and consequently, resulted in the successful infection.

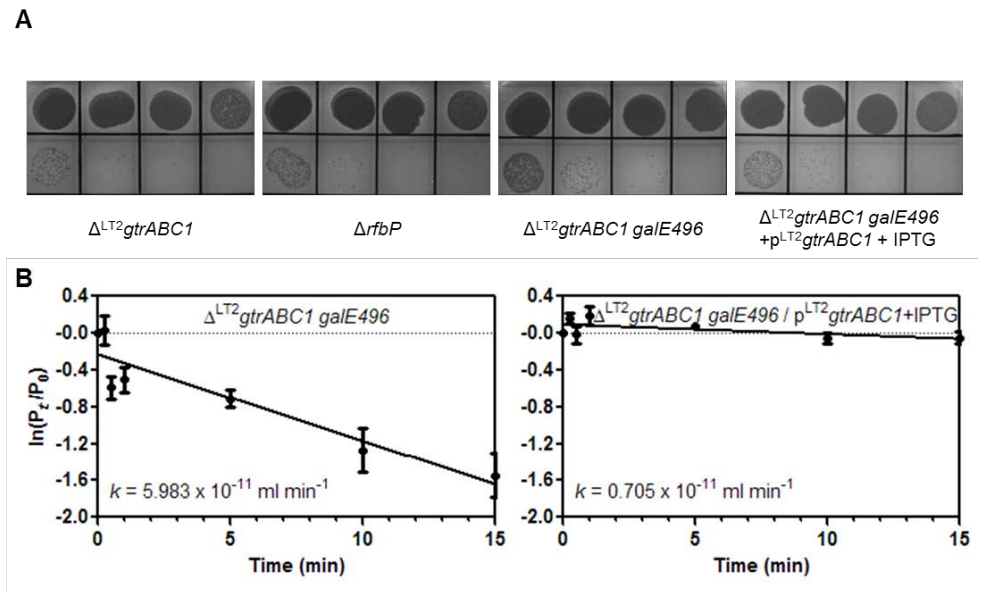


Fig. III-13. The BtuB exposure on the bacterial cell surface can overcome the loss of adsorption assisting function of O-antigen in the static culture condition.

A. The α-1,4-glucosylation of O-antigen did not significantly alter the SPC35 plaque turbidity or EOP on the *galE496* background. Ten-fold serial dilutions of SPC35 stock were spotted onto the lawns of Δ^{LT2}*gtrABC1*; Δ*rfbP*; Δ^{LT2}*gtrABC1 galE496*; and Δ^{LT2}*gtrABC1 galE496* + p^{LT2}*gtrABC1* induced with 1000 μM of IPTG.

B. The SPC35 adsorption constant (*k*) was dramatically decreased by the α-1,4-glucosylation of O-antigen on the *galE496* background.

In this study, the phase variable expression of the ^{LT2}*gtrABC1* cluster was monitored by ^{LT2}*gtrABC1-lacZ* fusion. It was reported that the O12-2-antigen variant is not predominant in nature (Makela, 1973), and that the frequency of an ON to OFF phase switch was reported to be approximately 10-fold higher than the frequency of an OFF to ON phase switch (Broadbent *et al.*, 2010). Therefore, the existence of a relatively small number of OFF (white) phase colonies among the ON (blue) phase daughter colonies of the SPC35-challenged ^{LT2}*gtrABC1-lacZ* strain (Fig. III-10C and Fig. III-11) indicates that SPC35 infection can select ON phase clones that are resistant to phage infection. Indeed, the observed reduction in the growth rate of phage infected-WT bacteria prior to becoming overgrown, as shown in Fig. III-3, occurred as a consequence of the decreasing cell viability (data not shown); the surviving population might comprised with ON phase cells that grow rapidly and eventually become SPC35-resistant. However, the SPC35-susceptible OFF phase cells can be easily regenerated from the ON phase cells through phase variable switching of the ^{LT2}*gtrABC1* cluster (Fig. III-12B), such that SPC35-resistance can transiently occur in SPC35-resistant mutants. The phase variable expression of ^{LT2}*gtrABC1* also explains how SPC35 could make turbid plaques rather than no plaques on ON (blue) phase WT *Salmonella* (Fig. III-10B) but not on *Salmonella* overexpressing ^{LT2}*gtrABC1* under the *lac* promoter (Fig. III-8B) in which the phase variation is impossible.

Several mechanisms were proposed to explain the odd phenomenon of rapidly generated transient phage-resistance, including physiological and/or genetic change(s) in the phage-treated *Salmonella* (Kocharunchitt *et al.*, 2009), the existence of a phage-resistant subpopulation in the initial culture (Carey-Smith *et al.*, 2006) and the clonal heterogeneity of *E. coli* O157:H7 (Fischer *et al.*, 2004). Phage-resistance through the temporary inactivation of genes associated with receptor biosynthesis has been previously demonstrated in *Campylobacter jejuni* (Sorensen *et al.*, 2011) and in *H. influenzae* (Zaleski *et al.*, 2005), where the slipped-strand mispairing causes translational truncation of the gene. In this study, however, the development of transient phage-resistance by phase variable ^{LT2}*gtrABC1* expression in *S*. Typhimurium differs from the above cases, as the target is the adsorption-assisting apparatus (LPS) rather than the receptor itself (BtuB). Furthermore, the ^{LT2}*gtrABC1* cluster is involved in the modification, but not the biosynthesis, of LPS. Therefore, it might actually be advantageous for *S*. Typhimurium to harbor the ^{LT2}*gtrABC1* cluster to obtain resistance against phages without experiencing any fitness costs, i.e., the loss of intracellular bacterial components. This phage defense-mediating gene cluster might have originated from phages because similar gene clusters have been observed to produce a superinfection-immunity in several temperate phages, including the P22-like *Salmonella* phages and the *Shigella flexneri* phages SFII, SFV and SFX (Allison

and Verma, 2000; Bogomolnaya *et al.*, 2008; Broadbent *et al.*, 2010). The fact that the ^{LT2}*gtrABC1* cluster is located within the novel pathogenicity island 16 (SPI-16) (Vernikos and Parkhill, 2006) further supports this possibility.

The ^{LT2}*gtrABC1* cluster-mediated phase variable O-antigen glucosylation is also associated with *Salmonella* virulence. Phase variation has been considered as a mechanism for bacterial evasion of the host innate and acquired immune systems (van der Woude, 2006). When the heterogeneous clonal population generated by phase variation colonizes a host, the host immune system targets one subpopulation, while other subpopulations presenting different surface antigens remain unaffected. Bogomolnaya *et al.* suggested that O-antigen glucosylation by the ^{LT2}*gtrABC1* cluster assists in the evasion of the host adaptive immune response because mutants incapable of O-antigen glucosylation exhibited defective persistence in a murine intestine model (Bogomolnaya *et al.*, 2008). Moreover, the fact that the ^{LT2}*gtrABC1* cluster is specific for the *S. enterica* subspecies I further strengthens the epidemiological significance of O-antigen glucosylation in *Salmonella* virulence (Bogomolnaya *et al.*, 2008). The advantages of the ^{LT2}*gtrABC1* cluster not only for *Salmonella*-phage interactions but also for *Salmonella*-animal host interactions might represent another driving force for the stable maintenance of this gene cluster within the *Salmonella* genome. However, the existence of the ^{LT2}*gtrABC1* cluster within the host genome could also be beneficial to the phages themselves; it

might save prospective prey from unintended attacks, allowing the phages to maintain their population in a rapidly changing environment. Therefore, this adaptation might be one of the final settlements of the ‘arms-race’ between bacteria and phages, resulting in their stable co-existence in nature.

Considering these points, transient phage-resistance through phase variable surface antigenic modifications might not be limited to *Salmonella*-SPC35, but could represent a widespread trait among other Gram-negative bacteria-phage systems. Indeed, to the best of my knowledge, temporal phage-resistance has been reported only in Gram-negative pathogens presenting surface antigens (LPS, LOS, CPS, etc.), as described above (Fischer *et al.*, 2004; Kocharunchitt *et al.*, 2009; Sorensen *et al.*, 2011; Zaleski *et al.*, 2005). In some cases, however, it is yet unclear whether the causal relationship could be developed between the transient phage-resistance and the phase variable modification of surface antigens. Further studies should concentrate on surface antigen modifications and their simultaneous effects on both the phage-resistance and virulence of Gram-negative pathogens. Understanding this relationship will significantly expand our knowledge of the complex predator-prey relationship between phage and bacteria and between bacteria and eukaryotic hosts.

Chapter IV.

Conversion of Temperate Phage to Virulent by Constitutive Expression of Novel Anti-Repressor

IV-1. Introduction

Bacteriophages (phages), natural viral predators of bacteria, multiply by infecting specific host bacteria. After recognition of specific host extracellular apparatuses (e.g., lipopolysaccharides, flagella, pili, outer membrane proteins) as the host receptors, phages inject their nucleic acids into the host cells to initiate replication by taking over the host metabolic processes and machinery. Phage genome replication occurs via two different developmental paths. In the lytic cycle, replication of the injected phage genome is initiated immediately in the host cells along with the expression of several proteins that are required for the assembly of phage capsids. The unavoidable final fate of the infected bacteria is the death by lysis proteins (i.e., endolysins) and the bursting out of newly synthesized infectious progeny into the surrounding environment. In the lysogenic cycle, however, phage genomes are maintained within the host cells for several generations as a part of host genomes, mostly by integrating into host chromosomes or, more rarely, by replicating as low-copy-number phage-plasmids (Girons *et al.*, 2000; Ravin, 2011). Until conditions that alter host physiology occur, the expression of phage genes necessary for progeny production, as well as host cell lysis, are tightly repressed by a phage regulatory system that includes a phage repressor. Physiological changes in the host induced by UV light irradiation or other DNA-damaging agents activate

the lytic cycle by disabling the phage repressor. The switch from the lysogenic to lytic state results in vigorous phage genome replication with the expression and assembly of phage genes and gene products, respectively, and a release of the resultant progeny along with host lysis. Based on this phage lifecycle, phages are categorized into two types: virulent phages that strictly go into the lytic cycle and temperate phages that can enter both the lytic and lysogenic cycle.

The lytic switch following lysogenic development in temperate phages has been well-investigated with phage lambda. Immediately after lambda infection, the concentration of lambda CII protein in the infected host cells determines the lambda lifecycle choice between lytic and lysogenic. CII is a transcriptional activator that acts on the promoter of the lambda repressor *cI* gene. Lysogeny will be favored if the cellular levels of CII are high enough (Court *et al.*, 1975). In addition, CII activates the transcription of the *int* gene, which encodes a phage integrase that is required for phage genome integration into the host chromosome (Katzir *et al.*, 1976). CIII, an inhibitor of the ATP-dependent protease FtsH, also contributes to lambda lysogen development because FtsH breaks down CII (Kobiler *et al.*, 2007; Shotland *et al.*, 1997). The synthesized CI repressors form dimers and later bind to the left and right operators (O_L and O_R, respectively) to prevent expression of the early genes and subsequent late genes (Echols and Green, 1971; Reichardt and Kaiser, 1971). Upon host DNA damage, however, the lambda

lifecycle is switched to lytic development via CI proteolysis. Similar to the inactivation process of LexA, the RecA and single-stranded DNA complex formed upon DNA damage stimulates the autoproteolysis of CI (Little, 1984; Little and Mount, 1982; Sauer *et al.*, 1982). Next, early and late genes repressed by CI are expressed and progeny are produced. This mechanism illustrates that lambda and other similar phages exploit the host cellular SOS response to escape quickly from a potentially dangerous shelter using the RecA-dependent cleavable repressor.

In 1971, Hershey (1971) defined the term clear plaque (*cp*) mutant as follows: "Lambda and other temperate phages form plaques that are turbid owing to growth of cells surviving the infection. Mutants forming clear plaques (clear mutants) establish immunity less efficiently and permit fewer cells to survive". Such *cp* mutants have been reported in several temperate phages, such as lambda, P22, P1, P7, phi80, and ε15 (Bronson and Levine, 1971; Pons, 1984; Reyes, 1985; Scott *et al.*, 1977; Scott and Kropf, 1977; Wright, 1971). Interestingly, most of the mutations that confer to these phages the clear plaque phenotype are closely related with lysogen establishment ability: mutations located in the coding sequence of the repressor or in the operators of the repressor. For example, when mutations were induced by 9-aminoacridine in wild-type (WT) *E. coli* K-12 lysogens of lambda, more than 90% of the *cp* mutants had specific mutations in two hot spots in the *cI* repressor gene (Pons, 1984).

The aim of the present study is an identification of the factor(s) that cause a phenotypic difference between two very similar podoviral phages SPC32H and SPC32N. I observed that a single nucleotide change at the LexA-binding site that overlaps a promoter of a novel phage anti-repressor gene causes constitutive expression of the anti-repressor Ant and consequent inhibition of phage repressor function in SPC32N. As a result, SPC32N could not establish lysogeny as in *cp* mutants. The LexA-dependent lytic/lysogenic switch involving the anti-repressor, rather than repressor proteolysis, has been reported previously in only siphon-and myoviral phages (Lemire *et al.*, 2011; Mardanov and Ravin, 2007; Shearwin *et al.*, 1998), and the Ant of podoviral SPC32H/N had no homology to the reported anti-repressors. A homology search of a protein database revealed the widespread presence of homologous proteins of Ant, suggesting the extensive use of the anti-repressor for induction among temperate phages in the order *Caudovirales*.

IV-2. Materials and Methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table IV-1. Unless mentioned otherwise, all *S.* Typhimurium mutants were derived from the prophage-cured *Salmonella enterica* serovar Typhimurium strain LT2 (referred to as LT2(c)) (Erickson *et al.*, 2009) and its derivative ^{LT2}*gtrABC1*-deletion strain (Δ^{LT2}*gtrABC1*; SR5003) (Kim and Ryu, 2012) to exclude the effect of other prophages and spontaneous phage-resistance via O-antigen glucosylation, respectively. Bacterial cells were grown with aeration in Luria-Bertani (LB) broth or on LB agar plates [1.5% (wt/vol) agar] at 37°C or 30°C. The media was supplemented with antibiotics or chemicals at the following concentrations, if required: ampicillin (Ap), 50 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; chloramphenicol (Cm), 25 μg ml^{-1}; 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 40 μg ml^{-1}; L-arabinose, 0.2% (final concentration); and isopropyl-β-D-thiogalactopyranoside (IPTG), 1000 μM (final concentration). For the disc diffusion assay, 6-mm-diameter filter paper discs were laid on the bacteria-inoculated solidified top agar [LB supplemented with 0.4% (wt/vol) agar; add X-gal, if necessary], soaked with the 10-μl of indicated concentrations of arabinose, antibiotics or mitomycin C (MMC), and incubated at 37°C for 8 hr.

Table IV-1. Bacterial strains and bacteriophages used in Chapter IV.

Strains	Relevant characteristics[a]	Reference or source
Bacterial strains		
Salmonella enterica serovar Typhimurium		
LT2(c)	Prophage cured strain LT2; wild-type; host for phage SPC32H and SPC32N	(Erickson *et al.*, 2009)
SR5016	LT2(c) with Δ*rfbP*	(Kim and Ryu, 2012)
SR5018	SR5016 with p*rfbP*; *rfbP* overexpression strain	(Kim and Ryu, 2012)
SR5003	LT2(c) with Δ^{LT2}*gtrABC1*	(Kim and Ryu, 2012)
SR5005	SR5003 with p^{LT2}*gtrABC1*; ^{LT2}*gtrABC1* overexpression strain	(Kim and Ryu, 2012)
SR5025	LT2(c) with Δ*oafA*	(Kim and Ryu, 2012)
SR5027	SR5025 with p*oafA*; *oafA* overexpression strain	(Kim and Ryu, 2012)
SR5028	LT2(c) with Δ^{LT2}*gtrABC1* Δ*oafA*	This study
SR5032	SR5003 with p*oac*	This study

SR5033	SR5028 with p*oac*	This study
SR5100	Δ^{LT2}*gtrABC1* (32H); SR5003 lysogenized by SPC32H	This study
SR5112	SR5003 with p*int*	This study
SR5167	Δ^{LT2}*gtrABC1* (32H Δ*int*); SR5003 lysogenized by SPC32H Δ*int*	This study
SR5121	SR5003 with pBAD24	This study
SR5115	SR5003 with p*rep*	This study
SR5140	Δ^{LT2}*gtrABC1* (32H Δ*ant*); SR5003 lysogenized by SPC32H Δ*ant*	This study
SR5122	SR5003 with p*ant*	This study
SR5141	SR5100 with pBAD24	This study
SR5142	SR5100 with p*ant*	This study
SR5150	SR5100 with p*ant**	This study
SR5143	SR5003 with pBBRlux	This study
SR5144	SR5003 with pP_{ant_H}::*lux*	This study

SR5145	SR5003 with pP_{ant_N}::*lux*	This study
SR5146	SR5100 with pBBRlux	This study
SR5147	SR5100 with pP_{ant_H}::*lux*	This study
SR5148	SR5100 with pP_{ant_N}::*lux*	This study
SR5158	LT2(c) with Δ^{LT2}*gtrABC1* Δ*sulA* Δ*lexA*	This study
SR5162	SR5158 with pP_{ant_H}::*lux*	This study
SR5163	SR5158 with pP_{ant_H}::*lux* and pBAD24	This study
SR5164	SR5158 with pP_{ant_H}::*lux* and p*lexA*	This study
SR5176	LT2(c) with Δ^{LT2}*gtrABC1* Δ*sulA lexA*(G85D); non-cleavable LexA mutant	This study
SR5190	SR5176 with pP_{ant_H}::*lux*	This study
SR5189	Δ^{LT2}*gtrABC1* (32H *recET::lacZ*); SR5003 lysogenized by SPC32H *recET::lacZ*	This study
SR5188	Δ^{LT2}*gtrABC1* Δ*sulA lexA*(G85D) (32H *recET::lacZ*); SR5176 lysogenized by SPC32H *recET::lacZ*	This study

SR5192	Δ^{LT2}*gtrABC1*(32H *rep*-HA); SR5003 lysogenized by SPC32H *rep*-HA	This study
SR5197	Δ^{LT2}*gtrABC1*(32H *rep*-HA *ant*-HA); SR5003 lysogenized by SPC32H *rep*-HA *ant*-HA	This study

Escherichia coli

DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ* Δ*M15*) *hsdR17 recA1 endA1 gyrA 96 thi1 relA1*; plasmid replication	Laboratory collection
S17-1 lambda *pir*	*recA thi hsdRM*$^+$ RP4::2-Tc::Mu::Km Tn7, lambda *pir*; Tp^r Str^r ; conjugal donor	(Simon *et al.*, 1983)
BTH101	F^- *cya-99 araD139 galE15 galK16 rpsL1*(Str^r) *hsdR2 mcrA1 mcrB1*; reporter strain in bacterial two-hybrid assay	(Karimova *et al.*, 1998)
BL21(DE3)	F^- *ompT hsdS* (r_B^- m_B^-) *gal* (DE3); protein overexpression	Laboratory collection

Bacteriophages

SPC32H	Infect *S*. Typhimurium; O-antigen-specific	This study
SPC32N	Infect *S*. Typhimurium; O-antigen-specific	This study
SPC32H Δ*int*	SPC32H derivative with Δ*int*	This study
SPC32H Δ*ant*	SPC32H derivative with Δ*ant*	This study
SPC32H *m1*	SPC32H derivative with one nucleotide substitution in *tsp* gene (C to T)	This study
SPC32H *m2*	SPC32H derivative with one nucleotide substitution in *ant* gene promoter (G to T)	This study
SPC32H *m12*	SPC32H derivative with two nucleotide substitutions in *tsp* gene (C to T) and *ant* gene promoter (G to T)	This study

[a] Tp^r, trimethoprim resistant; Str^r, Streptomycin resistant.

Bacteriophages. The bacteriophages used in this study are listed in Table IV-1. The phages were purified, propagated and concentrated as previously described (Kim and Ryu, 2011). *S*. Typhimurium LT2(c) was used as the host bacteria for phage propagation, and phage precipitation with PEG 6000 followed by CsCl-density gradient ultracentrifugation (78,500 x g for 2 hr at 4°C) was performed to prepare the concentrated phage stocks (ca. > 10^{11} PFU ml^{-1}). The phage stocks were stored at 4°C until further uses. To determine the phage infectivity and efficiency of plating (EOP) against specific host bacteria, the phage spotting assay and routine double-agar overlay assay were conducted with LB soft agar [0.4% (wt/vol) agar; supplemented with the indicated concentration of antibiotics, IPTG or L-arabinose, if necessary] as previously described (Kim and Ryu, 2011, 2012). The Red™ Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA) or KENOX #1 (Samsung Techwin Co. Ltd., South Korea) were used to capture plate images. For morphological analysis, transmission electron microscopy (TEM) was performed with phage stocks as previously described (Kim and Ryu, 2011).

Bacteriophage genome sequencing and analysis. The nucleic acid of phage SPC32H or SPC32N was manually extracted by the phenol/chloroform extraction methods with protease K/SDS treatment (Sambrook and Russell, 2001). The pyrosequencing of the extracted nucleic acids was conducted using a Genome

Sequencer FLX Titanium by Macrogen, Seoul, South Korea. The quality-filtered reads were assembled using GS De Novo assembler (v. 2.60), and the open reading frames (ORFs) that encode proteins more than 35 amino acids were predicted using GeneMarkS (Besemer *et al.*, 2001), Glimmer 3.02 (Delcher *et al.*, 2007) and FgenesV (Softberry, Inc., Mount Kisco, NY). The annotation of the predicted ORFs was performed based on the results of BlastP (Altschul *et al.*, 1990), InterProScan (Zdobnov and Apweiler, 2001) and NCBI Conserved Domain Database (Marchler-Bauer *et al.*, 2007) analysis. tRNAscan-SE (Lowe and Eddy, 1997) was used to predict the tRNA coding sequences. The putative promoter regions and transcription factor-binding sites of specific phage genes were predicted using BPROM (Softberry, Inc., Mount Kisco, NY). Genomic comparison at the DNA level was visualized using Easyfig. The sequences of the phage SPC32H genome and SPC32N genome are available at GenBank under accession numbers KC911856 and KC911857, respectively.

Construction of *Salmonella* mutant strains. For the in-frame deletion of specific gene(s) from the *S.* Typhimurium chromosome, the lambda Red recombination method (Datsenko and Wanner, 2000) was applied. In brief, a Km^r cassette flanked with the upstream and the downstream regions of the deletion-target site was amplified from plasmid pKD13 via PCR with appropriate

oligonucleotides. The plasmid pKD46-harboring *S*. Typhimurium strain was grown aerobically at 30°C with 100 mM L-arabinose to the early-exponential phase, and then transformed with the resulting PCR product via electroporation. The Km^r-transformants selected from kanamycin plate were transformed with the plasmids pCP20 to remove the Km^r cassette. The in-frame deletion of target gene(s) was confirmed with PCR and subsequent DNA sequencing. Human influenza hemagglutinin (HA) epitope-tagging to the 3'-end of specific prophage genes was also conducted with the same method using specific oligonucleotides containing a HA-tag sequence.

To introduce a point mutation (g254a in nucleotide sequence; G85D in amino acid sequence) into the *lexA* gene of the *Salmonella* chromosome, the following 3 steps of genetic manipulations were conducted as previously described (Kim and Ryu, 2012) with some modifications. First, a 471 nt-segment of the *lexA* gene region, which contains the targeting site of point mutation, was replaced with the Km^r-cassette by lambda Red recombination method (see above) in an in-frame *sulA* deletion mutant. As a second step, *lexA* gene from *S*. Typhimurium LT2 strain was cloned into the pGEM®-T easy vector system (Promega, USA) with oligonucleotides LT2-lexA-CF3 and LT2-lexA-CR3 according to the manufacturer's instructions. The resulting plasmid pGEM-T::*lexA* was subjected as a template plasmid to site-directed mutagenesis using a QuickChange II Site-

Directed Mutagenesis Kit (Agilent Technologies, USA) with oligonucleotides LT2-lexA-G85D-F1 and LT2-lexA-G85D-R1 according to the manufacturer's instruction manual. The sequence confirmed, point mutation-containing *lexA* gene was sub-cloned into the SphI-SacI digested suicide conjugal vector pDS132 (Philippe *et al.*, 2004) to construct plasmid pDS*lexA*(G85D). In the last step, the conjugation of *E. coli* S17-1 λ *pir* harboring pDS*lexA*(G85D) as a conjugal donor and *S.* Typhimurium LT2 Δ*sulA lexA*::Km^r from the first step as a conjugal recipient was conducted. The donor and recipient cells grown to the stationary phase in LB broth was harvested, washed with one-milliliter of 10 mM $MgSO_4$ for 3 times, resuspended and mixed with 100-μl of 10 mM $MgSO_4$, and spotted onto LB agar plate. The grown cells were resuspended in 1 ml of LB broth and a portion (100 μ) of the cell suspension was plated on LB agar plates contained Km and Cm. After overnight incubation at 37°C, the insertion of pDS*lexA*(G85D) into the *lexA* region via a primary homologous recombination was PCR confirmed, and the positive clones were subjected to sucrose challenge for a secondary homologous recombination. The overnight grown positive clones were inoculated (1:1000) into 5 ml LB broth containing 20% sucrose (final concentration), incubated for 10 hr at 37°C, and serially diluted in 10-fold with LB broth. One hundred microliters of each dilution was plated and incubated on LB agar supplemented with 5% sucrose and without NaCl overnight at 37°C. The sucrose-mediated counter-selected clones

were patched on each Cm, Km and plain LB agar plates. A Cm^s Km^s clone was selected, and the *lexA*(G85D) mutation was confirmed by PCR and subsequent DNA sequencing.

The SPC32H lysogen [Δ^{LT2}*gtrABC1*(32H)] was isolated through sequential streaking of SPC32H-resistant clones from the inside of a high-titer phage-spotting zone on Δ^{LT2}*gtrABC1*. The isolated lysogen was confirmed by immune testing against SPC32H infection and PCR amplification of the specific phage attachment site (*attR* site) using the oligonucleotides 32H-attP and 32H-attB.

The transcriptional *recET::lacZ* fusion in SPC32H lysogen was constructed previously described methods with some modifications (Ellermeier *et al.*, 2002). Briefly, the Km^r cassette from pKD13 was amplified via PCR with oligonucleotides 32H-recE-lacZ-F and 32H-recT-lacZ-R. The SPC32H lysogen-containing pKD46 was transformed with the resulting PCR product, and the Km^r cassette was removed from the transformant by introduction of pCP20. The integration of the *lacZY* genes into the FRT site on the scar region was promoted by transformation with pCE70 (Merighi *et al.*, 2005), resulting in a transcriptional fusion of *lacZ* to the *rec* operon of SPC32H. The proper integration at the target site was PCR and subsequently DNA sequencing confirmed. The oligonucleotides used in the construction of *Salmonella* mutants are listed in Table IV-2.

Table IV-2. The oligonucleotides used in Chapter IV.

Oligonucleotides	Sequences (5'→3')[a]	Purpose
LT-oafA-Red-F	ATG ATC TAC AAG AAA TTC AGA CTC GAT ATA AAT GGG CTA ATG TAG GCT GGA GCT GCT TCG	SR5028 construction
LT-oafA-Red-F	TTA TTT TGA AAT CTG CTT TTT CAC TTC CTC AAT AAA CCA CAT TCC GGG GAT CCG TCG ACC	SR5028 construction
32H-oac-CF4	AAA TAC CAA TCA TGG ATC CTG ATT CAT A	p*oac* construction
32H-oac-CR4	GTT CTT ACA AGA CAA AGC TTA ACA ACA T	p*oac* construction
32H-int-CF2	TAA AGG TAT ACT CTG GAT CCC GTC	p*int* construction
32H-int-CR2	TGA CCT ACA GCG AAG CTT TAT ACA	p*int* construction
32H-int-Red-F	ATG AAG CAC GTC ATT CTG GCT GAC AGT ACC CGC GCC ATG ATG TAG GCT GGA GCT GCT TCG	SR5167 construction

32H-int-Red-R	GTG CTC CCC GTT AAT CCA GGA ATC AAC CAT ATC AGC CCA CAT TCC GGG GAT CCG TCG ACC	SR5167 construction
32H-rep-CF2	AAT GGG CAA ATG AAT TCG CTA TGA AA	p*rep* construction
32H-rep-CR1	TGG TAA TTG CGT GTC GAC TGA G	p*rep* construction
32H-ant-Red-F	CAG TAA TCG AGA TGC GTT TGC AGA TCC TGC ATT GTC ATT TTG TAG GCT GGA GCT GCT TCG	SR5140 construction
32H-ant-Red-R	GCT GGA TAG TCA TGA TAT CTT CTG TTA CTG TAT GTT TAT AAT TCC GGG GAT CCG TCG ACC	SR5140 and *ant*::Km^{r} clone construction
32H-ant-CF1	TGT TTG CAT GGA GAA TTC GAG ATG C	p*ant* construction
32H-ant-CR1	GCC GCA GAG TCG ACC TTT TAT TTT T	P*ant*, P*ant** construction
32H-ant*-CF2	CAT GGA GAA TTC GAG ATG **A**CA ACG G	P*ant** construction
32s-Pant-CF1	TCA GTT GAG CTC GTC ATG TAA G	pP_{ant_H}::*lux* and pP_{ant_N}::*lux* construction

32s-Pant-CR1	GGT GAT ACT GCC ACT AGT TCT C	pP_{ant_H}::*lux* and pP_{ant_N}::*lux* construction
LT2-sulA-Red-F	ATG TAC ACT TCA GGT TAT GCA AAT CGT TCT TCG TCA TTT CTG TAG GCT GGA GCT GCT TCG	SR5158 construction
LT2-sulA-Red-R	CGC ACG TAC AGG GCG CAT GAT AAA CCC TAC CGC ATT ACC TAT TCC GGG GAT CCG TCG ACC	SR5158 construction
LT2-lexA-Red-F	CCA GAC AGG TAT GCC ACC GAC GCG TGC GGA AAT CGC GCA GTG TAG GCT GGA GCT GCT TCG	SR5158 construction
LT2-lexA-Red-R	ACT ACA ACC ATT CCC CGT TGC GAA TGA CGC CTA CCG CCA GAT TCC GGG GAT CCG TCG ACC	SR5158 construction
LT2-lexA-CF1	TAT ATA CAC CCA GAA TTC GGA ATG AAA GCG	p*lexA* construction
LT2-lexA-CR1	ATT GCC GGA TGT CGA CTT ACA AGG AG	p*lexA* construction
LT2-lexA-CF3	AAA GCG GCA GGA TAT TCC TG	pGEM-T::*lexA* construction

LT2-lexA-CR3	ATT TCA AGG AAG CGT CTC GC	pGEM-T::*lexA* construction
LT2-lexA-G85D-F1	CGT GTC GCG GCG GAT GAA CCG CTT CTG	Site-directed mutagenesis at *lexA*(G85D)
LT2-lexA-G85D-R1	CAG AAG CGG TTC ATC CGC CGC GAC ACG	Site-directed mutagenesis at *lexA*(G85D)
32H-recE-lacZ-Red-F	AGT AAG TCA CAA CTG GAT ATG GTG GCC AAG AAC CCT TCC CTG TAG GCT GGA GCT GCT TCG	SR5188 and SR5189 construction
32H-recT-lacZ-Red-R	TCT TTG TTG CAG GTG TGG CGC CGT GGC GCC ACG GTG GTG A AT TCC GGG GAT CCG TCG ACC	SR5188 and SR5189 construction
32H-rep-HA-Red-F	CTC ATT CAT AAA CTT CGT GTT TGA GCA GAA CAA AAG CAA G*TA TCC GTA TGA TGT TCC TGA TTA TGC TAG CCT C*TA ATG TAG GCT GGA GCT GCT TCG	SR5192 and SR5197 construction
32H-rep-HA-Red-R	ACC GCC ATC GGG CAG GTA AAG CGT CAG AAT GGC AGG GGA TAT TCC GGG GAT CCG TCG ACC	SR5192 and SR5197 construction

32H-ant-HA-Red-F	TCT CGA TTA CTG TGC AGA ACA GTT ACG AAA ACA AAC CAC A*TA TCC GTA TGA TGT TCC TGA TTA TGC TAG CCT C*TA ATG TAG GCT GGA GCT GCT TCG	SR5197 construction
32H-ant-HA-Red-R	TGT CAT AGC ATG AAT GTG ACA TGT CAC GAG GCC GCA GAA GAT TCC GGG GAT CCG TCG ACC	SR5197 construction
pKT25-32H-rep-CF1	AAC TGC AGG GAT GAA AAG TAT TTA TGA CAT	pKT25-rep construction
pKT25-32H-rep-CR1	CGG GAT CCT TAC TTG CTT TTG TTC TG	pKT25-rep and pUT18C-rep construction
pUT18C-32H-ant-CF1	AAC TGC AGG ATG CAA CGG CAG TAT CA	pUT18C-ant construction
pUT18C-32H-ant-CR1	CGG GAT CCT TAT GTG GTT TGT TTT CGT AAC	pUT18C-ant and pKT25-ant construction
pKT25-32H-ant-CF1	AAC TGC AGG GAT GCA ACG GCA GTA TCA	pKT25-ant construction
pUT18C-32H-rep-CF1	AAC TGC AGG ATG AAA AGT ATT TAT GAC ATA AG	pUT18C-rep construction
pHIS-LT2-lexA-CF2	TAT ATA CAC CCC ATG GGC GGA ATG AAA G	pHIS-LexA construction

pHIS-LT2-lexA-CR2	ATT GCC GGA TCT CGA GTT ACA AGG AG	pHIS-LexA construction
pHIS-32H-Rep-CF1	AAT ACC ATG GCT ATG AAA AGT ATT TAT GAC ATA AGA CGC	pHIS-Rep construction
pHIS-32H-Rep-CR1	AAA GCT CGA GAA TGG CAG GGG ATT ACT TGC	pHIS-Rep construction
pHIS-32H-Ant-CF4	CAT AAG CCA TGG GGA TGC AAC GGC AGT ATC AC	pHIS-Ant construction
pHIS-32H-Ant-CR3	GCC GCA GAC TCG AGC TTT TAT TTT TCA TTA TGT GG	pHIS-Ant construction
32s-APR-CF1	CTT CAG TTG AGA CCG TCA TG	PCR for APR_H and APR_N
32s-APR-CR1	TAA GAT GTG AGT CCT CCA CC	PCR for APR_H and APR_N
32H-tsp-Red-F	GCG CAT AAG CAA CCT GGA GAT ACT TAC ATG ACG GTC TCA ATG TAG GCT GGA GCT GCT TCG	*tsp*::Km^r clone construction
32H-tsp-Red-R	GTA AAG TTG GGA ATG AGG ATG TCT TTA TCA TCA ACT CTT GAT TCC GGG GAT CCG TCG ACC	*tsp*::Km^r clone construction
32N-tsp-CF1	TCT CTT CTT CTC CAT GCA AAC ACT G	pGEM-T::tsp_N construction
32N-tsp-CR1	GTA GAG GGT TCA GTT ATC CAC TCC C	pGEM-T::tsp_N construction

32H-ant-Red-F1	CTT CAA ATC CTT CTT CCA GCG GAT GGT GAT ACT GCC GTT GTG TAG GCT GGA GCT GCT TCG	*ant*::Km^r clone construction
32N-ant-CF3	TAT TTT CTG CAG TGT GGT TTG TTT TCG TAA	pDS*ant*$_N$ construction
32N-ant-CR2	TCC TGT GAG CTC TCC CGC AC	pDS*ant*$_N$ construction

[a] Restriction enzyme sites and HA-tag coding sequences are underlined and italicized, respectively. Bold case indicates the artificially inserted redundant nucleotide to generate the frame-shift in *ant* gene.

Construction of phage mutants. To obtain the mutant phages, the SPC32H lysogen [Δ^{LT2}*gtrABC1*(32H)] was genetically engineered using lambda Red recombination and double-homologous recombination-based counter selection, and then the resulting recombinant mutant prophages were induced with MMC (1 μg ml^{-1}, final concentration) or spontaneously, purified, and propagated.

To generate the SPC32H *m1* mutant, the SPC32H lysogen-containing pKD46 was aerobically grown with 100 mM L-arabinose, harvested, and transformed with Km^r-cassettes amplified from pKD13 with oligonucleotides 32H-tsp-Red-F and 32H-tsp-Red-R. The resulting truncated tailspike gene (*tsp*::Km^r) was replaced with the *m1*-containing *tsp* gene (tsp_N) by double-homologous recombination-based counter selection similar to the method described above [see the section of *lexA*(G85D) mutant construction in "*Construction of* Salmonella *mutant strains*"] with appropriate oligonucleotides and recombinant plasmids. The recombinant SPC32H *m1* mutant prophage was spontaneously induced from the resulting lysogen at stationary growth phase, and the mutation *m1* in the phage genome was confirmed by DNA sequencing.

For the SPC32H *m2* mutant construction, similar methods were applied. Notably, however, the mutation *m2* appeared to trigger the lysogen lysis, which might be due to the constitutive expression of the anti-repressor. Therefore, genetic engineering strategies were carefully employed to ensure that the intermediate

engineered strains did not constitutively express the anti-repressor. The *ant* gene in the recipient *S.* Typhimurium SPC32H lysogen was truncated by Km^r cassette insertion [Δ^{LT2}*gtrABC1* (32H *ant*::Km^r)], and the *ant* gene in the *m2*-containing conjugal plasmid, pDSant_N, was also constructed as a truncated form missing the stop codon. The positive clone resulting from the primary homologous recombination [Δ^{LT2}*gtrABC1* (32H *ant*::Km^r) (pDSant_N)] was challenged by sucrose, and then the spontaneously induced recombinant mutant phages in supernatant was screened using double-agar overlay assay with Δ^{LT2}*gtrABC1* and subsequent specific DNA sequencing of single plaques. Fortuitously, the SPC32H *m12* mutant as well as SPC32H *m2* mutant was simultaneously screened out and used in this study. The oligonucleotides used in construction of phage mutants are listed in Table IV-2. See Fig. IV-1 for schematic representation of the construction of the mutant phage.

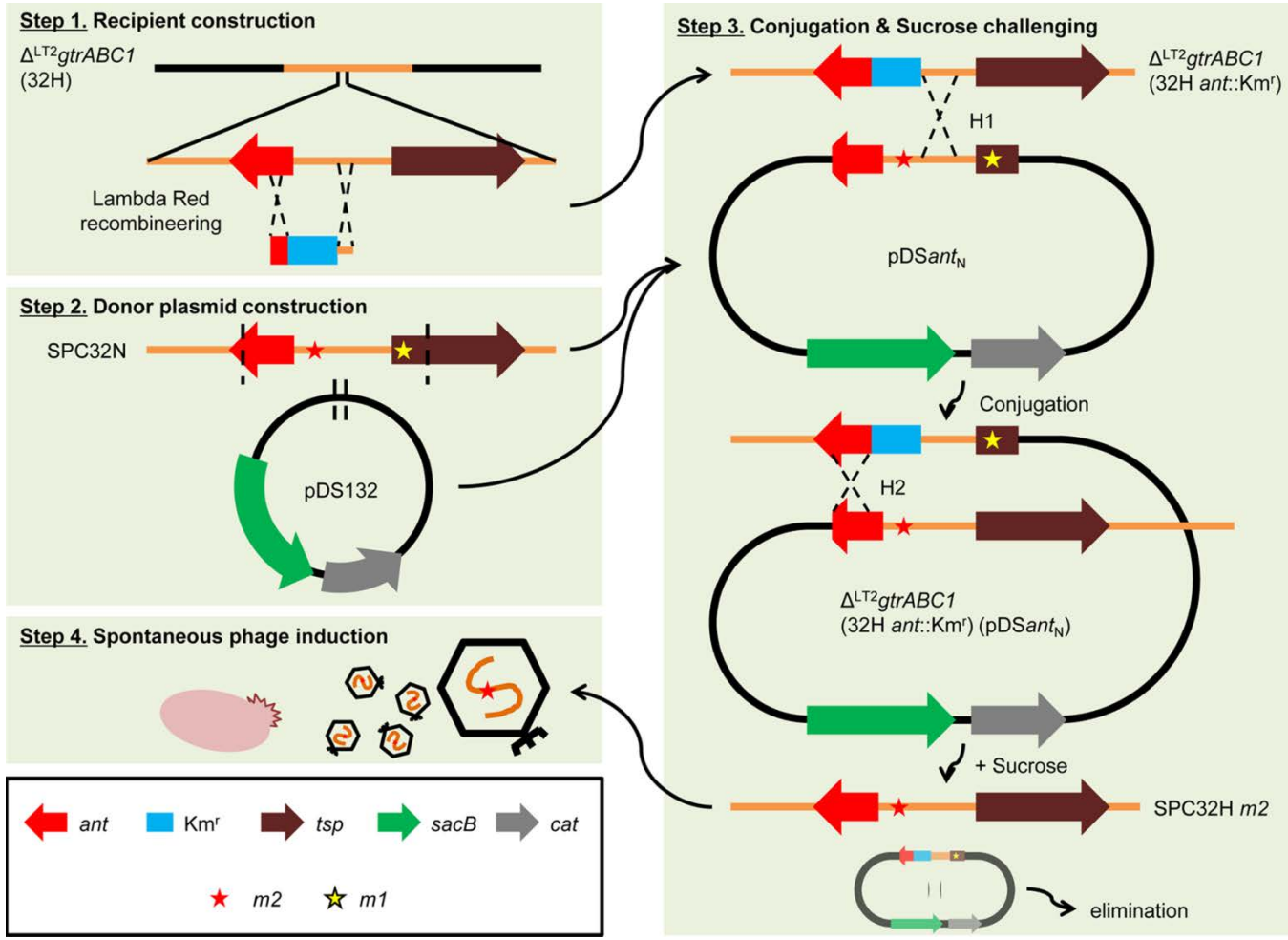

Step 1. Recipient construction
ΔLT2gtrABC1
(32H)
Lambda Red
recombineering
Step 2. Donor plasmid construction
SPC32N
pDS132
Step 4. Spontaneous phage induction
ant
Kmr
tsp
sacB
cat
m2
m1
Step 3. Conjugation & Sucrose challenging
ΔLT2gtrABC1
(32H ant::Kmr)
H1
pDSantN
Conjugation
H2
ΔLT2gtrABC1
(32H ant::Kmr) (pDSantN)
+ Sucrose
SPC32H m2
elimination

Fig. IV-1. Schematic representation of mutant phage construction. The procedures for genetic manipulation for SPC32H *m2* mutant construction are presented. Step 1, construct the conjugal recipient lysogen by lambda Red recombination. Step 2, construct the suicide conjugal donor plasmid that contains the substitution-target region by general cloning. Step 3, conjugate the constructed donor plasmid-containing *E. coli* and the recipient *Salmonella* lysogen, and challenge the selected conjugants with sucrose. Step 4, screen the mutant phages from the culture supernatants. H1, primary homologous recombination; H2, secondary homologous recombination. SPC32H *m12* would be obtained if H1 occurrs downstream of *m1*.

Plasmids construction. The plasmids used in this study are listed in Table IV-3. Plasmid p*oac* expressing the SPC32H *oac* (*O-ac*etyltransferase) gene under the control of the *lac* promoter was constructed as follows. The *oac* gene from extracted SPC32H DNA was PCR amplified using the oligonucleotides 32H-oac-CF4 and 32H-oac-CR4. The purified PCR product was digested with BamHI and HindIII, and cloned into the pUHE21-2 *lacI*^{q} plasmid vector (Soncini *et al.*, 1995), which also digested with the same pair of restriction enzymes. Similar genetic techniques with appropriate oligonucleotides were applied to construct a plasmid p*int*, but vector pBAD24 (Guzman *et al.*, 1995) and enzymes EcoRI and SalI were used for p*rep* and p*ant*. The oligonucleotides 32H-ant*-CF2 instead of 32H-ant-CF1 was used to construct the plasmid p*ant**, which contains an *ant* gene frame-shifted by a single bp (adenine) insertion next to the start codon of *ant*. The plasmid p*lexA* was similarly constructed in a pBAD24 vector backbone using *S.* Typhimurium LT2 gnomic DNA as a PCR template.

The plasmid pP_{ant_H}::*lux* and pP_{ant_N}::*lux* were constructed for the bioluminescence reporter assay. A 197-bp fragment upstream of the *ant* gene in SPC32H (designated P_{ant_H}) or SPC32N (designated P_{ant_N}) was PCR amplified using oligonucleotides 32s-Pant-CF1 and 32s-Pant-CR1, digested with the restriction enzymes SacI and SpeI, and ligated between the SacI and SpeI sites of promoterless *luxCDABE* reporter plasmid pBBRlux (Lenz *et al.*, 2004), resulting in

Table IV-3. Plasmids used in Chapter IV.

Plasmids	Relevant characteristics[a]	Reference or source
Lambda Red recombineering		
pKD46	P_{BAD}-*gam-beta-exo oriR101 repA101*^{ts}; Ap^r	(Datsenko and Wanner, 2000)
pKD13	FRT Km^r FRT PS1 PS4 *oriR6Kγ*; Ap^r	(Datsenko and Wanner, 2000)
pCP20	*cI*857 λP_R*flp oripSC101*^{ts}; Ap^r Cm^r	(Datsenko and Wanner, 2000)
pCE70	FRT *tnpR lacZY*$^+$ *oriR6Kγ*; Km^r	(Merighi *et al.*, 2005)
Gene complementation and/or overexpression		
pUHE21-2 *lacI*^{q}	rep_{pMB1} *lacI*^{q}; Ap^r	(Soncini *et al.*, 1995)
p*rfbP*	pUHE21-2 *lacI*^{q}-*rfbP*; Ap^r	(Kim and Ryu, 2012)
p*oafA*	pUHE21-2 *lacI*^{q}-*oafA*; Ap^r	(Kim and Ryu, 2012)
p^{LT2}*gtrABC1*	pUHE21-2 *lacI*^{q}-^{LT2}*gtrABC1*; Ap^r	(Kim and Ryu, 2012)
p*oac*	pUHE21-2 *lacI*^{q}-*oac*; Ap^r	This study
p*int*	pUHE21-2 *lacI*^{q}-*int*; Ap^r	This study

pBAD24	General expression vector with the P_{BAD} promoter; Ap^r	(Guzman *et al.*, 1995)
p*rep*	pBAD24-*rep*; Ap^r	This study
p*ant*	pBAD24-*ant*; Ap^r	This study
P*ant**	pBAD24-*ant**; encoding frame-shifted *ant*;Ap^r	This study
p*lexA*	pBAD24-*lexA*; Ap^r	This study
Luciferase reporter assay		
pBBRlux	A derivative of broad-host-range cloning vector pBBR1MCS containing a promoterless *luxCDABE*; Cm^r	(Lenz *et al.*, 2004)
pP_{ant_H}::*lux*	pBBRlux with *ant* promoter of SPC32H; Cm^r	This study
pP_{ant_N}::*lux*	pBBRlux with *ant* promoter of SPC32N; Cm^r	This study
Bacterial two-hybrid assay		
pKT25	Encode T25 fragment of adenylate cyclase; Km^r	(Karimova *et al.*, 1998)
pUT18C	Encode T18 fragment of adenylate cyclase; Ap^r	(Karimova *et al.*, 1998)
pKT25-zip	pKT25 with *zip*; Km^r	(Karimova *et al.*, 1998)

pUT18C-zip	pUT18C with *zip*; Ap^r	(Karimova *et al.*, 1998)
pKT25-rep	pKT25 with *rep*; Km^r	This study
pUT18C-ant	pUT18C with *ant*; Ap^r	This study
Protein expression		
pHIS-parallel1	Protein expression vector; allowing N-terminal His_6-tagging with a TEV cleavage site; Ap^r	(Sheffield *et al.*, 1999)
pHIS-LexA	pHIS-parallel1 with *lexA*; Ap^r	This study
pHIS-Rep	pHIS-parallel1 with *rep*; Ap^r	This study
pHIS-Ant	pHIS-parallel1 with *ant*; Ap^r	This study
Subcloning or conjugation		
pGEM-T® easy	PCR product cloning vector; Ap^r	Promega
pDS132	*R6K ori mobRP4 cat sacB*; conjugal suicide vector; Cm^r	(Philippe *et al.*, 2004)
pDS*lexA*(G85D)	pDS132-*lexA*(G85D); Cm^r	This study
pDSant_N	pDS132-ant_N; Cm^r	This study

[a] Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant

the transcriptional fusion of the operon *luxCDABE* to putative promoter of *ant* gene.

To construct the protein expression plasmids pHIS-LexA, pHIS-Rep and pHIS-Ant, the expression vector pHIS-parallel1 containing a recombinant tobacco etch virus (rTEV) protease cleavage site (Sheffield *et al.*, 1999) was used. The *lexA* gene from *S*. Typhimurium LT2 or the *rep* or *ant* gene from SPC32H was amplified via PCR with appropriate oligonucleotides, digested with NcoI and XhoI, and cloned into the same pair of restriction enzyme-digested pHIS-parallel1, resulting in N-terminal His_6 peptide-tagged cloned protein. The oligonucleotides used for plasmid construction are listed in Table IV-2.

Bioluminescence reporter assay. *S*. Typhimurium strains harboring the reporter plasmid were aerobically overnight cultured at 37°C in LB broth supplemented with appropriate antibiotics. The cultures were inoculated (1:100) into 200 μl fresh media in 96-well plate and incubated at 37°C with aeration. The cellular bioluminescence of the culture as well as absorbance at 600 nm (A_{600}) was measured periodically using an Infinite® 200 PRO (Tecan), and the results were expressed in arbitrary relative light units (RLU). To trigger the SOS responses, MMC (1 μg ml^{-1}, final concentration) was added to the culture after a 3-hr incubation. The assays were performed three times independently with triple technical replications.

Western blot analysis. HA epitope-tagged *S*. Typhimurium strains were aerobically cultured in LB broth at 37°C for 2 hr. Incubation was continued after a MMC treatment (1 μg ml^{-1}, final concentration), and portions of the culture (1.5 ml) were sampled at the indicated time points. The cells were harvested by centrifugation at 16,000 x g, 4°C for 2 min, and pellets were immediately frozen at -80°C until lysed with B-PER reagent (Thermo Scientific, USA) according to the manufacturer's instructions. Soluble proteins (10 μg) from the supernatant of cell lysates were mix with 4X Laemmli SDS-sample buffer, boiled for 10 min, and then loaded onto 15% SDS-polyacrylamide slab gel. After electrophoresis, the separated proteins were electro-transferred to PVDF membrane. The membrane was blocked with TBST buffer (10 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20, adjusted pH 8.0) supplemented with 0.45% skim milk using a SNAP i.d.™ Protein Detection System (Millipore), and then the membrane was incubated for 10 min with a TBST buffer supplemented with primary anti-HA or anti-DnaK antibodies. After 3 washes with 20 ml TBST buffer, secondary antibody solution [TBST buffer supplemented with 0.45% skim milk and the goat anti-mouse IgG-HRP (Santa Cruz Biotechnology)] was applied for 10 min, and another wash with TBST buffer was performed. The chemiluminescence signals were developed with the WEST-ZOL® plus Western Blot Detection System (iNtRON Biotechnology, South Korea) according to the manufacturer's instructions, and then X-ray film was exposed to

the light to detect the signals.

Bacterial two-hybrid assay. The protein-protein interaction was determined by the recovery of the adenylate cyclase (CyaA) activity through heterodimerization of fusion proteins. The gene *rep* was PCR amplified from the genomic DNA of the phage SPC32H using the oligonucleotides pair pKT25-32H-rep-CF1/pKT25-32H-rep-CR1. The resulting PCR product was digested with PstI and BamHI, and ligated between the PstI and BamHI sites of pKT25 to fuse Rep at the C-terminal end of T25 fragment, generating pKT25-rep. To construct other fusion plasmids (i.e., pUT18C-ant, pKT25-ant and pUT18C-rep), the same procedures with the appropriate oligonucleotide pairs and backbone plasmids (pKT25 or pUT18C) were conducted. *Escherichia coli* BTH101 reporter strain (*cyaA*$^{-}$) was co-transformed with the combinations of the fusion plasmids (e.g., pKT25-rep and pUT18c-ant), and positive transformants were selected on LB agar containing Km and Ap. Strain BTH101 was also co-transformed with pKT25/pUT18C or pKT25-zip/pUT18C-zip plasmids pair as negative or positive control, respectively. The transformants were streaked on LB agar supplemented with Km, Ap and X-gal to determine the Rep-Ant interaction by monitoring of colony color. Otherwise, the interaction was quantitatively examined using a β-galactosidase assay according to the previously described methods (Kim and Ryu, 2012), and the results were

expressed in Miller units (Miller, 1972).

Purification of proteins, rTEV protease treatment and analytical size-exclusion chromatography. The *E. coli* BL21 (DE3) harboring pHIS-LexA, pHIS-Rep or pHIS-Ant was aerobically grown to an OD_{600} = ~ 0.15 at 37°C, and then expression of His_6-tagged *S*. Typhimurium LexA or SPC32H Rep or Ant protein was induced with 100 μM IPTG. After additional 4 hr incubation at 25°C, the cells were harvested and washed once with ice-cold PBS. The cells were resuspended in 5-ml of lysis buffer (20 mM Tris-Cl, 500 mM NaCl and 20 mM imidazole, adjusted to pH 8.0) and lyzed by sonication on ice, centrifuged at 16,000 x g for 30 min at 4°C, and then the filtered (0.22 μm-pore-size filter; Millipore) supernatant was subjected to lysis buffer-equilibrated nickel chelated nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen, CA, USA). After incubation with gentle agitation at 4°C for 1 hr, protein-bound Ni-NTA resin was washed thrice with 10 times the resin volume of washing buffer (20 mM Tris-Cl, 500 mM NaCl and 30 mM imidazole, adjusted to pH 8.0). The His_6-tagged target proteins were eluted with elution buffer (20 mM Tris-Cl, 500 mM NaCl and 250 mM imidazole, adjusted to pH 8.0) and concentrated with Vivaspin 20 (3,000 MWCO PES; Sartorius Stedim Biotech, Germany) according to the manufacturer's instructions. The buffer was changed to a storage buffer (20 mM Tris-Cl, 500 mM

NaCl and 50% glycerol) using PD midiTrap^{TM} G-25 (GE healthcare, UK) according to manufacturer's instructions, and then aliquots of proteins were stored at -80°C, until further uses.

To remove the His_6 tag from the purified proteins, rTEV protease (1:5 ratios in concentration) was added at 4°C for 6 hr in cleavage buffer (10 mM Tris-Cl, 150 mM NaCl, 0.5 mM EDTA, 100 mM DTT, adjusted to pH 8.0). The His_6-tag-free proteins in the flow-through fraction of the Ni-NTA affinity chromatography were concentrated, buffer changed, and stored, as above.

For analytical size-exclusion chromatography of SPC32H Rep and Ant, a Superdex 200 10/300 GL column (GE Healthcare, USA) was used. The column was equilibrated with a buffer consisting of 500 mM NaCl and 20 mM Tris-HCl pH 8.0, and then a purified His_6-Rep or His_6-Ant protein (500 μl of 0.8 μg μl^{-1}) was loaded on to the column at a flow rate of 0.5 ml min^{-1}.

Electrophoretic mobility shift assay (EMSA). The 313-bp *ant* gene promoter region (APR), containing the putative repressor-binding site as well as LexA-binding site, was PCR amplified from SPC32H or SPC32N using the oligonucleotides 32s-APR-CF1 and 32s-APR-CR1, and the resulting PCR product was labeled with γ-^{32}P using T4 polynucleotide kinase (Takara Bio Inc., Japan) according to manufacturer's instructions. The labeled DNA fragment

(approximately 4 nM) was incubated with varying concentrations of purified LexA for 30 min at 37°C in a 20-µl reaction mixture containing 1 X binding buffer [10 mM HEPES-NaOH (pH 8.0), 10 mM Tris-Cl (pH 8.0), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 5% glycerol] and 1.1 µg of poly(dI-dC) (Sigma). For determination of Rep binding to labeled DNA, various amounts of purified Rep was incubated for 15 min at 20°C with the 4 nM of labeled DNA fragment in the 20-µl reaction mixture. When appropriate, Rep was pre-incubated with various concentrations of Ant for 30 min at 20°C prior to the incubation with labeled DNA as above. The samples were electrophoresed at 100 V, 4°C for 1.5 hr on a 6% native polyacrylamide slab gel (29:1 acrylamide:bisacrylamide), which was pre-ran at 200 V, 4°C for 7.5 min in 0.5 X TBE buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.3). The gels were vacuum-dried, and the radioactivity was analyzed using BAS2500 system (Fuji film).

IV-3. Results

Phenotypic differences between SPC32H and SPC32N. Previously, I isolated nine phages specific for *Salmonella enterica* serovar Typhimurium or *Eschrichia coli* from several chicken fecal samples (Kim and Ryu, 2011). Among these phages, two phages, originating from a same sample collection, exhibited discriminative plaque morphologies on host *S.* Typhimurium lawn: one phage (SPC32H) formed clear plaques surrounded by a halo, but the other (SPC32N) formed clearer plaques without a halo (Fig. IV-2A). However, transmission electron microscopy (TEM) analysis revealed that both phages belonged to the family *Podoviridae* and possessed an icosahedral head with a short tail (Fig. IV-2B). The restriction fragment length polymorphism (RFLP) analysis of two phage genomes with EcoRV and NdeI were indistinguishable (Fig. IV-3A), suggesting that SPC32H and SPC32N might be genetically close. In addition, the host ranges of these two phages were identical (Table IV-4), and both phages use the O-antigens on *Salmonella* surface as the host receptor (Fig. IV-3B). To determine the factor(s) causing the differences in plaque morphology between SPC32H and SPC32N despite several common features, I sequenced the whole genomes of both phages.

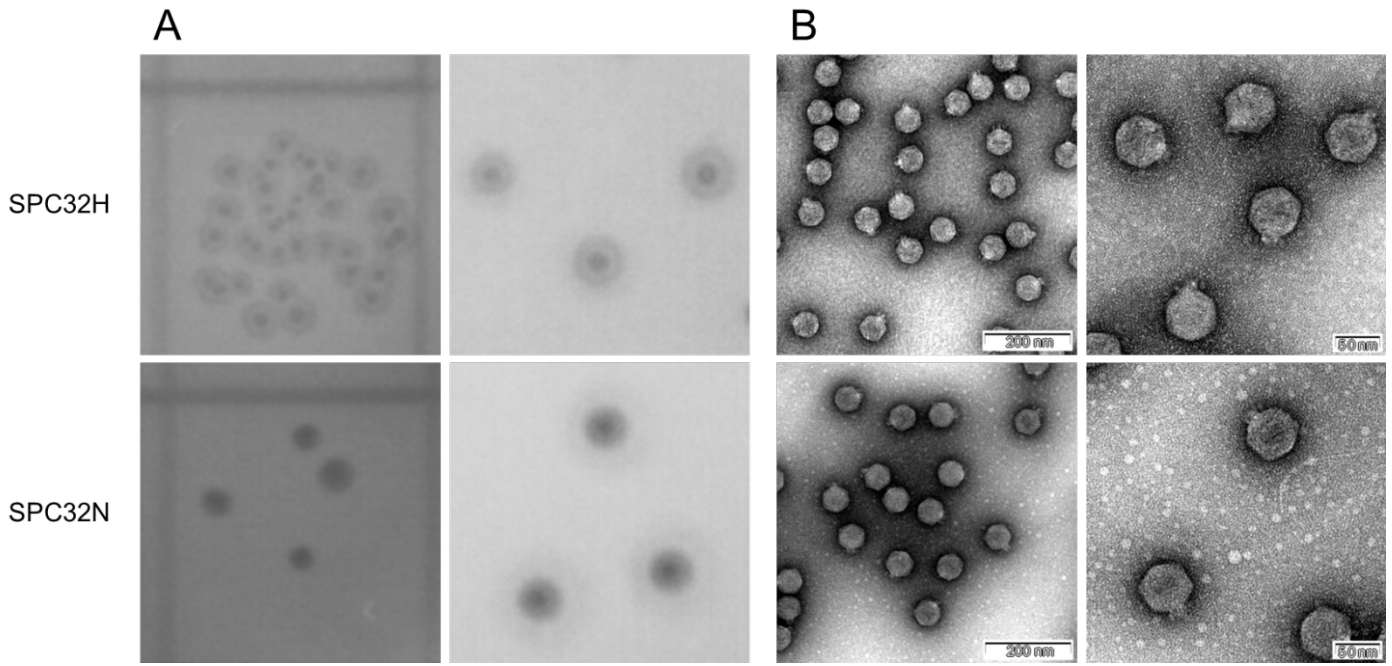


Fig. IV-2. The phages SPC32H and SPC32N, belong to the family *Podoviridae*, produce morphologically distinct plaques.

A. Plaque morphology of SPC32H (upper panel) and SPC32N (lower panel). Dilutions (10 μl) of each phage stock were spotted on a lawn of *S*. Typhimurium LT2(c) (left panel) or the strain Δ^{LT2}*gtrABC1* (right panel).

B. TEM images of SPC32H (upper panel) and SPC32N (lower panel). A scale bar is at the right-bottom corner of each image.

Table IV-4. Host range of SPC32H and SPC32N

Host	Phage plaques[a]	
	SPC32H	SPC32N
Salmonella Typhimurium		
LT2	C	C
UK1	C	C
SL1344	C	C
14028s	C	C
ATCC 19586	C	C
ATCC 43174	C	C
DT104	C	C
S. Enteritidis		
ATCC 13078	C	C
S. Typhi		
Ty2-b	-	-
S. Paratyphi		
A 1B 211	-	-
B 1B 231	T	T
C 1B 216	T	T
S. Dublin		
1B 2973	-	-
S. enterica subsp. *arizonae*		
KCCM 41035	-	-
KCCM 41575	-	-
KCCM 41651	-	-
S. enterica subsp. *indica*		
KCCM 41759	-	-
S. enterica subsp. *houtenae*		
KCCM 41760	-	-
S. enterica subsp. *diarizonae*		
KCCM 41761	-	-
S. enterica subsp. *salamae*		
KCCM 41762	-	-
S. enterica isolates		
3068	T	T

3605	-	-
3792	-	-
4509	T	T
Escherichia coli		
MG1655	-	-
MC4100	-	-
DH5α	-	-
DH10B	-	-
O157:H7 ATCC 35150	-	-
O157:H7 ATCC 43888	-	-
O157:H7 ATCC 43890	-	-
O157:H7 ATCC 43894	-	-
O157:H7 ATCC 43895	-	-
O157:NM 3204-92	-	-
O157:NM H-0482	-	-

a, C, clear plaques; T, turbid plaques; -, no plaques.

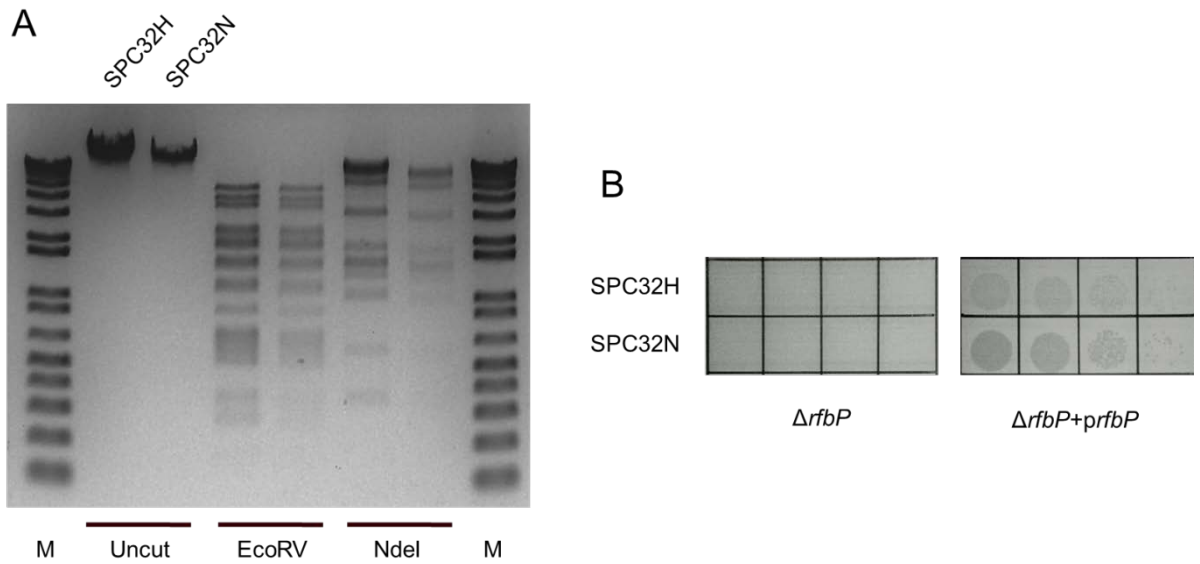


Fig. IV-3. Identical properties of SPC32H and SPC32N.

A. Genomes of SPC32H and SPC32N exhibit the same RFLP patterns. Each phage's genomic DNA was extracted, digested with EcoRV or NdeI at 37°C for 2 hr, and then electrophoresed in a 1% agarose gel along with non-digested DNA. The inverse image of EtBr-stained gel is presented. M, 1 kb^+ DNA ladder (Invitrogen).

B. Both SPC32H and SPC32N utilize the *Salmonella* O-antigens as host receptor. Ten-fold serial dilutions (10 μl) of each phage were spotted on a lawn of Δ*rfbP* mutant (SR5016; absence of O-antigens) or a complementation strain (SR5018, *rfbP* gene under the *lac* promoter was induced with 1 mM.IPTG).

Two SNPs between SPC32H and SPC32N genome. The genomic DNA of SPC32H and SPC32N were extracted with a phenol/chloroform extraction method (Sambrook and Russell, 2001) and completely sequenced by pyrosequencing. The quality filter-through reads were assembled into one contig with a 38,689-bp length for both phages, supporting the genomic resemblance. The double-stranded genomic DNA contained a G+C content of 50.16% and 51 predictable open reading frames (ORFs) with one tRNA coding sequence for arginine. About half of the ORFs (24 ORFs) were annotated as hypothetical proteins, whereas the other specifically annotated proteins were classified into the following modules: DNA packaging, virion structure morphogenesis, lysogenic conversion, host lysis, and DNA replication/recombination (Fig. IV-4). A phage integrase as well as a putative repressor were predicted in the genome, indicating that both phages might be temperate phages. BlastP searches revealed that the genomes of SPC32H and SPC32N highly resembled *Salmonella* phage ε15 and other ε15-like phages (Kropinski *et al.*, 2007). Indeed, whole genome comparisons made at the DNA level revealed a significant degree of synteny between genomes of SPC32H, ε15, and ε15-like phage phiV1 (Fig. IV-4), supporting assignment of SPC32H and SPC32N in the ε15-like phages.

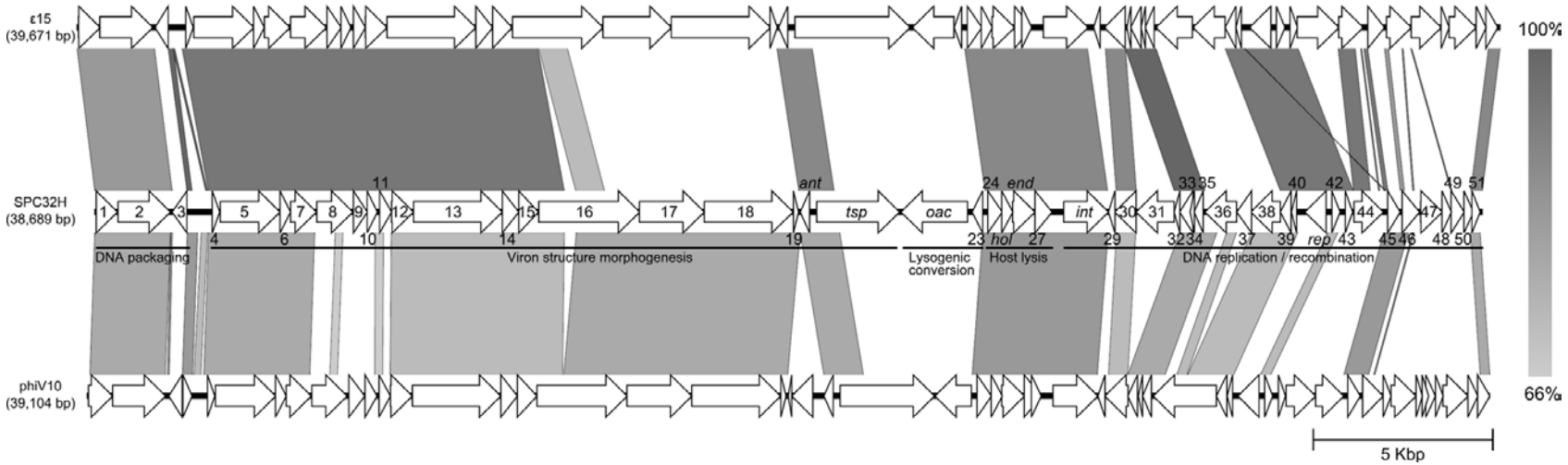
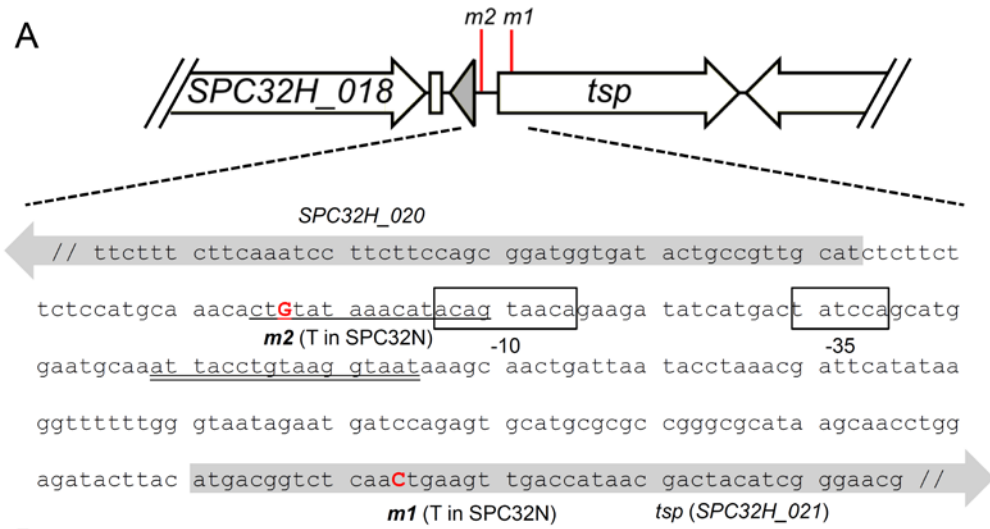


Fig. IV-4. DNA-level alignment of the genomes of phage ε15 (NC_004775.1), SPC32H, and phiV10 (NC_007804.2) using Easyfig. High DNA sequence similarity between the genomes is indicated by gray regions. ORFs of SPC32H are indicated by numbered or annotated arrows. Phage functional modules are indicated under the arrows. *ant*, anti-repressor; *tsp*, tailspike; *oac*, *O*-acetyltransferase; *hol*, holin; *end*, endolysin; *int*, integrase; *rep*, repressor. Note that the genome of SPC32N was identical to that of SPC32H except the two single nucleotide differences (see Fig. IV-5A).

Interestingly, a comparison of the whole genome sequences of SPC32H and SPC32N revealed only two nucleotide differences. One SNP, *m1*, was located within the *tsp* gene (*SPC32H_021*) encoding phage the tailspike, and the other SNP, *m2*, was located in the intergenic region between the gene (*SPC32H_020*) encoding a hypothetical protein and the *tsp* gene (Fig. IV-5A). Therefore, relations between these two SNPs and the phenotypic difference in SPC32H and SPC32N were investigated in detail.

***m1* in *tsp* gene is not responsible for the phenotypic differences.** As described above, *m1* was located within the coding sequence (CDS) for tailspike while *m2* in a non-coding region, allowing me to assume that *m1*, rather than *m2*, might affect the phenotypes of these two phages. This assumption was further supported by the fact that *m1* was a non-synonymous SNP; the 5^{th} codon from the start codon of tailspike protein in SPC32H encodes a threonine whereas that in SPC32N encodes an isoleucine (Fig. IV-5B). Since the tailspike protein is one of the virion structural proteins that involved in the recognition of host receptors, I hypothesized that the different phage adsorption ability owing to the differential tailspike structure might cause the distinct lysis zone turbidity/plaque morphology between SPC32H and SPC32N.

A



B

Tailspike

	atg	acg	gtc	tca	aCt	gaa	gtt	gac	cat	aac	gac	tac	atc	//
SPC32H	M	T	V	S	**T**	E	V	D	H	N	D	Y	I	//
	atg	acg	gtc	tca	a**T**t	gaa	gtt	gac	cat	aac	gac	tac	atc	//
SPC32N	M	T	V	S	**I**	E	V	D	H	N	D	Y	I	//

C

LexA-binding site (SOS box)

E. coli	**CTG**nnnnnnnnn**CAG**
SPC32H	**CTG**tatgtttata**CAG**
SPC32N	**CTG**tatgtttata**AAG**
ε15	**CTG**tatgtttata**CAG**
phiV10	**CTG**tgtatttata**CAG**

Fig. IV-5. There are two nucleotide differences between genomes of SPC32H and SPC32N.

A. Schematic representation of the location of two nucleotide differences, *m1* and *m2*. The partial SPC32H genome sequence around the two nucleotide differences is presented. *m1* (located within the *tsp* gene) and *m2* (located at the intergenic region between the *SPC32H_020* gene and *tsp* gene) are indicated using red and upper-case letters. The predicted -10 and -35 sites of the putative promoter for *SPC32H_020* gene are boxed. The putative LexA-binding site (SOS box) and putative repressor-binding site are underlined and double-underlined, respectively.

B. 5'-nucleotide sequence for *tsp* gene of SPC32H and SPC32N, and the relevant amino acid sequence for tailspike protein. *m1* as well as the corresponding amino acid polymorphism were indicated as red and upper-case letters.

C. Consensus sequence of the LexA-binding site from *E. coli* (Fernandez De Henestrosa *et al.*, 2000; Lewis *et al.*, 1994b; Little and Mount, 1982) and putative LexA-binding sites from ε15-like phages SPC32H, SPC32N, ε15 (GenBank accession number NC_004775.1), and phiV10 (NC_007804.2). *m2* is indicated in red and upper-case letters. Note that the represented LexA-binding site sequence of SPC32H and SPC32N are reverse-complements to that in panel A.

Previously, I revealed that the structural modification of *Salmonella* lipopolysaccharides (LPS), which occurred via phase variable expression of O-antigen glucosylating genes ^{LT2}*gtrABC1*, causes a dramatic increasing of resistance against *Salmonella* phage SPC35 (Kim and Ryu, 2012). I speculated that the similar phenomenon might affect the infectivity of O-antigen-specific phage SPC32H and SPC32N. I hypothesized, however, SPC32N could escape this resistant mechanism by recognizing the modified O-antigens as well as unmodified O-antigens via the tailspike protein variant, such that produce clearer plaques than SPC32H. This hypothesis was checked by phage spotting assay on lawns of various *Salmonella* strains possessing modified O-antigens. However, the ^{LT2}*gtrABC1* overexpression strain that produces O12-2-antigens formed extremely turbid plaques of both SPC32H and SPC32N, and the *oafA* overexpression strain that produces O5-antigens exhibited the same lysis patterns to WT (clear with halo and clearer without halo by SPC32H and SPC32N, respectively) (Fig. IV-6). These results indicated that SPC32H and SPC32N equally recognize the O5-antigens but not the O12-2-antigens. Because ^{LT2}*gtrABC1* and *oafA* genes were encoded within the *Salmonella* chromosome, I next checked the effect of phage-originated O-antigen modification. SPC32H (and SPC32N) contains the *O*-acetyltransferase gene (*oac*) in the lysogenic conversion module (Fig. IV-4) to prevent the superinfection of other phages. Thus, there was another possibility that SPC32N could recognize

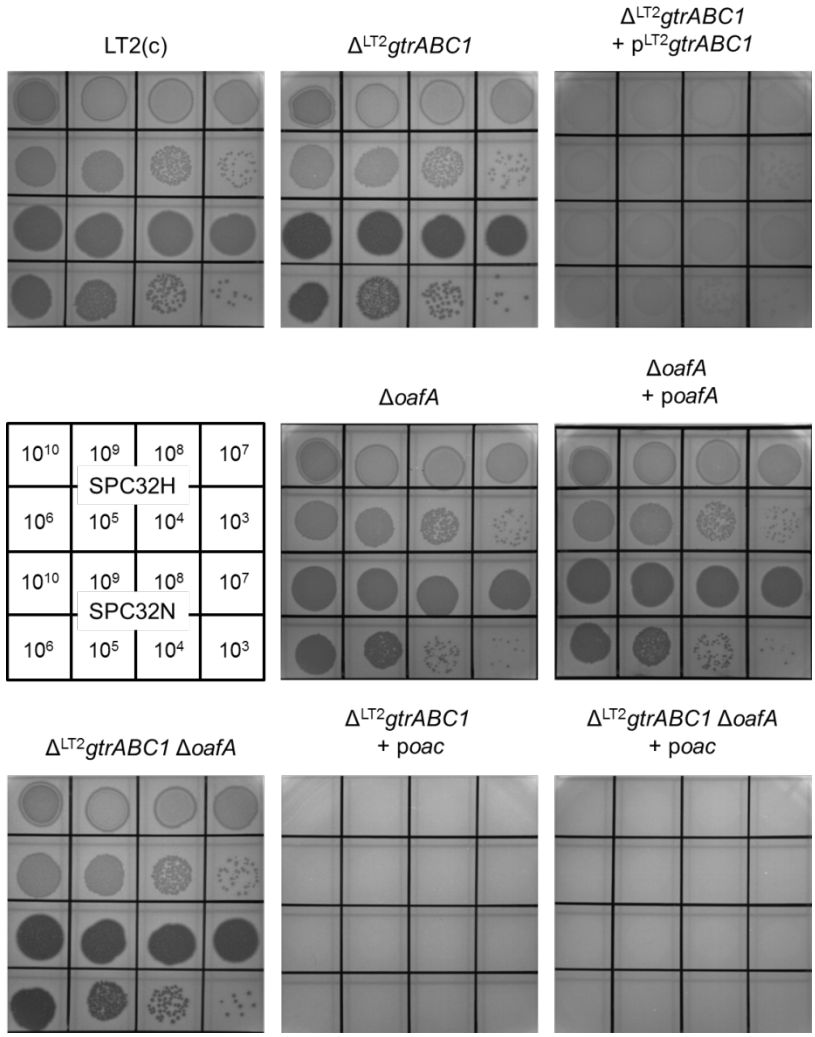


Fig. IV-6. O-antigen-recognition spectrums of SPC32H and SPC32N are equal despite the *m1* in *tsp* gene. Ten-fold serial dilutions (10 μl) of each phage were spotted on the following *Salmonella* strains: LT2(c), wild-type strain; Δ^{LT2}*gtrABC1*, O12-2-antigen-deficient strain; Δ^{LT2}*gtrABC1*+p^{LT2}*gtrABC1*, O12-2-antigen-overexpressing strain; Δ*oafA*, O5-antigen-deficient strain; Δ*oafA*+p*oafA*, O5-antigen-overexpressing strain; Δ^{LT2}*gtrABC1* Δ*oafA*, both O12-2- and O5-antigen-deficient strain; Δ^{LT2}*gtrABC1*+p*oac* and Δ^{LT2}*gtrABC1* Δ*oafA*+p*oac*, *O*-acetylated-antigen-overexpressing strains. Phage name and approximate titer in each grid were indicated at the middle left boxes.

both the normal O-antigens generated by WT *Salmonella* and the *O*-acetylated O-antigens by lysogens via the variant tailspike protein, so that superinfect and lyse the lysogenized host with clearer plaques. However, no plaques were produced by both phages on the *oac* overexpression strains (Fig. IV-6). Altogether, these results suggested that SPC32H and SPC32N equivalently utilize the O-antigens as the host receptor, rather than SPC32N could recognize more various O-antigens. Therefore, the differential plaque forming by SPC32H and SPC32N might be originated from other factor(s) than *m1*.

Repressor complements the lysogenic development of lytic cycle-biased SPC32N. It is well known that lysogen formation is normally associated with plaque morphology. The lysogenization ability of SPC32H and SPC32N was investigated with a primer pair that could specifically amplify the right end of the phage genome attachment site (*attR* site) in the lysogen. Integrases of SPC32H and ε15 share a 93% identity in amino acid level and both phages contain the highly conserved common core regions and arm-type binding sequences that are required for phage genome integration (Kropinski *et al.*, 2007), suggesting that both phages may integrate their genome into the same attachment site, near the end of *Salmonella guaA* gene. Therefore, a primer pair consisting of a forward primer annealing to the upstream region of phage's integrase gene (*int*) and a reverse

primer annealing within the *guaA* gene was used for PCR to determine the lysogen formation by SPC32H or SPC32N. The specific *attR* band was amplified with DNA from the SPC32H lysis zone, whereas no band appeared with DNA from the SPC32N lysis zone (Fig. IV-7A). The putative lysogen of SPC32H [Δ^{LT2}*gtrABC1* (32H)] purified from the SPC32H lysis zone also generated the specific band through colony or genomic DNA PCR, but none of the DNA from the parental *Salmonella*, SPC32H, and SPC32N displayed the specific PCR band (Fig. IV-7A). Furthermore, the purified SPC32H lysogen spontaneously produced phages that formed the halo-plaques (Fig. IV-7B) during a prolonged incubation. These results clearly demonstrate that host *Salmonella* can be lysogenized by SPC32H but not by SPC32N.

Accordingly, I hypothesized that this differential lysogenization ability might be due to the some problem(s) during the recombination between *Salmonella* chromosome and SPC32N genome. As shown in Fig. IV-8A, the SPC32N plaques on the integrase-overexpression strain still clearer than SPC32H plaques, and produce no PCR band of *attR* site, indicating that supplementation of integrase could not complements the lysogenic development of SPC32N. Curiously, the deletion of integrase gene from the SPC32H eliminated the plaque's halo although the clarity of lysis zone was still significant than SPC32H (Fig. IV-8B). It suggested that the halo of SPC32H plaque was an outcome of the lysogenization,

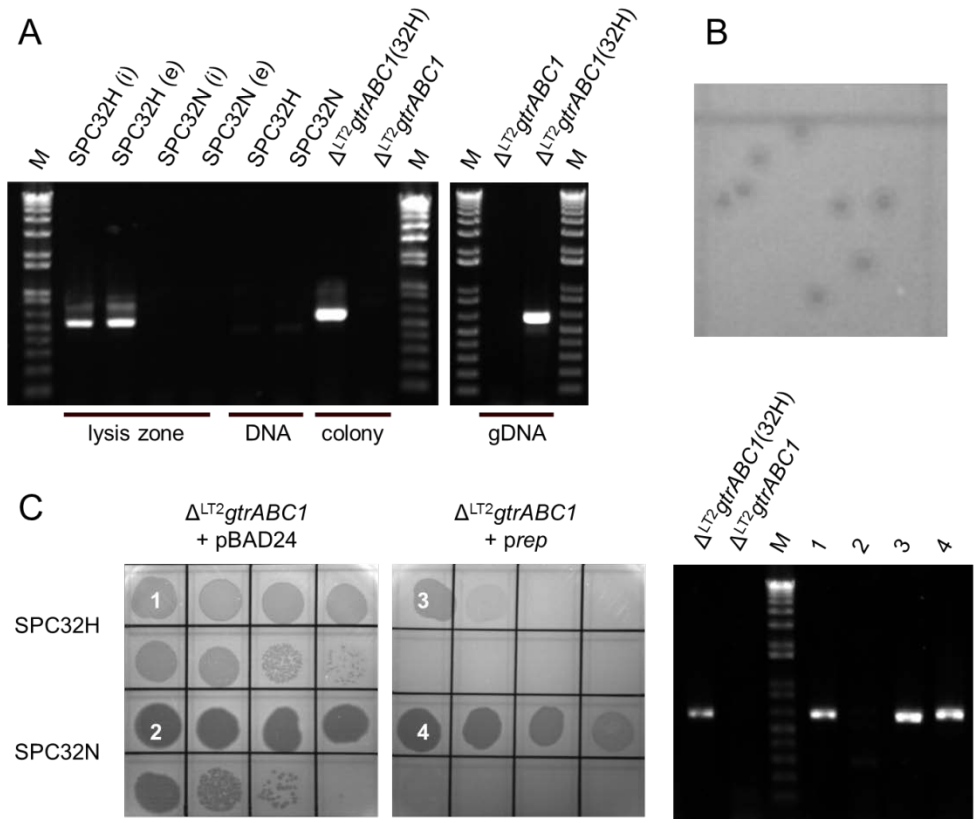


Fig. IV-7. The supplementation of putative repressor leads the lysogenic development of the lytic cycle-biased SPC32N.

A. SPC32H can lysogenize host *Salmonella*, whereas SPC32N is biased to the lytic cycle. Various template samples were PCR amplified with an *attR* site-specific primer pair. i, inner part of lysis zone; e, edge of lysis zone; gDNA, genomic DNA.

B. Halo-forming plaques of the spontaneously induced phages from the SPC32H lysogen. Supernatant of overnight-cultured Δ^{LT2}*gtrABC1* (32H) was diluted and spotted on the Δ^{LT2}*gtrABC1* lawn. Note that the plaque morphology was similar to SPC32H plaques (Fig. IV-2A).

C. SPC32N lysogenizes the host *Salmonella* that expresses the putative repressor. *Salmonella* strains transformed with a putative repressor-overexpressing plasmid (p*rep*) or control plasmid (pBAD24) were infected by both SPC32H and SPC32N, and the samples from each indicated lysis zone were subjected to the PCR amplification for *attR* site. Note that no arabinose was added for the *rep* expression.

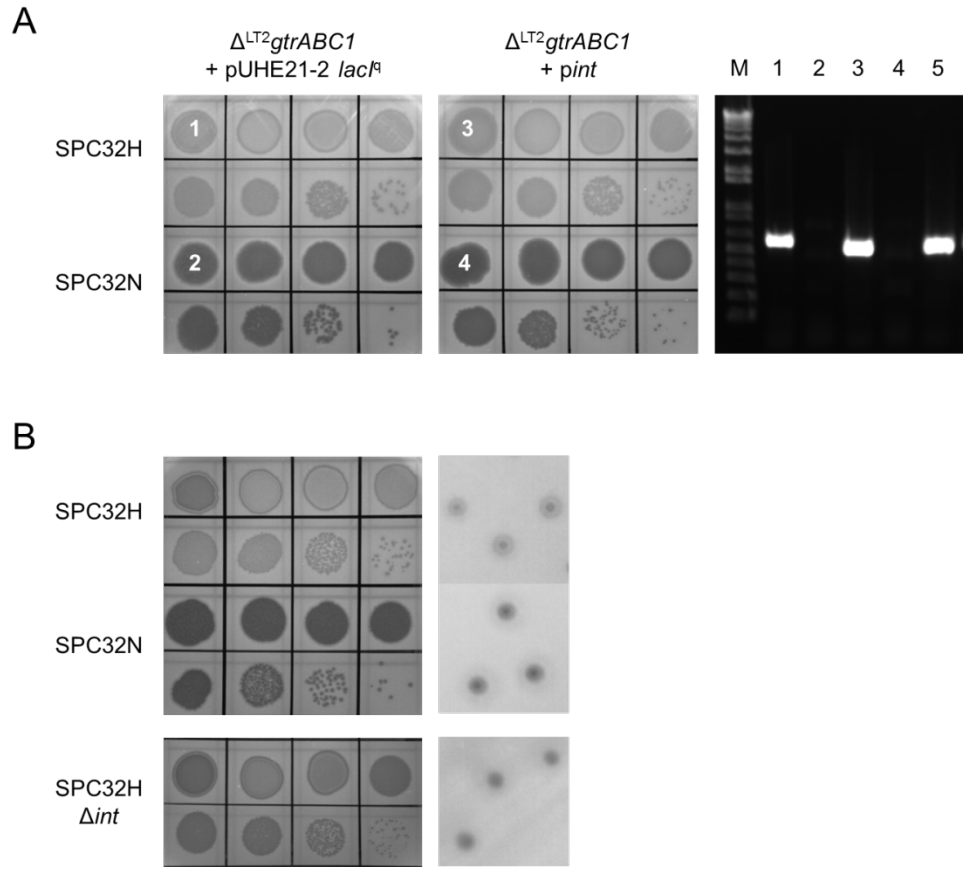


Fig. IV-8. SPC32N is deficient in the lysogenic development and beyond.

A. Serially diluted SPC32H and SPC32N were spotted on the lawns of *Salmonella* strains that transformed with a phage integrase-overexpressing plasmid (p*int*) or control plasmid (pUHE21-2 *lacI*^{q}), and samples from each indicated lysis zone were subjected to the *attR* site-specific PCR. For the *int* expression, 1 mM IPTG was supplemented. Lane 5, Δ^{LT2}*gtrABC1*(32H), a SPC32H lysogen.

B. Comparison of lysis zone turbidity (left) and plaque morphology (right) of the *int* gene-deleted SPC32H (SPC32H Δ*int*) and parental SPC32H and SPC32N. Each phage dilutes were spotted on the Δ^{LT2}*gtrABC1* lawn. The images of lysis zones and single plaques for SPC32H and SPC32N are reproduced from Fig. IV-6 and IV-2A, respectively, for comparison. Although the plaque halo was disappeared, lysis zone was not cleared significantly by *int* gene deletion.

and the clearer, non-halo plaque of SPC32N was resulted from the deficient not only in lysogenic development but also beyond of that, such as incapable of lytic genes repression.

Because the phage repressor plays a critical role in the maintenance of lysogenic state by repressing the expression of lytic genes and both phages have a putative repressor gene, I checked the repression deficiency of SPC32N. The putative repressor gene (*rep*) of SPC32H under an arabinose-inducible promoter was introduced into the host *Salmonella*, and SPC32H and SPC32N were spotted on the lawns of the constructed strain with or without arabinose. Interestingly, the EOP (efficiency of plating) of both phages was reduced significantly ($<10^{-5}$ for SPC32H and $<10^{-2}$ for SPC32N) and the lysis zones were more turbid than the control, even without the arabinose induction (Fig. IV-7C). Moreover, the specific PCR band of the *attR* site appeared with the lysis zone of SPC32N as well as SPC32H, suggesting that the supplementation of repressor can assist the lysogenic development in SPC32N. These results imply that SPC32N was defective in the maintenance of lysogeny due to an insufficient amount of the active repressor. Curiously, the induction of the *rep* gene with 0.01% of arabinose resulted in cell lysis, indicating that an excessive foreign phage repressor is harmful to host *Salmonella* (Miroux and Walker, 1996), and thus inferring a need for precise control of repressor expression and/or its activity in the lysogen.

Novel anti-repressor encoded by SPC32H_020 governs the lytic switch. The location of the second SNP, *m2*, was 24-bp upstream of the start codon of hypothetical protein SPC32H_020, suggesting the possibility that *m2* causes the phenotypic differences by affecting the expression of this hypothetical protein. The SPC32H_020 is a small protein consisting of 86 amino acids, and a BlastP search of proteins displaying high homology with SPC32H_020 revealed no protein with known function but did reveal 40 hypothetical proteins without any known function with more than 64% of identity. These include proteins from the *Enterobacteriaceae* family bacteria, such as *E. coli*, *Salmonella* spp., *Klebsiella* spp., *Citrobacter* spp., and *Cronobacter* spp. and from the ε15-like phages including ε15, TL-2011b, phiV10, SPN1S, and SPN9TCW (Table IV-5). Interestingly, some larger size-proteins (>218 amino acids) with a relatively low identity (<42%) were annotated as putative anti-repressors, suggesting the possibility of an anti-repressor role for SPC32H_020.

The phage anti-repressor binds to and interferes with its cognate repressor, resulting in the activation of the lytic switch following prophage induction. To determine whether SPC32H_020 functions in the lytic switch as an anti-repressor, a *Salmonella* strain harboring an SPC32H without *SPC32H_020* [designated Δ^{LT2}*gtrABC1* (32H Δ*ant*)] was constructed, and the efficiency of prophage

Table IV-5. SPC32H_020 resembling proteins from BlastP

Accession number	Annotation	Strains	Length (amino acids)	E-value	Max identity (%)
Hypothetical proteins from bacteriophages					
NP_848227.1	hypothetical protein ε15p19	Enterobacteria phage ε15	86	2e-54	95
AEW24598.1	hypothetical protein	*Escherichia* phage TL-2011b	85	2e-48	88
YP_512278.1	hypothetical protein PhiV10p24	*Escherichia* phage phiV10	85	9e-47	88
YP_005097996.1	unnamed protein product	*Salmonella* phage SPN1S	127	7e-56	100
AFH20853.1	hypothetical protein SPN9TCW_020	*Salmonella phage* SPN9TCW	54	9e-31	100
Hypothetical proteins from bacteria					
ZP_22998610.1	hypothetical protein H262_19093	*Citrobacter freundii* GTC 09479	86	2e-56	100
YP_002330238.1	hypothetical protein E2348C_2740	*Escherichia coli* O127:H6 str. E2348/69	86	6e-56	99
ZP_13804664.1	hypothetical protein ECDEC10B_3832	*Escherichia coli* DEC10B	54	8e-29	94
ZP_11928201.1	hypothetical protein ECSTEC7V_3016	*Escherichia coli* STEC_7v	54	5e-29	93
ZP_11966801.1	hypothetical protein EC12741_1213	*Escherichia coli* 1.2741	54	3e-28	92
YP_006134832.1	hypothetical protein UMNK88_3111	*Escherichia coli* UMNK88	54	3e-28	92
ZP_12285649.1	hypothetical protein ECSTECS1191_3363	*Escherichia coli* STEC_S1191	54	2e-28	91
ZP_14801383.1	hypothetical protein ECTW09109_3808	*Escherichia coli* TW09109	54	1e-27	91
ZP_11642153.1	hypothetical protein SDB_02389	*Shigella dysenteriae* CDC 74-1112	54	6e-27	90

ZP_13401462.1	hypothetical protein SEEN443_21095	*Salmonella enterica* subsp. *enterica* serovar Newport str. CVM 19443	86	8e-50	90
ZP_20163457.1	hypothetical protein A15S_00476	*Escherichia coli* KTE209	85	8e-49	89
YP_006141911.1	hypothetical protein ECNA114_4788	*Escherichia coli* NA114	85	4e-48	89
ZP_16460384.1	hypothetical protein HMPREF9532_01728	*Escherichia coli* MS 57-2	90	1e-47	88
ZP_07189897.1	conserved hypothetical protein	*Escherichia coli* MS 196-1	85	1e-47	88
ZP_19751991.1	hypothetical protein A17M_02631	*Escherichia coli* KTE224	85	2e-47	88
ZP_17680965.1	hypothetical protein ESTG_01058	*Escherichia coli* B799	85	2e-47	88
YP_002408599.1	hypothetical protein ECIAI39_2658	*Escherichia coli* IAI39	126	2e-47	88
ZP_07116787.1	conserved hypothetical protein	*Escherichia coli* MS 198-1	126	3e-47	88
ZP_19839339.1	hypothetical protein A1U5_02945	*Escherichia coli* KTE66	85	8e-47	88
ZP_19755740.1	hypothetical protein A17U_01523	*Escherichia coli* KTE228	85	1e-46	87
EGP24357.1	hypothetical protein PPECC33_23630	*Escherichia coli* PCN033	85	4e-47	87
EHV05286.1	hypothetical protein ECDEC4D_3231	*Escherichia coli* DEC4D	85	6e-47	87
ZP_06991300.1	conserved hypothetical protein	*Escherichia coli* FVEC1302	91	1e-46	87
YP_853609.1	hypothetical protein APECO1_4057	*Escherichia coli* APEC O1	126	1e-46	87
ZP_08349251.1	conserved hypothetical protein	*Escherichia coli* M605	85	2e-46	87
ZP_22007930.1	hypothetical protein TW14425_RS05350	*Escherichia coli* TW14425	85	2e-45	86
EJA85653.1	hypothetical protein SEEN470_12756	*Salmonella enterica* subsp. *enterica* serovar Newport str. CVM 19470	86	1e-45	81
ZP_19162567.1	hypothetical protein BN132_608	*Cronobacter turicensis 564*	64	5e-20	67
ENY54520.1	hypothetical protein C210_30672	*Klebsiella pneumoniae* subsp. *pneumoniae* KpMDU1	86	1e-32	65

ZP_23428121.1	hypothetical protein MTE1_1400	*Klebsiella pneumoniae* JHCK1	86	1e-32	65
ZP_08305916.1	hypothetical protein HMPREF9538_03605	*Klebsiella* sp. MS 92-3	98	1e-32	65
EJJ32557.1	hypothetical protein KPNIH1_26325	*Klebsiella pneumoniae* subsp. *pneumoniae* KPNIH1	98	1e-32	65
ZP_06016658.1	conserved hypothetical protein	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC 13884	91	1e-32	65
ZP_23922371.1	hypothetical protein G000_15769	*Klebsiella pneumoniae* ATCC BAA-2146	98	1e-31	65
EJJ32616.1	hypothetical protein KPNIH1_26220	*Klebsiella pneumoniae* subsp. *pneumoniae* KPNIH1	98	2e-32	64
Putative anti-repressor proteins from bacteria					
EJJ32560.1	putative anti-repressor protein	*Klebsiella pneumoniae* subsp. *pneumoniae* KPNIH1	231	5e-14	41
EJJ32617.1	putative anti-repressor protein	*Klebsiella pneumoniae* subsp. *pneumoniae* KPNIH1	229	6e-14	38
EJA85655.1	antirepressor-like protein	*Salmonella enterica* subsp. *enterica* serovar Newport str. CVM 19470	313	3e-11	42
EJA65774.1	antirepressor-like protein	*Salmonella enterica* subsp. *enterica* serovar Newport str. CVM 19443	313	3e-11	42
EHW66624.1	antA/AntB antirepressor family protein	*Escherichia coli* DEC10B	218	1e-08	40
YP_002408600.1	putative phage anti-repressor protein	*Escherichia coli* IAI39	218	1e-08	38
EIL74143.1	putative anti-repressor protein	*Escherichia coli* 576-1	228	4e-08	38
YP_006116175.1	putative phage anti repressor protein	*Escherichia coli* ETEC H10407	230	4e-08	40
ZP_06991301.1	anti-repressor protein	*Escherichia coli* FVEC1302	241	5e-08	40
EIA35792.1	anti-repressor protein	*Escherichia coli* SCI-07	236	5e-08	40

induction was measured. Compared with the WT SPC32H phage, the spontaneous induction rate of the lysogen containing *SPC32H_020*-deleted mutant phage was significantly lower (ca. 6 x 10^{-6}-fold lower than WT phage; see Table IV-6), indicating the critical role of SPC32H_020 in normal prophage induction. Furthermore, treatment with mitomycin C (MMC) did not cause a notable enhancement of prophage induction (1.11-fold increase) of the mutant phage lysogen, whereas MMC caused a 78.65-fold increased induction of the WT phage lysogen (Table IV-6). Because the EOPs of both the WT and mutant phage were similar (1.8 x 10^7 PFU ml^{-1} and 4.0 x 10^7 PFU ml^{-1}, respectively) against the Δ^{LT2}*gtrABC1* strain, these results imply that SPC32H_020 might act as an anti-repressor. The function of the *SPC32H_020* gene was tested again with a phage spotting assay with *Salmonella* harboring a plasmid overexpressing the *SPC32H_020* (p*ant*) with the pBAD promoter. Expectedly, both SPC32H and SPC32N generated clearer lysis zone/plaques, and the lysogen-specific *attR* fragment was not amplified via PCR with the samples from the SPC32H lysis zone (Fig. IV-9A). These results allowed me to conclude that SPC32H_020 is an anti-repressor acting in the lytic switch. To verify the function of SPC32H_020 in lytic switching and prophage induction, *Salmonella* (Δ^{LT2}*gtrABC1*) and SPC32H lysogen of *Salmonella* [Δ^{LT2}*gtrABC1* (32H)] both harboring p*ant* were subjected to an arabinose disc diffusion assay. The SPC32H lysogen of the *Salmonella*

Table IV-6. Comparison of prophage induction efficiency

Strain	Phage titer (PFU ml^{-1})		Fold change
	- MMC	+ MMC (1 μg ml^{-1})	**(fold)**
Δ^{LT2}*gtrABC1* (32H)	4.45 x 10^{8}	3.50 x 10^{10}	78.65
Δ^{LT2}*gtrABC1* (32H Δ*ant*)	2.65 x 10^{3}	2.95 x 10^{3}	1.11

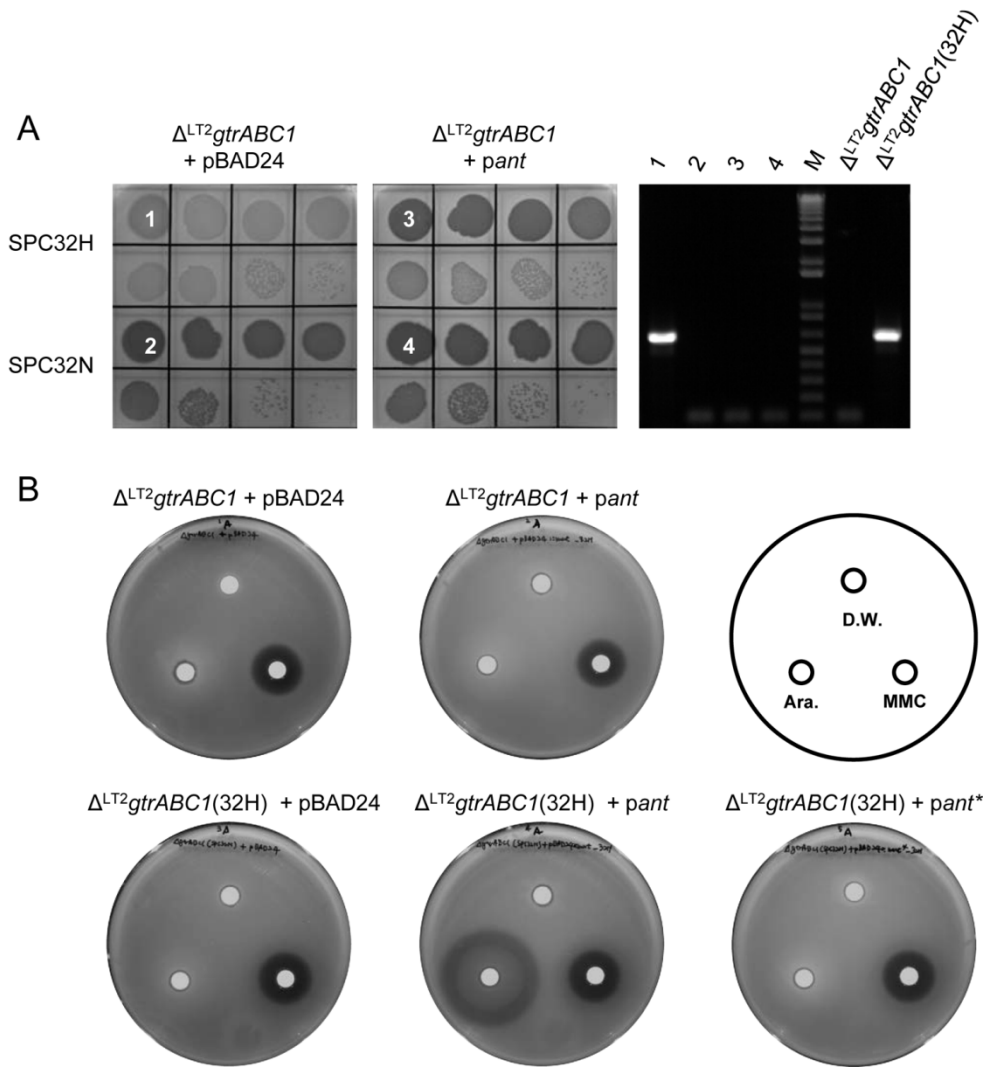

A
ΔLT2gtrABC1 + pBAD24
ΔLT2gtrABC1 + pant
SPC32H
SPC32N
1
2
3
4
1 2 3 4 M ΔLT2gtrABC1 ΔLT2gtrABC1(32H)
B
ΔLT2gtrABC1 + pBAD24
ΔLT2gtrABC1 + pant
D.W.
Ara.
MMC
ΔLT2gtrABC1(32H) + pBAD24
ΔLT2gtrABC1(32H) + pant
ΔLT2gtrABC1(32H) + pant*

Fig. IV-9. The putative anti-repressor, encoded by *SPC32H_020*, induces the lytic development of SPC32H.

A. SPC32H cannot lysogenize the SPC32H_020-overexpressing *Salmonella* strain. Serially diluted SPC32H and SPC32N were spotted on the lawns of the *Salmonella* strain harboring a SPC32H_020-overexpressing plasmid (p*ant*) or control plasmid (pBAD24), and the *attR* site was PCR amplified from each sample of the indicated lysis zones. L-arabinose (0.2%, final concentration) was added for the induction of SPC32H_020 from p*ant*.

B. The expression of SPC32H_020 protein promotes the switch from lysogenic to lytic development. The SPC32H lysogen [Δ^{LT2}*gtrABC1* (32H)] and non-lysogen (Δ^{LT2}*gtrABC1*) were transformed with the p*ant* or control plasmid (pBAD24), and the resulting strains were subjected to a disc diffusion assay with 10 µl of following solutions: D.W., distilled water; Ara., 15% L-arabinose; and MMC, 0.5 mg ml^{-1} mitomycin C. p*ant** indicates the plasmid encoding a frame-shifted *ant* gene. Arabinose-induced bacterial lysis was observed only in the SPC32H lysogen harboring the p*ant*.

[Δ^{LT2}*gtrABC1* (32H)] harboring p*ant* underwent lysis in the presence of 15% arabinose, but no lysis was observed without p*ant* (Fig. IV-9B). When the arabinose-inducible plasmid contained a frame-shifted *ant* gene, which was generated by an insertion of one additional nucleotide (adenine) next to the start codon of *SPC32H_020* gene, the arabinose treatment did not cause the lysis of a lysogenized strain (Fig. IV-9B), implying that *SPC32H_020* accomplishes its lytic-switching role as a protein rather than as an RNA. Therefore, all the results strongly suggested that *SPC32H_020* encodes a novel anti-repressor protein playing a significant role in lysogenic to lytic switching. I annotated *SPC32H_020* gene as *ant* (*ant*i-repressor) and its gene product as Ant.

***m2* in the SOS box causes constitutive expression of anti-repressor from SPC32N.** The above results indicate that *m2* might allow the overexpression of *ant* gene in SPC32N. A putative promoter and transcription factor-binding sites within the upstream region of *ant* gene were predicted using the BPROM program (Softberry, Inc., Mount Kisco, NY). As indicated in Fig. IV-5A, the -10 and -35 sites of the putative promoter and one LexA-binding site, also known as the SOS box, overlapping the predicted -10 site were found. Intriguingly, *m2* is located in the consensus sequence of the LexA-binding site (Fernandez De Henestrosa *et al.*, 2000; Lewis *et al.*, 1994b; Little and Mount, 1982) (Fig. IV-5A). LexA is a

transcriptional repressor that represses various SOS regulons, including LexA itself and RecA protein, via binding to the SOS box. DNA damage induces the formation of activated RecA nucleoprotein filaments that promote autocleavage of LexA and consequent derepression of SOS regulons (Little and Mount, 1982). Therefore, I hypothesized that the *ant* gene is another SOS regulon that is controlled by the LexA protein, and the *m2* in a consensus sequence of LexA-binding site might prevent the LexA-mediated repression of *ant* gene. As considered above, the derepressed Ant synthesis most likely causes the lytic cycle-bias during SPC32N proliferation.

To prove my hypothesis, I first examined the promoter activity of the *ant* gene from both SPC32H and SPC32N via a bioluminescence reporter assay. The 197-bp DNA fragment containing the putative *ant* gene promoter region was fused with the promoterless *luxCDABE* in reporter plasmid pBBRlux, and then the bioluminescence of various *Salmonella* strains harboring the reporter plasmid was compared. In contrast to the low RLU (relative light units) with the *ant* promoter from SPC32H (P_{ant_H}), the promoter from SPC32N (P_{ant_N}) presented approximately 2-log higher values (Fig. IV-10A and B). MMC treatment (1 μg ml^{-1}, final concentration) induced a significant increase of RLU in a clone harboring a pP_{ant_H}::*lux*, but not in a clone harboring a pP_{ant_N}::*lux* (Fig. IV-10A and B), suggesting that the *ant* promoter of SPC32H was activated by DNA damage,

whereas expression of SPC32N was constitutive independent of DNA damage. To elucidate whether these responses were associated with the LexA repressor, *Salmonella* mutants missing the *lexA* gene or expressing non-cleavable form of LexA [*lexA*(G85D)] were constructed, and the bioluminescence from the reporter plasmid pP_{ant_H}::*lux* was measured in these mutants. Both mutants were constructed in a Δ*sulA* background to suppress the lethality of the *lexA* deletion in *Salmonella* (Bunny *et al.*, 2002), and this *sulA* deletion did not affect the reporter gene expression (data not shown). Without the *lexA* gene, the activity of P_{ant_H} was comparable to that observed in the $lexA^+$ background with MMC and was not affected by MMC treatment (Fig. IV-10B). When the *lexA* gene under the arabinose-inducible promoter was complemented, the phenotype of $lexA^+$ was partially recovered by 0.2% arabinose induction (Fig. IV-10C). In contrast, the replacement of LexA with LexA(G85D) prevented promoter activation by MMC treatment (Fig. IV-10B), indicating that DNA damage activates the *ant* gene promoter through LexA proteolysis. In addition, no other factors from SPC32H appeared to be involved in *ant* gene regulation because the assay results were similar regardless of the presence of the SPC32H prophage (Fig. IV-10B).

To experimentally prove the binding ability of LexA to the SOS box within P_{ant_H} or P_{ant_N}, an electrophoretic mobility shift assay (EMSA) was performed. When the radio-labeled DNA fragment of the *ant* gene promoter region from

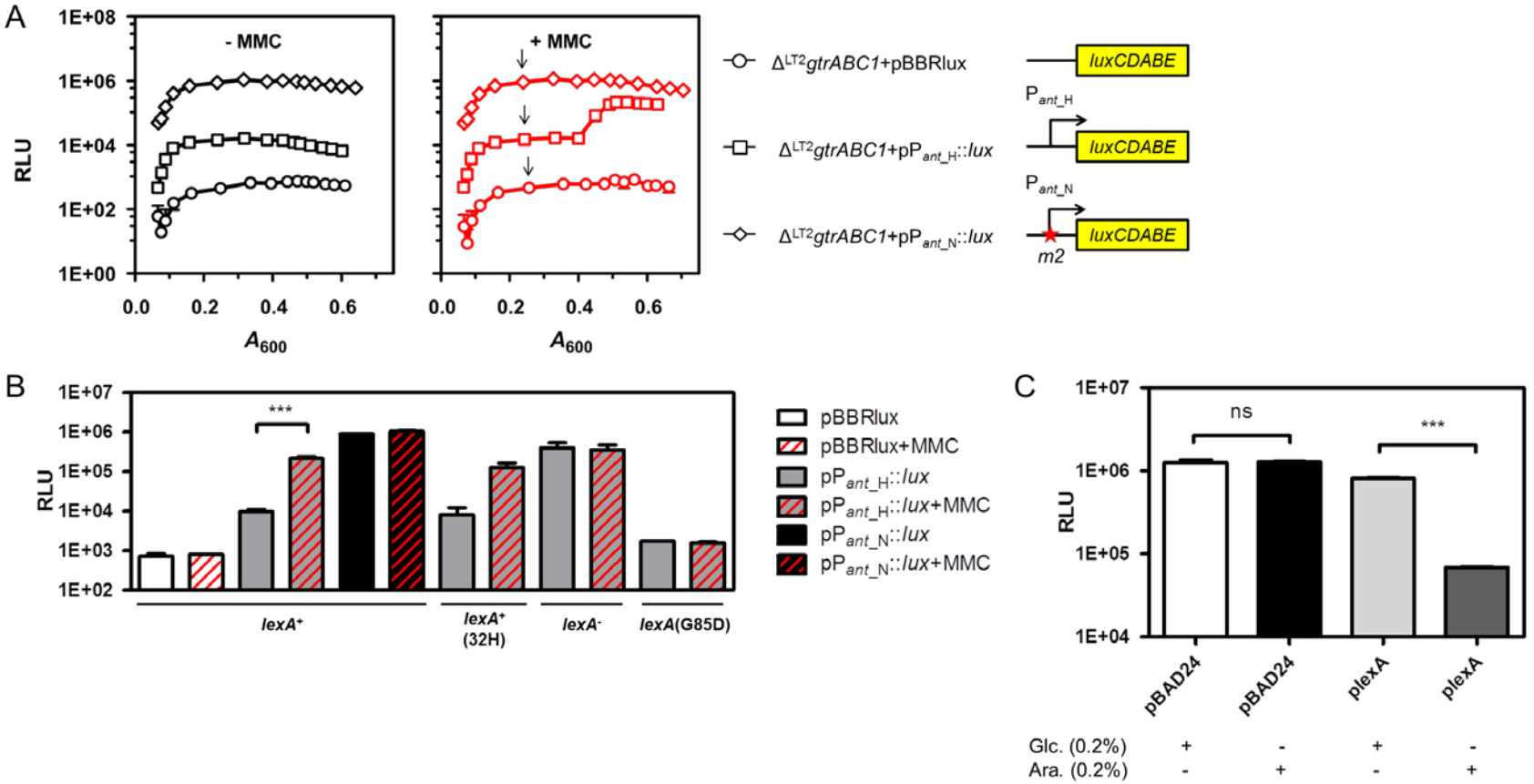

A
- MMC
+ MMC
RLU
1E+08
1E+06
1E+04
1E+02
1E+00
0.0
0.2
0.4
0.6
A600
ΔLT2gtrABC1+pBBRlux
ΔLT2gtrABC1+pPant_H::lux
ΔLT2gtrABC1+pPant_N::lux
luxCDABE
Pant_H
Pant_N
m2
B
RLU
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
***
lexA+
lexA+ (32H)
lexA-
lexA(G85D)
pBBRlux
pBBRlux+MMC
pPant_H::lux
pPant_H::lux+MMC
pPant_N::lux
pPant_N::lux+MMC
C
RLU
1E+07
1E+06
1E+05
1E+04
ns
***
pBAD24
pBAD24
plexA
plexA
Glc. (0.2%) + - + -
Ara. (0.2%) - + - +

Fig. IV-10. The *ant* promoter of SPC32H is activated by DNA damage via LexA proteolysis, whereas that of SPC32N is constitutively activated independent of DNA damage. The RLU (relative light units) were calculated by dividing the measured bioluminescence by A_{600} and represented using a log-scaled Y-axis. The results present the means with the SD of three independent assays.

A. Time-course observation of the *ant* promoter activity with or without DNA damage. *Salmonella* strains harboring the bioluminescence plasmid pP_{ant_H}::*lux* (*luxCDABE* fused to the putative *ant* promoter of SPC32H) or pP_{ant_N}::*lux* (*luxCDABE* fused to the putative *ant* promoter of SPC32N) were incubated at 37°C, and the bioluminescence, as well as A_{600} of the culture, was measured every half hour. The vertical arrows indicate MMC treatment (1 μg ml^{-1}, final concentration; at 3 hr after incubation).

B. The *ant* promoter activity of the various *Salmonella* strains harboring the bioluminescence plasmid at A_{600} = ~0.6. MMC (1 μg ml^{-1}, final concentration) was added at 3 hr after incubation. *lexA*$^{+}$, Δ^{LT2}*gtrABC1*; *lexA*$^{+}$(32H), Δ^{LT2}*gtrABC1*(32H); *lexA*$^{-}$, Δ^{LT2}*gtrABC1* Δ*sulA* Δ*lexA*; *lexA*(G85D), Δ^{LT2}*gtrABC1* Δ*sulA lexA*(G85D). ***, $P < 0.001$.

C. The activity of the SPC32H *ant* promoter in a *Salmonella* Δ^{LT2}*gtrABC1* strain harboring both the bioluminescence plasmid pP_{ant_H}::*lux* and *lexA* complementation plasmid (p*lexA*) or control plasmid (pBAD24). Bioluminescence was measured at A_{600} = ~0.6 and the RLU (relative light units) are presented as bioluminescence/A_{600}. D-Glucose (Glc.) and L-arabionose (Ara.) were added for the repression and induction of the *lexA* gene, respectively. ns, not significant; ***, $P < 0.001$.

SPC32H (APR_H*) was incubated with an increasing amount of purified *Salmonella* LexA, a specific mobility shift was observed, and the APR_H* fragment was released by the addition of unlabeled competing cold probe APR_H (Fig. IV-11). In contrast, the unlabeled cold probe APR_N (*ant* gene promoter region from SPC32N) was unable to compete with the APR_H* for LexA, and the APR_N* fragment was not shifted with the LexA (Fig. IV-11), confirming that LexA could not repress the *ant* gene expression in SPC32N due to an inability of LexA to bind to the SOS box containing an SNP (*m2*).

Accordingly, the hypothesized overall cascade of SPC32H induction was investigated with the *Salmonella* strains lysogenized by SPC32H containing a transcriptional *lacZ* fusion to putative *recET* genes. Because the phage *recE* and *recT* genes, encoding 5'→3' exonuclease and single-strand DNA binding/annealing protein, respectively, promote homologous recombination to mediate the integration/excision of phage genome to/from host chromosome (Carter and Radding, 1971; Kmiec and Holloman, 1981; Li *et al.*, 1998), the expression of *recET* (and its orthologous genes) can be a reporter for prophage induction. As shown in the Fig. IV-12, the treatment with MMC rather than other antibiotics activated the *recET::lacZ* fusion in the $lexA^+$ background but not in the *lexA*(G85D) background, indicating that the DNA damage generated by MMC induces SPC32H induction dependent on LexA proteolysis. Obviously, the

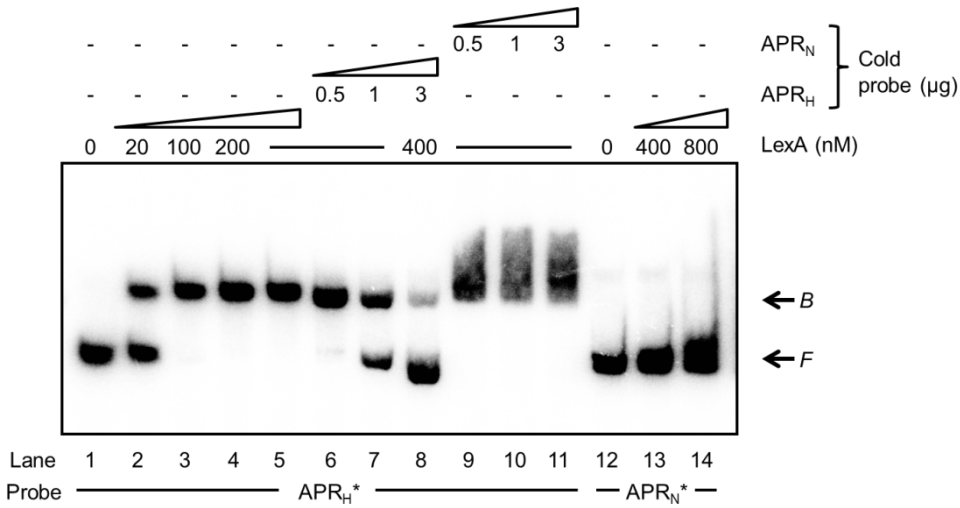


Fig. IV-11. LexA specifically binds to the putative *ant* gene promoter region of SPC32H but not to that of SPC32N containing *m2*. The γ-^{32}P-labeled DNA fragment of the *ant* gene promoter region from SPC32H (APR_H*) or that from SPC32N (APR_N*) was incubated with the indicated amounts of purified *Salmonella* LexA, and was subjected to an electrophoretic mobility shift assay (EMSA). The same sequence of DNA fragments, but unlabeled, (APR_H and APR_N) were additionally used for competition analysis. The position of the unbound fragments (*F*) and retarded fragments by LexA (*B*) are indicated.

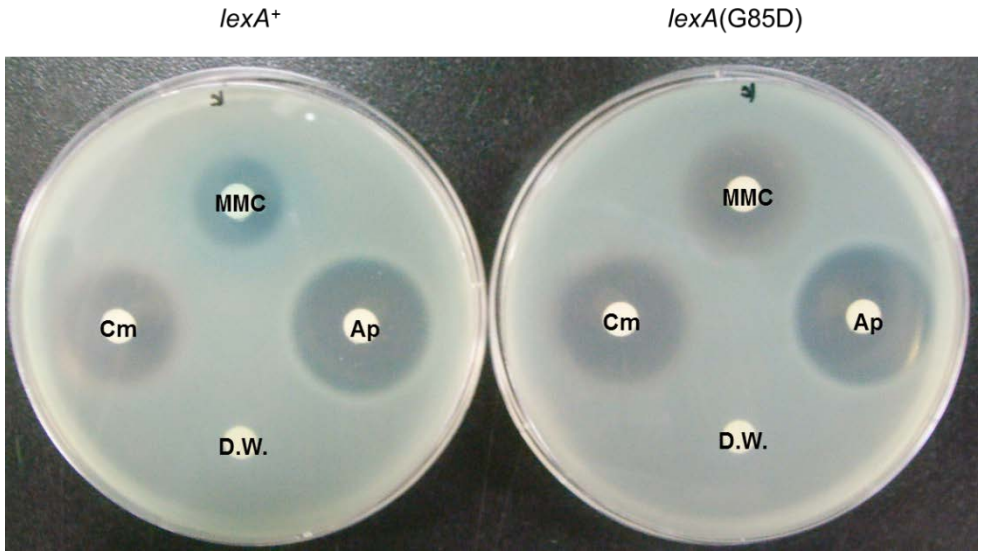


Fig. IV-12. The DNA damage-induced LexA proteolysis causes the switching to lytic development. Transcriptional *lacZ* fusion to the putative *recET* genes was introduced into the SPC32H lysogens harboring an intact (*lexA*$^{+}$) or non-cleavable [*lexA*(G85D)] LexA, and the resulting strains were subjected to a disc diffusion assay with the following solutions: MMC, 0.5 mg ml^{-1} mitomycin C; Cm, 2.5 mg ml^{-1} chloramphenicol; Ap, 10 mg ml^{-1} ampicillin; and D.W., distilled water. Note that the blue zone appeared surrounding the MMC disc with the *lexA*$^{+}$ lysogen only.

expression of the Ant protein resulting in lysis by the SPC32H lysogen specifically (Fig. IV-9B), demonstrating that derepression of *ant* gene via LexA proteolysis induced this phage induction. All together, these results strongly demonstrated that the *ant* gene of SPC32H was negatively controlled by LexA and that the SNP *m2* in the SOS box causes the dramatic phenotype differences between SPC32H and SPC32N by influencing expression of the *ant* gene.

The anti-repressor Ant interacts directly with the repressor Rep. Although I assumed that the anti-repressor expressed from the *ant* gene inhibits the activity of the repressor, the mechanism was unclear. There could be two possible modes of anti-repressor action: one mode is direct binding of the anti-repressor to its cognate repressor and inactivation of repressor function, and the other mode is competing with the repressor for the repressor-binding site on DNA. The putative repressor of SPC32H (designated Rep), encoded by *SPC32H_041*, is a 198-amino acids protein and possesses a helix-turn-helix motif, which is considered a DNA-binding domain. It was noticeable that the RecA-mediated autocleavage site (Ala-Gly or Cys-Gly), a highly conserved site of cleavable repressors such as lambda CI (Daniels *et al.*, 1983), was not observed in the putative repressor of SPC32H (Rep), strongly supporting the notion that the inhibition of Rep through means other than autocleavage assisted by RecA nucleofilaments occurs during the prophage

induction. The immunodetection of Human influenza hemagglutinin (HA) epitope-tagged Rep demonstrated that the expression level of Rep was almost constant without cleavage throughout a one-hour treatment with MMC (Fig. IV-13). Undoubtedly, the lytic switch was turned on in this experiment by MMC because HA-tagged Ant appeared and accumulated dependent on the MMC treatment (Fig. IV-13B).

To determine the possible interaction between Rep and Ant, I performed a bacterial two-hybrid assay based on the restoration of β-galactosidase activity in *E. coli cyaA* mutant strain BTH101 (Karimova *et al.*, 1998). Rep and Ant were fused with the T25 or T18 fragments of the adenylate cyclase catalytic domain from *Bordetella pertussis*. The reporter strain *E. coli* BTH101 expressing the combination of these hybrid proteins (i.e., T25-Rep/T18-Ant or T25-Ant/T18-Rep) produced blue colonies on the X-gal plates (data not shown), indicating a heterodimerization of the hybrid proteins via interaction between Rep and Ant. This functional complementation of adenylate cyclase was also quantitatively measured through a β-galactosidase assay. As shown in Fig. IV-14A, the β-galactosidase activity of *E. coli* BTH101 harboring the Rep::Ant combinations was significantly higher (approximately 10 to 50-fold) than that of the negative control (*E. coli* BTH101 harboring the plain T18 and T25 fragments). The basal activities were also obtained with the clones containing one fusion protein only, supporting

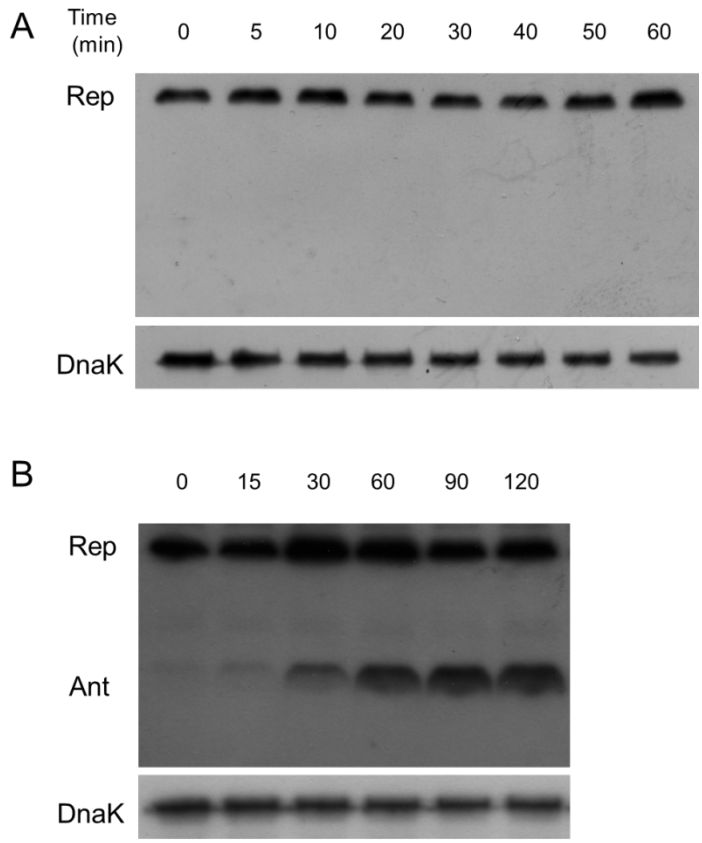


Fig. IV-13. DNA damage induces the accumulation of Ant, but not the cleavage of Rep. *Salmonella* strains lysogenized by SPC32H containing a HA-tagged Rep [A; Δ^{LT2}*gtrABC1* (32H *rep*-HA)] or both HA-tagged Rep and HA-tagged Ant [B; Δ^{LT2}*gtrABC1* (32H *rep*-HA *ant*-HA)] were exposed to MMC for 1 or 2 hr, respectively. The MMC-treated bacterial cultures were sampled at the indicated time points and subjected to western blotting to immunodetect the HA-tagged proteins. DnaK was used as an internal control.

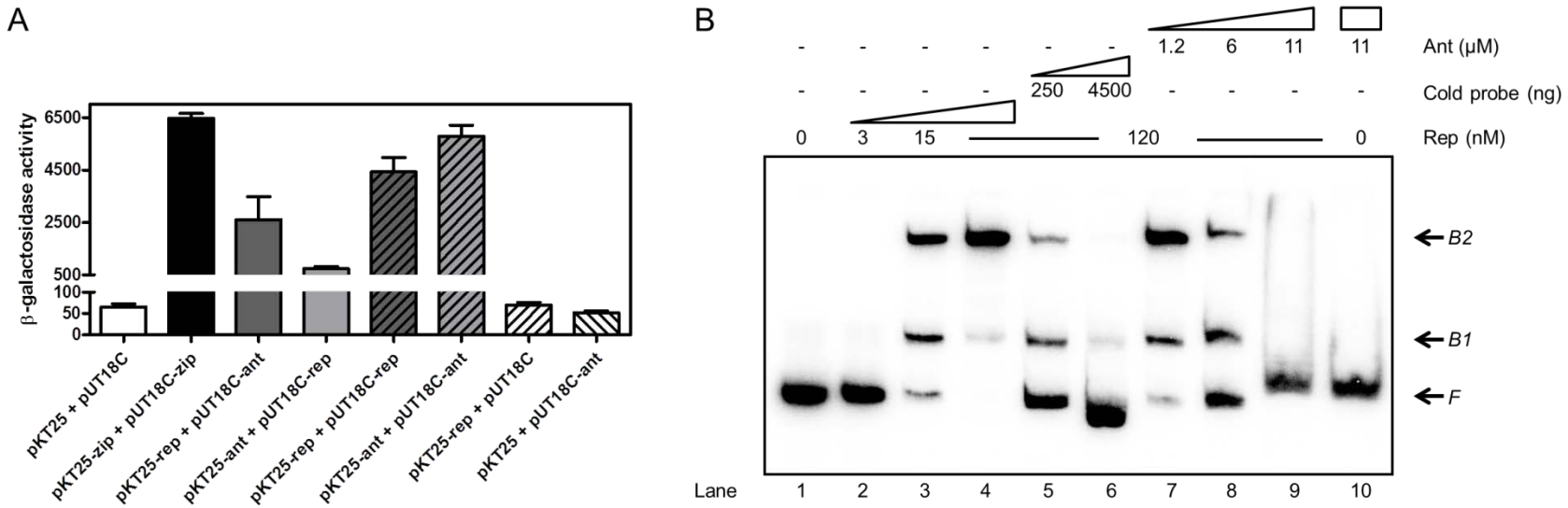


Fig. IV-14. Ant inhibits the binding of the cognate repressor Rep to the specific operator by direct binding to Rep.

A. A bacterial two-hybrid assay reveals the direct binding of Ant to Rep. *E. coli* BTH101 reporter strains harboring the indicated plasmids pair were subjected to a β-galactosidase assay to quantitatively measure the β-galactosidase activity. The activities are presented in Miller units.

B. EMSA with purified Rep and Ant elucidates the Ant-mediated inhibition of Rep-binding to its operator. The mixture of APR_H* and the indicated amounts of Rep were incubated at 20°C for 15 min within the 1 X binding buffer supplemented with 1.1 μg of poly(dI-dC) and then electrophoresed on the 6% native acrylamide slab gel for EMSA. For competition analysis, the unlabeled APR_H fragments were added as cold probes to the mixture. When appropriate, Rep was pre-incubated with the indicated amounts of Ant at 20°C for 30 min, and further incubated with APR_H* as described above. The position of unbound fragments (*F*) and retarded fragments by Rep (*B1* and *B2*) are indicated.

that the Rep::Ant interaction specifically complemented the β-galactosidase activity. Remarkably, strong Rep::Rep and Ant::Ant interactions were also observed, implying the possibility of multimer formation by each protein. Indeed, the results of analytical size-exclusion chromatography demonstrated that Rep and Ant were able to dimerize and tetramerize, respectively (Fig. IV-15). Altogether, these results suggest that significant interactions are possible for each protein between themselves as well as between each other.

An EMSA using purified Rep and Ant revealed inhibition by Ant of Rep target site binding. The high homology (97% identity) between Rep of SPC32H and the repressor of ε15 revealed by the BlastP search suggests that Rep might recognize the same DNA sequence as the ε15 repressor. The putative repressor-binding sequence (5'- ATTACCNNNNNGGTAAT -3') is scattered throughout the SPC32H genome as in ε15: upstream of terminase small/large subunit genes, upstream of phage structural protein module, upstream of the tailspike gene, upstream/downstream of the repressor gene and upstream of the putative restriction endonuclease gene. Among these areas, the upstream region of tailspike gene is the intergenic region between the *ant* and *tsp* genes (Fig. IV-5A), thus radiolabelled APR_H* including the putative repressor binding site as well as the SOS box was also used in this assay. Two DNA-protein complex bands with different mobility appeared when purified Rep was incubated with APR_H* (Fig. IV-14B), implying

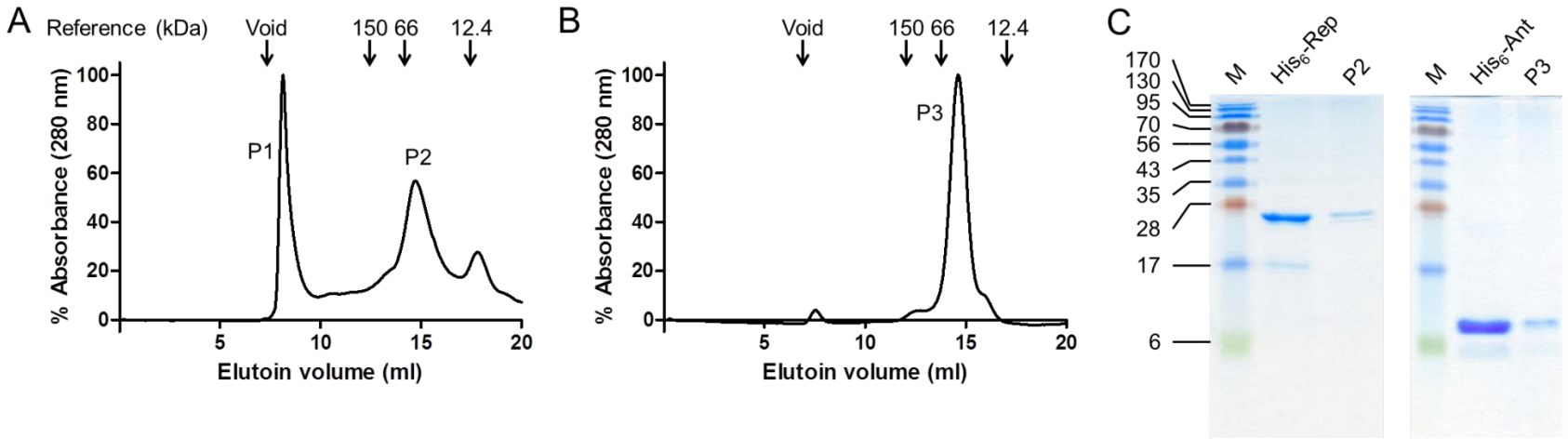


Fig. IV-15. Analytical size-exclusion chromatography of His_6-Rep (A) and His_6-Ant (B). The elution profiles imply the dimeric (P2) and tetrameric structure (P3) of His_6-Rep [monomeric molecular weight (M.W.) is approximately 25.8 kDa] and His_6-Ant (monomeric M.W. is approximately 13.3 kDa), respectively. The elution volumes for the M.W. standards are also presented in kDa units. Note that His_6-Rep tended to aggregate at the near void volume (P1). Each peak was confirmed by SDS-PAGE (C) as the corresponding specific protein.

that APR may have two Rep binding sites with different affinities for Rep. The specificity of Rep binding to APR was demonstrated by the competition assay with a non-labeled cold probe. Noticeably, the pre-incubation of Rep with purified Ant prevents the retardation of APR_H* fragment in an Ant concentration-dependent manner, suggesting that the specific interaction between Ant and its cognate repressor Rep interferes with the Rep-binding to its target DNA. Note that the protein concentrations indicated were expressed on the basis of assumption that the Rep and Ant in stocks existed as fully active dimers and tetramers, respectively. Obviously, the APR_H* fragment was not retarded by Ant alone (Fig. IV-14B lane 10), allowing the exclusion of the possibility that Ant interrupts Rep activity by competing for the Rep-binding site with Rep.

Introduction of *m2* to SPC32H generates the lytic cycle-biased clear plaque mutants. Considering the above results, I concluded that the constitutive expression of Ant due to the *m2* in SOS box can triggers the derepression of lytic genes through a direct inhibition of Rep-binding to target DNA. To verify whether *m1*, *m2*, or both single nucleotide differences were responsible for the differences between SPC32H and SPC32N, I introduced these different nucleotide sequences of SPC32N into the SPC32H. The stepwise genetic manipulations, including lambda Red recombination and SacB-mediated sucrose counter selection (see

Experimental procedures), were performed to construct mutant SPC32H phages, and the resulting phages were subjected to a spotting assay. As shown in Fig. IV-16, I could observe no significant changes in the turbidity of the lysis zone when the *m1* of the SPC32H was changed to that of the SPC32N. However, the halo and turbidity disappeared from the plaque and decreased in the lysis zone, respectively, when the *m2* of SPC32H was replaced by that of SPC32N. Obviously, the specific *attR* fragment was PCR amplified from the lysis zone of SPC32H *m1*, confirming that *m1* in *tsp* gene is not the reason for phenotypic differences. Therefore, the cascade started by a SNP *m2* in SOS box actually did flow, resulting in lytic cycle-bias in SPC32H similar to SPC32N.

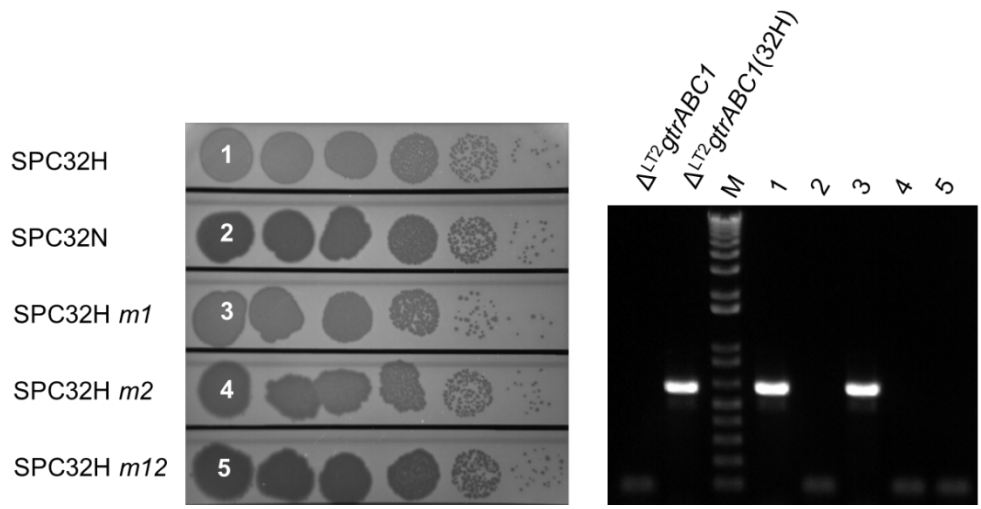


Fig. IV-16. Introducing *m2* from SPC32N into SPC32H causes lytic cycle-bias. Ten-fold serial dilutions of SPC32H, SPC32N and three mutant phages of SPC32H were spotted on the lawn of the strain Δ^{LT2}*gtrABC1*, and the produced lysis zones were subjected to the PCR of *attR* site for determination of lysogenization.

IV-4. Discussion

In this work, I sought to reveal the cause of phenotypic differences between two highly similar phages, SPC32H and SPC32N. I observed two nucleotide differences throughout the whole of the phage genomes, but only one, located within the non-coding region, was revealed as the reason for the differences. This nucleotide difference, designated *m2*, was actually positioned at the consensus sequence of a LexA-binding site that overlaps the -10 site of promoter for *SPC32H_020*, which encodes a hypothetical protein (Fig. IV-5A and C). Consequently, LexA could not bind to its binding site (Fig. IV-11), and it allows the constitutive expression of a small hypothetical protein (Fig. IV-10), which was identified as a novel anti-repressor. This anti-repressor inhibited the binding of its cognate phage repressor to regulatory regions (Fig. IV-14B), resulting in a switch of the phage lifecycle from lysogenic to lytic. Therefore, the formation of clearer, non-halo plaques of SPC32N with no lysogen development (Fig. IV-2A and 7A) was originated by the single nucleotide difference, *m2*.

To date, at least two categories of anti-repression system have been identified in the temperate phages. The first system is represented by the Cro protein of several lambdoid phages, such as phage lambda, HK022, and HK97. In this system, the binding of the Cro protein to target operator sites prevents expression of the *cI* gene

encoding the phage repressor CI (Freiedman and Gottesman, 1983). In contrast, the second system controls the repressor activity at the protein level. For example, the anti-repressor Tum of myoviral coliphage 186 directly binds to the phage repressor CI, preventing CI from binding to its operator sites (Shearwin *et al.*, 1998). Notably, the latter system has been reported in only a few temperate phages. Sipoviral coliphage N15 possesses an anti-repressor AntC that directly acts on the phage repressor CB (Mardanov and Ravin, 2007), and siphoviral prophages Gifsy-1and Gifsy-3 in *S*. Typhimurium strain 14028 express the anti-repressors GfoA and GfhA, respectively, to regulate the activity of each cognate repressor GfoR and GfhR by direct binding (Lemire *et al.*, 2011). The present study elucidated the regulation and mode of action of anti-repressor categorizing as the second system from the rest family (i.e., the family *Podoviridae*) of the order *Caudovirales*.

A common feature of the phages belonging to the second system is the LexA-regulated initiation of anti-repressor expression: all of these temperate phages, including SPC32H, are signaled to express the anti-repressor by a host SOS-response global regulator, LexA. Specifically, the promoter of each anti-repressor gene overlaps the LexA-binding site (Lemire *et al.*, 2011; Mardanov and Ravin, 2007; Shearwin *et al.*, 1998), such that DNA damage induces the RecA-mediated proteolysis of LexA and the consequent derepression of the anti-repressor gene. As a result, phage repressors are inactivated by the direct binding of the synthesized

anti-repressors, allowing phages to switch to the lytic cycle. In fact, connecting the host SOS response into the lytic switch is a fundamental strategy of prophages to escape from the host being in danger. As well-studied with phage lambda, the host RecA nucleoprotein, which is activated by DNA damage, causes the autoproteolysis of the phage repressor CI, resulting in entry into the lytic cycle (Freiedman and Gottesman, 1983; Little, 1984).

Compared with the RecA-dependent cleavable repressor system, the anti-repressor system appears to be more advantageous to the prophages. If host bacteria are able to repair DNA damages before prophage induction and survive (Kuzminov, 1999), escaping from the host cells would be the wrong choice for prophages. In this case, the induced phages should re-establish the prophage state in the new host bacterial cells to stably maintain their genome as a part of host genome. Obviously, this superfluous step would be prevented by expressing the anti-repressor in a LexA cleavage-dependent manner. As anti-repressor levels are reduced by the replenished LexA pool, the lysogenic development could resume because inactivated, rather than degraded, repressors restore their function by dissociating the anti-repressor. Although the reversible binding of Ant and the recovery of Rep activity after the Ant dissociation were not identified in the present study, the Rep levels were stably maintained without degradations during the MMC treatment (Fig. IV-13), suggesting that Rep could be recycled in the resumed

SPC32H lysogenic development. Indeed, the anti-repressor Tum/repressor CI pair from coliphage 186 exhibits reversible Tum-binding and the recovery of CI activity after dissociation of Tum (Shearwin *et al.*, 1998). I am now attempting to elucidate this issue by investigating the structure of the Rep-Ant complex as well as each protein. When considering the advantages in the rapid resumption of the regulatory circuit, this repressor/anti-repressor system might be widespread among the temperate phages. Remarkably, several homologues of SPC32H Ant were identified in other *Podoviridae* phages and various bacteria in the family *Enterobacteriaceae* (Table IV-5), most likely as a gene product of prophages. Moreover, the phage anti-repressors Tum (from *Myoviridae* coliphage 186), AntC (from *Siphoviridae* coliphage N15), GfoA (from *Siphoviridae* phage Gifsy-1) and Ant (from *Podoviridae* phaeg SPC32H) are distinct from each other at the amino acid sequence level (Fig. IV-17), suggesting that diverse repressor/anti-repressor pairs are present in the order *Caudovirales* to allow for more prudent control of their lytic/lysogenic switching. Therefore, as suggested by Mardanov and Ravin, the cleavable repressor system might not be the rule for lytic/lysogenic regulation in temperate phages (Mardanov and Ravin, 2007).

In accordance with the previously reported *cp* mutants of several phages (Bronson and Levine, 1971; Pons, 1984; Scott *et al.*, 1977; Scott and Kropf, 1977), SPC32N, which is a *cp* mutant of SPC32H, has a deficiency in lysogen

Fig. IV-17. Sequence alignments of the phage anti-repressors. The amino acid sequences of Tum (from coliphage 186), AntC (from coliphage N15), GfoA (from Gifsy-1) and Ant (from SPC32H) (A) or those except the Tum (B) were aligned using ClustalW2. No noticeable conserved residues were observed.

establishment. However, the deficiency originates from the *m2*-derived constitutive expression of the anti-repressor rather than from the typical mutations in the repressor or its binding sites. Similar to SPC32N, base substitutions or single-nucleotide deletions at the putative LexA-binding site in *Bacillus thuringiensis*-specific linear temperate phage GIL01 also generate some *cp* mutants (Fornelos *et al.*, 2011). Although the aforementioned study suggested that host LexA regulates the lytic switch of GIL01 with other phage-borne factors, what and how the gene products located at the downstream of the LexA-binding site produced the clear phenotypes were not clearly revealed. When considering also the results of the present study, certain protein(s) encoded in the LexA regulons of GIL01 might be constitutively expressed by the mutations at LexA-binding site and most likely inhibit(s) the regulatory circuit of GIL01 via an anti-repressor-like mechanism.

Generally, virulent phages have been studied as alternatives to the antibiotics to control antibiotic-resistant bacteria, whereas temperate phages are not considered because their potential risk of leaving lysogens. Contrarily, recent two proof-of-concept studies utilized the lysogenization ability, rather than the bactericidal capacity, of temperate phages to control *E. coli*. One study used modified M13 phages which overexpressing proteins that target nonessential host genes, such as SOS regulons, to enhance bacterial killing by antibiotics (Lu and Collins, 2009). The other study used engineered lambda phages to reverse bacterial antibiotic-

resistance to susceptible, by introducing *rpsL* and *gyrA* genes that conferring sensitivity to streptomycin and nalidixic acid, respectively (Edgar *et al.*, 2012). Given the results of present study, temperate phages also could be engineered to one of the promising candidate for biocontrol agents similar to virulent phages. By introducing the *m2* into the temperate phage SPC32H, I successfully constructed the *cp* mutant phage that did not enter the lysogenic cycles (Fig. IV-16). Conceptually, deletion of key elements for the temperate nature (i.e., integrase, repressor, etc.) would be another simple way to construct the mutant temperate phages that are biased to lytic mode. Indeed, deletion of integrase gene in SPC32H resulted in the disappearance of plaque halo, a signature of temperate phage (Fig. IV-8B), and conferred the inability to form lysogens (data not shown). A potential concern with the use of these engineered temperate phages is the emergence of revertant phages resulting from spontaneous mutations or recombination with the functional genes of other similar phages. However, the use of phage cocktail that composed of different kinds of phages will eliminate the potential lysogens of the revertant phage. Additionally, using phage cocktails in conjunction with sub-lethal antibiotics that cause bacterial DNA damages will further ensure the bactericidal efficacy. For example, in the case of using phage cocktail that contains SPC32N, SPC32H *m2* or SPC32H (Δ*int*), use of quinolones (e.g., ciprofloxacin, ofloxacin, nalidixic acid etc.) will resulting in the bursting out of the potential lysogens of

revertant phages because these phages are naturally turn on their lytic switch by the DNA damage-induced host SOS-response. Surely, the genetic engineering methods (i.e. lambda Red recombination and SacB-mediated sucrose counter selection) used in the present study also would be applied to construct the engineered SPC32H that, similar to the above two proof-of-concept studies, delivers *Salmonella* some genes enhancing antibiotic's efficacy or reversing resistance against antibiotics. In any case, the genetic engineering of temperate phages will enlarge our phage pool that could be developed as alternatives or supplement biocontrol agents for pathogens.

In conclusion, I have identified that the nucleotide difference *m2* is responsible for the phenotypic differences between temperate phage SPC32H and its *cp* mutant SPC32N. *m2* is a mutation at the consensus sequence of LexA-binding site that causes a constitutive expression of the novel phage anti-repressor Ant and, consequently, biases SPC32N toward the lytic development by directly interrupting the function of the cognate repressor Rep. Because Ant is a novel class of phage anti-repressor that is structurally distinct from previously reported anti-repressors, and numerous homologues of Ant are present in the putative prophage regions of the family *Enterobacteriaceae* as well as in ε15-like phages, the anti-repression system exemplified here with Ant and Rep might be widespread among temperate phages to allow for more prudent control their lytic/lysogenic switching. In addition, I successfully engineered SPC32H to do not enter the lysogenic cycle by

introduction of *m2* or deletion of *int* gene, and thus, the resulting phages as well as the engineering methods used could be applied to the fields of alternative antibiotics that utilize phages.

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국문 초록

박테리오파지 (파지)는 숙주 세균을 특이적으로 감염하여 죽이는 세균 바이러스이다. 최근 항생제 내성균들의 만연으로 인하여 박테리오파지를 대안적인 생물방제제 (biocontrol agent)로 사용하고자 하는 가능성이 재고되고 있다. 이에 다양한 닭의 분변 및 내장기관 샘플들로부터 살모넬라 또는 대장균 종에 특이적인 9종류의 파지를 분리하였다. 이들 중, SPC35라 명명한 독성 파지 (virulent phage)는 살모넬라 티피뮤리움과 대장균을 모두 감염시킬 수 있는 독특한 특성을 보여 심층적인 연구를 수행하였다. 투과전자현미경을 이용한 형태학적 분석과 118,351-bp로 이루이진 유전체 분석 결과 SPC35는 시포비리대 과 (family *Siphoviridae*)에 속하는 T5-유사 파지로 밝혀졌으며, 비타민 B_{12} 수용 외막 단백질인 BtuB가 SPC35의 숙주 수용체 (host receptor)로 밝혀졌다. 흥미롭게도, SPC35를 대장균과 살모넬라 티피뮤리움 배양액에 감염시켰을 때 예상보다 빠르게 파지 저항성 돌연변이체 (phage resistant mutant)가 나타났다. *btuB* 유전자를 살펴본 결과, 대부분의 파지 저항성 대장균 돌연변이체들은 삽입서열 2 (insertion sequence 2; IS*2*)에 의해 *btuB* 유전자가 망가져있었다. 이와는 대조적으로 파지 저항성 살모넬라 티피뮤리움 돌연변이체들은 *btuB* 돌연변이를 가지고 있지 않았으며 SPC35가 없을 때 쉽게 SPC35-감수성 (SPC35-susceptibility)을 회복하여, 이들은 상변이적인 (phase variable) 파지 저항성을 가지고 있음을 추측케하였다.

비록 'BtuB의 파손'과 같이 숙주 수용체를 변화시켜 파지의 흡착을 완전히 막는 방법이 파지의 감염에 저항하는 가장 일반적인 방어기작이기는 하지만, 이는 해당 세균에게 잠재적인 적합 대가(fitness cost)를 치르게 한다. 이러한 점에서, 본 연구에서는 살모넬라 티피뮤리움이 O-항원의 상 변이적인 개조를 통해 적합 대가가 없는 일시적인 SPC35-저항성을 나타낼 수 있음을 밝혔다. 일반적으로 파지 SPC35는 숙주 수용체로써 BtuB를 필요로하지만 살모넬라 티피뮤리움을 성공적으로 감염시키기 위해 살모넬라의 O12-항원 또한 흡착-도움 기구 (adsorption-assisting apparatus)로써 사용함을 알아내었다. 살모넬라는 ^{LT2}*gtrABC1*이라 명명한 O-항원 글루코실화 유전자를 상 변이적으로 발현하여, O12-항원의 갈락토오스 잔기를 α-1,4 결합으로 글루코실화시킴으로써 O12-항원의 흡착 도움 작용을 막으며, 이로써 *btuB* 유전자의 어떠한 돌연변이도 없이 일시적으로 SPC35-저항성을 나타내게 된다. 항원 변이 (antigenic variation)는 병원균의 병원성에 있어서도 이점이 되므로, 위와 같은 상 변이적 항원 개조를 통한 일시적인 파지 흡착의 중단은 아마도 다양한 그람 음성 병원균-파지 관계에 널리 퍼져있을 것이라 생각한다. 이러한 결과들은 성공적인 생물방제를 위해 단일 파지보다 병원균의 서로 다른 수용체를 인지하는 다양한 파지들로 구성된 혼합물 (cocktail)이 더욱 효과적으로 작용할 것임을 말해준다.

온건성 파지 (temperate phage)에 의한 용원성 감염 (lysogenic infection)은 숙주의 SOS-반응에 감응하여 용균성 (lytic) 양식으로 바뀌게 된다. 이러한 변화를 관장하는 궁극적 요소는 파지의 억제자

(repressor)로, 이는 대부분의 온건성 파지들에서 숙주 RecA 단백질에 의존적인 자동-벽개성 (autocleavable) 단백질로 알려져 있다. 살모넬라에 특이적인 포도비리대 과 (*Podoviridae* family)의 온건성 파지 SPC32H와 SPC32N은 용균반(plaque)의 형태가 서로 매우 다른 표현형적 차이가 있었지만, 이들의 전체 유전체에서는 오직 두 군데에서만 단일 뉴클레오티드 다형성 (single nucleotide polymorphism; SNP)이 존재했다. 이 단일 뉴클레오티드 다형성이 표현형적 차이를 초래하는 이유를 연구하는 과정에서, 파지 용균성 생활환 전환 기작에 관여하는 새로운 클래스의 항-억제자 (anti-repressor)를 발견하였다. SPC32H의 억제자 (repressor; Rep)는 SOS 반응에 의해 잘리지 않았으며, 대신 숙주 LexA 단백질에 의해 그 발현이 음성적으로 조절되는 작은 항-억제자 단백질 (Ant)에 의해 불활성화 되었다. *ant* 프로모터는 LexA-결합 부위(LexA-binding site)와 겹쳐져 있었는데, SPC32N의 경우, 이 LexA-결합 부위의 보존적 서열 (consensus sequence)에 단일 뉴클레오티드 돌연변이가 위치하고 있었다. 이에 따라 LexA 결합에 의한 *ant* 발현의 억제가 불가능하여 Ant가 지속적으로 합성되기 때문에, 파지 SPC32N는 용균성 생활환으로만 편향되어 결과적으로 더 투명한 용균반을 형성함을 알 수 있었다. 아미노산 서열 분석 결과 Ant는 이전에 보고되었던 다른 시포비리대 (*Siphoviridae*) 및 미오비리대 (*Myoviridae*) 파지의 항-억제자와 구조적으로 차이가 있었으며, 데이터베이스에서 다수의 Ant 동족체 (homologue)들을 다양한 포도비리대 파지 및 추정 프로파지들 (putative prophages)에서 발견할 수 있었다. 따라서, 보다 신중한

프로파지 유도를 위하여 이러한 항-억제자를 매개로하는 항-억제 시스템이 온건성 파지들 사이에서 널리 퍼져있을 것이라 생각한다. 한편, 본 연구 과정에서 만든 용균성 생활환으로 편향된 돌연변이 온건성 파지들과 여기에 사용된 유전자 조작 기법들은 항생제 대체제 개발 분야에서 다양하게 응용될 수 있을 것이다. 결론적으로, 본 연구에서 분리한 독성 및 온건성 파지들은 살모넬라를 제어하기 위한 새로운 생물방제제로써 실질적으로 사용할 수 있을 것이라 기대된다. 또한, 본 연구를 통해 새롭게 밝힌 세균-파지 상호작용 기작들, 즉 숙주 세균에서 파지 저항성이 나타나는 기작 및 프로파지의 용균성 생활환 전환 기작은 파지를 이용해 병원균을 제어하기 위한 전략을 개발함에 있어서 보다 효과적이고 개선된 방향을 제시하는 데에 기초 자료로 활용될 수 있을 것이다.

주제어 : 박테리오파지, 파지 응용 생물방제, 살모넬라 종, 숙주-파지 상호작용, 파지 저항성, 항-억제자

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