Doctoral thesis

Two modes of host recognition by bacteriophage T4

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General introduction

Discovery of bacteriophage and history of phage therapy

Bacteriophage is a virus that infects bacteria. Bacteriophage was discovered by Félix D'Herelle in 1917 as invisible microbe endowed with antagonistic effects on *Shiga Bacillus*. Since then, phages had been used agents against bacterial disease and achieved certain results. This method called phage therapy. However, after the widespread use of antibiotics in 1940s, phage therapy had fallen into disuse.

Today, antibiotics are losing potency because of increasing antibiotic resistance of bacteria and the lack of new types of antibiotics. This situation spotlights an urgent resurgence of phage therapy. In contrast to antibiotics that are effective against a wide range of bacterial species, phages infect only a limited range of bacteria. Because of its strict host specificity, phages can kill only pathogenic bacteria without affecting resident microbiota. On the other hand, when a new pathogenic bacterium appears, existent phage would not be able to infect it. Thus a naturally isolated phage that can infect the bacterium is necessary and its safety should be established before use for therapy. Although this approach takes much time, labor and cost, isolation of useful phage is not guaranteed. In addition, it is well known that bacterial receptors for phages frequently change by mutation (Morita *et al.*, 2002; Mizoguchi *et al.*, 2003). Therefore, it would be impractical to prepare in advance a stock of therapeutic phages for an epidemic of unexpected pathogenic bacteria. This problem might be alleviated or solved if the host specificity of a known phage is controlled to infect a given strain, even if it is not a natural host. Host specificity is mainly governed by the step of adsorption, or the attachment of phage virions to a susceptible host cell. This step is triggered by a process in which phage tail fibers or attachment proteins recognize specific receptors on the host cell surface (Goldberg *et al.*, 1994). In order to establish a method to manipulate host

specificity, it is important to understand the mechanism of adsorption. However, its precise mechanism remains unclear.

T4 phage is a virus that infects limited strains of *Escherichia* and *Shigella* But T4 can produce progeny on various bacteria except for these strains. Thus, T4 is an appropriate candidates for phage therapy. Therefore, I analyzed adsorption mechanism of bacteriophage T4 to understand its host range determinant. Extending from this work, as part of application of T4 in phage therapy, I also tried to modify host range of bacteriophage T4.

Chapter1

Two modes of host recognition by bacteriophage T4

Abstract

Bacteriophages have strict host specificity, which is governed by the step of adsorption. Here, I systematically examined the interaction between the *Escherichia coli* receptors lipopolysaccharide (LPS) and outer membrane protein C (OmpC), and the tail fibers of bacteriophage T4. Using a variety of LPS and OmpC mutants of B, K-12, and O157 strains, I demonstrated that T4 adsorbs to *E. coli* via two different modes, OmpC-dependent and OmpC-independent. T4 has no specificity for the sugar sequence of the outer core (one of three LPS regions) in the presence of OmpC but, in the absence of OmpC, T4 can adsorb to a specific LPS which has only one or two unbranched glucose residues. Characterization of the T4 mutants Nik (No infection to K-12 strain) and Nib (No infection to B strain) identified some amino acids that are involved in binding to OmpC and LPS. The result suggested that the two different modes for adsorption use different surfaces of long tail fibers: The top surface of distal tip in T4 long tail fibers interacts with LPS and the lateral surface of distal tip interacts with OmpC.

Introduction

Bacteriophage T4 was isolated in 1945 by M. Demerec and Fano from a phage cocktail that T. Rakieten possessed. Phages in the cocktail were divided into 7 phages by host range and shape of plaques. The 7 phages isolated were named T1 to T7 (Demerec and Fano, 1945). T4 is one of the best characterized phages and has been studied for decades in the field of molecular biology as a model organism.

T4 has strict host specificity like other phages and infects only B and K-12 strains of *E. coli* in addition to some strains of *Shigella*, illustrating that the host specificity of T4 can discriminate between strains of *E. coli*. Nevertheless, T4 can produce progeny in some bacteria that are not natural hosts, such as *Salmonella*, *Aerobacter*, *Proteus*, *Serratia* and *Yersinia*, when T4 DNA is introduced into spheroplasts of these bacteria (Wais and Goldberg, 1969; Dawes, 1975). These results illustrate that host range of T4 is determined at the first step of adsorption against these bacteria.

Adsorption step of T4 consists of four steps. First, long tail fibers (LTF) of T4 bind to receptors of host cell reversibly. When at least three of six long tail fibers attach to receptors, short tail fibers (STF) bind to receptors irreversibly. Then the hexagonal baseplate changes into star-shaped (Crawford and Goldberg, 1980). Lastly, the sheath is contracted and the inner tube penetrates outer membrane of host cell, resulting in injection of T4 DNA into host cell (Hu *et al.*, 2015).

T4 LTF consists of four proteins, Gp34 (gene product of *34*), Gp35, Gp36 and Gp37. Gp34 is located on the proximal side of LTF and binds to the baseplate. Gp35 is a knee-cap protein, and Gp36 and Gp37 form the distal part of LTF, the farthest distal part of which is Gp37. When T4 adsorbs to *Escherichia coli* cells, the distal tips (DT) of LTF bind to receptors on the cell surface (Montag *et al.*, 1990). DT is formed by the C terminal part of Gp37. Gp37 is a homotrimer. Each chain twists around a neighboring chain, forming a fiber like alpha helix (Bartual *et al.*, 2010). In

previous research, Krisch and his colleagues isolated a host range mutant of T4 and identified this mutation locus within the DT-encoding part of gene *37* (Tétart *et al.*, 1996). These results indicate that a host range of T4 is determined by the binding between DT and receptors.

Lipopolysaccharide (LPS) and outer membrane protein C (OmpC) are defined as receptors when T4 adsorbs to *E. coli* K-12 strain (Yu and Mizushima, 1982; Montag *et al.*, 1990). Another susceptible host is *E. coli* B strain, which has a deletion in the genomic sequence for OmpC and T4 uses only LPS as a receptor in this strain (Wilson *et al.*, 1970; Montag *et al.*, 1990). LPS is a component of the outer leaflet of the outer membrane in Gram-negative bacteria and is composed of three regions, lipid A, inner core, and outer core. Mizushima and his colleagues found that the outer core was not required for T4 adsorption in the presence of OmpC and that T4 could adsorb to a strain that has a glucose in outer core in the absence of OmpC (Yu and Mizushima, 1982). Similarly, analysis of T4 adsorption to B strain demonstrated that T4 adsorbed to strains, LPSs of which have one or two glucose(s) in outer core (Prehm *et al.*, 1976) . Thus, T4 exhibits two different modes of adsorption, one of which depends on OmpC and the other is independent of OmpC. These observations suggest that DT of T4 LTF can recognize a different type of molecules, sugar chain (LPS) and protein (OmpC). Alternatively, T4 can adsorb to K-12 and B strains by a common mechanism to recognize LPS, but the difference in LPS structure between these two strains might result in different requirements for OmpC as a co-factor. However, this fundamental question has been unanswered. In addition which amino acids of DT is used in each modes of adsorption and whether those amino acids are same in two modes of adsorption were unknown.

In this study, I systematically examined the role of LPS in T4 adsorption dependent on or independent of OmpC. My results show that T4 has no specificity for the sugar sequence of the outer core in adsorption dependent on OmpC. On the other hand, T4 has strict specificity in adsorption independent of OmpC and binds

only to a specific LPS, the outer core of which has only glucose(s) that are not branched. In addition, I could isolate the T4 mutants Nik (No infection to K-12 strain) and Nib (No infection to B strain), helping to identify the amino acids in DT required for the recognition of LPS or OmpC, and strongly suggested that the top surface of the DT head domain interacts with LPS and its lateral surface does with OmpC.

Materials and methods

E. coli strains and phages

Escherichia coli strains used in this chapter are listed in Table 1. Wild-type phage is T4D (Doermann and Hill, 1953).

Construction of *E. coli* mutants

The deletion mutants of ompC were constructed as follows. First, the kanamycin-resistance cassette was removed from JW3601, JW3602 and JW3606 by yeast Flp recombinase expressed from pCP20 (Cherepanov and Wackernagel, 1995). Next, the deletion mutants of ompC (TY0721, TY0722, TY0726 and TY0723) were constructed by T4 GT7 phage transduction of the kanamycin-resistance cassette from JW2203 (Wilson *et al.*, 1979). TY0703, TY0707 and TY0708 were constructed as described (Datsenko and Wanner, 2000). Briefly, a fragment containing a chloramphenicol-resistance cassette flanked with the sequences upstream and downstream of the gene which should be deleted was amplified by PCR with pKD3 as a template. Primers used for PCR were: for TY0703, 5′-GTCAACGATTGTTTCTGATTTTATAGACAAATAAAACCGTTAAAACAGTGGTGTAGGCTGGAGCTGCTTC and 5′-TTATATCATTACTTTATAGTTTCCCAGTTTTAATGCTTTATCTTTTCAATATGGGAATTAGCCATGGTCC; for TY0707, 5′-TAACGGAATACATGGCCTGGCTGAATCGCGACGCATAAGAGCTCTGCATGGTGTAGGCTGGAGCTGCTTC and 5′-GATGTTTTAACGATCAAAACCCGCATCCGTCAGGCTTCCTCTTGTAACAAATGGGAATTAGCCATGGTCC; for TY0708, 5′-TACTGGAAGAACTCAACGCGCTATTGTTACAAGAGGAAGCCTGACGGATGGT

GTAGGCTGGAGCTGCTTC and 5′-AAGTTTAAAGGATGTTAGCATGTTTTACCTTTATAATGATGATAACTTTTATGGGAATTAGCCATGGTCC. The amplified fragment was introduced into BW25113 harboring pKD46, which encodes λ phage Red recombinase. Then chloramphenicol-resistant colonies were screened by PCR with 5′-GTCAACGATTGTTTCTGATT and 5′-ACTTTATAGTTTCCCAGTTT for TY0703, 5′-TAACGGAATACATGGCCTGG and 5′-CGATCAAAACCCGCATCCGT for TY0707 and 5′-TACTGGAAGAACTCAACGCG and 5′-AGTTTAAAGGATGTTAGCAT for TY0708 to select cells with a deleted target gene. Also, TY0731, TY0732, and TY0733 were constructed as described above. The PCR product was introduced into O157:H7 harboring pKD46. Primers used were: for TY0731, 5′-CGTTGTAACCCGTAACTTATTTTTGCCAAAATTTTGGATACAGAATAAATGTGTAGGCTGGAGCTGCTTC and 5′-CATTTATCGTTTTATTATATATCATAATGAATTATTTTACCTTAAATCTATGGGAATTAGCCATGGTCC; for TY0732, 5′-TCAAACTAGGATTGAAGAGCAAATAATGATTATAATGAAGGGTAATTAAAGTGTAGGCTGGAGCTGCTTC and 5′-TGAAAAAATAGGAGTAACCGTAAATATTATTTTATCAACCATCTCGTATAATGGGAATTAGCCATGGTCC; for TY0733, 5′-ATGAAATATATACCAGTTTACCAACCGTCATTGACAGGAAAAGAAAAAGAGTGTAGGCTGGAGCTGCTTC and 5′-CTATTTATCACTATAAAATTCGTTAATAGATTCACAAATATAAATAACTTATGGGAATTAGCCATGGTCC. In the deletion of ompC from the genome of O157:H7 wild-type, TY0731 or TY0732, the DNA fragment was amplified by PCR using JW2203 DNA as the template and the primers 5′-CCGGTACCTAAAAAAGCAAATAAAGGCA and 5′-CCAAGCTTTGTACGCTGAAAACAATG, was introduced in O157:H7 wild-type,

TY0731 or TY0732 harboring pKD46. Kanamycin-resistant colonies were screened by PCR with same primers to select TY0750, TY0751 and TY0752.

Construction of a plasmid

To clone *E. coli* K-12 *ompC*, a DNA fragment containing *ompC* was amplified by polymerase chain reaction (PCR) using *E. coli* W3110 DNA as the template and the primers 5′-CCGGTACCTAAAAAAGCAAATAAAGGCA and 5′-CCAAGCTTTGTACGCTGAAAACAATG, digested with *Kpn*I and *Hin*dIII and ligated into the corresponding sites of pBAD33 (Guzman *et al.*, 1995) to construct pBAD33-$ompC_{K\text{-}12}$.

To construct pLTF, a DNA fragment containing gene *37* and gene *38* was amplified by PCR using T4 phage particles as the template and the primers FR80 and FR81 (Tétart *et al.*, 1998), digested with *Eco*RV and ligated into the corresponding site of pBluescript II SK+. pLTF-Nik1, pLTF-Nik2, pLTF-Nik8 and pLTF-Nib, each of which has a single base substitution of gene *37*, were constructed using a KOD-Plus-Mutagenesis Kit (TOYOBO) with pLTF as the template. Primers used for mutagenesis were: for pLTF-Nik1, 5′-GAAGGTGGTAATAAGATGTCATCATATGCCATAT and 5′-ACCAGTACCATTCCATGCCTCGATG; for pLTF-Nik2, 5′-GAGGGTGGGAGTAACACTAATGCAGCAGGG and 5′-CCTGTATGATATGGCATATGATGA; for pLTF-Nik8, 5′-GTAGGTAGTAATAAGATGTCATCATATGCCATAT and 5′-ACCAGTACCATTCCATGCCTCGATG; for pLTF-Nib, 5′-TGGTATGGTGTAGGTGGTAATAAGATGTCATCA and 5′-TTCCATGCCTCGATGTAGTGGCTGT.

To construct pBRSupD, a DNA fragment containing *serU* was amplified by polymerase chain reaction (PCR) using *E. coli* B40su1 DNA as the template and the

primers 5′-AACCAGTTCAAAACGATAGG and 5′-TTTCCACTATCAACAAGGAG, digested with *EcoR*V and ligated into the corresponding sites of pBR322 (Sutcliffe, 1979).

Bacteriophage adsorption assay

E. coli cells were grown in Luria–Bertani (LB) medium at 37°C until the density reached 3 x 10^8 cells ml^{-1} and T4 was added at a multiplicity of infection (m.o.i) of 0.01. At appropriate times after phage addition, an aliquot was withdrawn and diluted with BS buffer (8% NaCl and 10 mM potassium phosphate buffer, pH 7.4) saturated with chloroform. The number of unadsorbed phage particles was determined by plating with BW25113 for T4 wild-type and Nib or BB for Nik as an indicator cell. The fraction of unadsorbed phage particles was calculated with the number of input phage particles set to 100%. Adsorption assays were performed three times independently and data points represent the mean ± SD of triplicate measurements.

Purification of lipopolysaccharides (LPS) from E. coli

LPS of B40su1, BW25113, Δ*waaR,* Δ*waaO*, Δ*waaOB and* Δ*waaG* was purified as follows. Dried *E. coli* cells were prepared according to the method of (Inagaki *et al.*, 1995) with slight modification. Briefly, 7 L of overnight cultures of *E. coli* cells in LB medium were centrifuged at 4,300 *g* for 15 min and cell pellets were washed successively with 10-15 ml of ethanol, acetone, and 30-45 ml of diethyl ether. After washing, cells were dried under a reduced pressure over $SiO_2 \cdot nH_2O$ for 2 or 3 days. LPS was extracted from the dried cells by the phenol-chloroform-petroleum ether method (Galanos *et al.*, 1969), and dissolved with 50 mM Tris-HCl (pH 7.0). To check the integrity and purity of extracted LPS, LPS was separated by

Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) through 20% gels as described previously (Schägger, 2006) and visualized by silver staining.

LPS of Δ*waaF,* Δ*waaC,* O157, O157 Δ*waaJ,* O157 Δ*waaI and* O157 Δ*per* were prepared according to methods described previously (Moller *et al.*, 2003; Sheng *et al.*, 2008) with slight modification. Briefly, bacteria were incubated till OD_{600} reach 1.0. Cells were collected by centrifugation at 5,000 rpm for 5 min 4 °C and resuspended with 1/3 volume of PBS. Cells were incubated at 60 °C for 30 min then centrifuged at 11,700 rpm for 30 min at 4 °C. The supernatant was mixed with equal amount of 2 x SDS PAGE sample buffer then boiled for 10 min. Proteins were degraded by proteinase K (final concentration at 0.4 μg/ml) at 60 °C for 1 hour. LPS in the supernatant was stored at 4 °C. LPS was separated by Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) through 20% gels as described previously (Schägger, 2006) and visualized by silver staining.

Purification of OmpC from E. coli

OmpC was prepared according to the method of (Crowlesmith *et al.*, 1981) with slightly modification. Briefly, cells were incubated in 2 ml of LB at 37 °C till OD_{600} reach 0.5. Cells were harvested by centrifugation at 5,000 rpm for 5 min at 4 °C and resuspended with 3 ml of 10 mM Tris-HCl (pH 7.5). Harvested cells were broken by sonication for about 3 min. Sonicated lysate was centrifuged at 5,000 rpm for 5 min at 4 °C to remove residual whole cells. The supernatant was centrifuged at 20,000 g (14,900 rpm) at 4 °C for 60 min and the pellet was resuspended with 100 μl of 10 mM Tris-HCl (pH 7.5). OmpC from each cells were separated by 15 % SDS-PAGE at 20 mA for 150 min and visualized by CBB staining. In the case that OmpC was induced from plasmid by L-arabinose, add L-arabinose at 0.2 % (final concentration) at OD_{600} is 0.3 and incubate at 37 ℃ with shaking till OD_{600} reach 0.5.

Inactivation assay of T4 phage by purified LPS

35 μM of LPS were mixed with 3 x 10^7 particles of phage in 1 ml of M9C medium (M9-glucose medium supplemented with 0.3 % casamino acids, 1 μg ml^{-1} thiamine and 20 μg ml^{-1} tryptophan) containing 25 mM Tris-HCl (pH 7.0). At 10 min after phage addition, the mixture was diluted with BS buffer and the number of infective phage particles was determined by plating with appropriate cells. The relative numbers of infective phage particles in different LPSs was calculated with the number of infective phage particles in the absence of LPS set to 100%. Experiments were performed three times independently and data points represent the mean ± SD of triplicate measurements.

Inactivation assay of T4 phage by purified LPS from two strains

10 μM of B40su1 or *Δ waaOB* LPS and different concentration of BW25113 LPS were prepared in 50mM Tris-HCl (pH 7.5). 800 μl of this LPS solution were boiled and mixed with 200 μl of 5 x M9C. Inactivation assay of T4 by this LPS solution was performed as described above.

Isolation of Nik and Nib mutant phages

To isolate Nik (No infection to *E. coli* K-12 strain) a mutant phage which have lost the ability to absorb to K-12 cells, four cycles of subtraction were performed using a stock of T4 phage particles prepared on BB cells as follows: BW25113 cells were incubated in 100 ml of LB medium at 37 ºC until the cell density reached 1 x 10^8 ml^{-1}, collected by centrifugation at 2,300 *g* for 5 min, and suspended with 0.8 ml of fresh LB medium. The suspension was incubated with 1.2 ml of Dil2 buffer (0.01 M

Tris-HCl pH 7.5, 1 mM $MgCl_2$, 0.5% NaCl, 0.001% gelatin) containing 10^{11} particles of T4 for 5 min at 37 ºC. To remove adsorbed phage particles, *E. coli* cells were killed by the addition of chloroform and centrifuged at 4,400 *g* for 5 min. The supernatant containing unadsorbed phage particles was used for a second cycle of subtraction. In the second cycle, 10^{10} cells of growing BW25113 cells concentrated in 18 ml of LB medium by centrifugation were mixed with 2 ml of the supernatant after the first cycle of subtraction. After incubation at 37 ºC for 5 min, unadsorbed phage particles were again recovered by centrifugation, precipitated with polyethylene glycol as described previously (Yamamoto *et al.*, 1970), and suspended with 1 ml of Dil2 buffer. In the third cycle of subtraction, 1 ml of the suspension after the second cycle of subtraction was incubated with 0.5 ml of LB containing 10^{10} cells of growing BW25113 at 37ºC for 5 min. After centrifugation, the supernatant containing unadsorbed phage particles was subjected to the fourth cycle of subtraction. Finally, 1.5 ml of the stock solution containing unadsorbed phage particles (5.4×10^8 ml^{-1}) were obtained. After plating the stock solution with BB cells as an indicator, phage plaques were recovered and transferred onto plates seeded with BB or BW25113 cells. 0.3 % of phage particles in the stock solution could not form a plaque on BW25113 cells. Nib (No infection to *E. coli* B strain) mutant phage that had lost the ability to absorb to B cells was isolated by the same method as for Nik mutant phage except that BW25113 cells were substituted for BB cells. Finally, 1ml of the stock solution of Nik (2.6×10^6 ml^{-1}) was obtained. 26.2 % of phage particles in the stock solution could not form a plaque on BB cells. Sequence analysis for DT region of Nik and Nib mutants were performed using two primers, 5′-AAGTCCGCATATCCAAAGTTAGCTGTTGC and 5′-TATATTTTCATATTTAGAAGGGCCGAAGC.

Marker rescue test

The marker-rescue test was performed as below. The double mutant phage, *amN91 amSE31*, which an amber mutation in genes *37* and *38* respectively, was propagated on BW25113 cells harboring pLTF, pLTF-Nik1, pLTF-Nik2, pLTF-Nik8, or pLTF-Nib. During propagation, the DNA fragment between genes 37 and 38 containing each mutation was transferred into the T4 genome via homologous recombination between plasmid and T4 DNA. To pick up recombinant phages, the progeny was plated on BB cells for pLTF, pLTF-Nik1, pLTF-Nik2, and pLTF-Nik8, and BW25113 cells for pLTF-Nib. At the same time, the total number of the progeny was calculated by plating on B40su1 or BW25113 cells harboring pBRSupD. Plaques on BB or BW25113 were randomly picked up and the number of plaques showing same host range as Nik or Nib was counted by plating on BB and BW25113 cells. The emergence rate (%) shows the ratio of the number of phage plaques showing same host range as Nik or Nib to the total number of the progeny. Each value indicates the mean of three independent experiments.

Results

T4 adsorption dependent on OmpC

T4 phage uses both LPS and OmpC to adsorb to the *E. coli* K-12 strain (Henning and Jann, 1979). LPS is composed of three regions, lipid A, inner core, and outer core. Lipid A is predominantly buried in a lipid bilayer of the outer membrane. The inner core is an oligosaccharide attached to the extracellular side of lipid A, and the outer core is also an oligosaccharide extended from the inner core. While the structures of Lipid A is highly conserved among *E. coli* strains, those of the outer core and the inner core are diverse and are classified into five types (Schnaitman and Klena, 1993; Heinrichs *et al.*, 1998b; Amor *et al.*, 2000). The outer core of K-12 LPS is a K-12 type whose main chain is composed of Glc I, Glc II and Glc III (Fig.1). Branches of this strain are Gal attached to Glc I and Hep IV-GlcNAc to Glc III.

I first attempted to identify which structure of LPS is required for T4 adsorption dependent on OmpC. For this purpose, I employed a variety of sugar transferase-deficient mutants (Fig. 1 and 2). WaaO is the Glc II transferase and WaaB is the Gal transferase. Therefore, these deletion mutants, JW3602 (Δ*waaO*) and TY0703 (Δ*waaOB*) cannot attach GlcII and Gal and synthesize Glc I branched with Gal and only Glc I in the outer core, respectively (Fig. 2). WaaG is the Glc I transferase and JW3606 (Δ*waaG*) completely eliminates the outer core (Fig. 2) (Heinrichs *et al.*, 1998b). Δ*waaR* lacks Glc III transferase and its outer core is expected to have two forms, one with Glc II at the outer terminus and the other with GlcNAc-Hep IV branched to Glc II (Fig. 2) (Pradel *et al.*, 1992). The LPS synthesized in each mutant was extracted and analyzed by Tricine-SDS-PAGE and silver staining (Fig. 5A). In this analysis, the mobility of LPS depends on the mass of the polysaccharide and the relative migration rate of each LPS was consistent with its mass. LPS isolated from Δ*waaR* revealed two bands and their migration rates

were consistent with the above notion.

First, I carried out spot-test analysis to test the growth ability of T4 on these LPS mutants (Fig. 7A). T4 grew normally on all outer core mutants at an efficiency of plating (e.o.p) similar to that on wild-type. Next, the rate of T4 adsorption to each mutant was measured by plaque forming assays. After T4 was added to *E. coli* cultures, an aliquot at an appropriate time was treated with chloroform and the number of phage particles was counted by plating with BW25113 (K-12 wild-type) cells as an indicator. Since chloroform kills *E. coli* cells including those infected with T4, only unadsorbed phages can form plaques. Therefore, the reduction of plaque number along with time reflects the kinetics of T4 adsorption. As shown in Fig. 7B, T4 adsorbed efficiently and more than 90 % of T4 adsorbed to all outer-core mutants at 15 min, although the efficiency was slightly lower in Δ*waaG* compared to the other mutants. This observation suggested that T4 had no specificity for the sugar sequence of the outer core in the presence of OmpC.

To further address this possibility, I adopted *E. coli* strain O157. The outer core of the O157 LPS belongs to the R3 type and it is quite different from that of K-12. The main chain of the outer core is Glc I, Gal I and Glc II with GlcNAc branch attached to Gal I and Glc III branch to Glc II (Fig. 1 and 4). In addition, O157 LPS has repetitive units of oligosaccharides (O-antigen) on the extracellular side of the outer core (Shimizu *et al.*, 1999; Currie and Poxton, 1999). *waaJ* is a Glc II transferase and its deletion mutant, TY0732 (O157 Δ*waaJ*) eliminated O-antigen, leaving Glc I and Gal I branched with GlcNAc in the outer core (Fig. 4). I analyzed the LPS lengths of O157 and its mutants by the same method as described. As shown in Fig. 5C, many bands were observed in O157 wild-type and these bands were attributable to the repetitive units of O-antigen. O157 Δ*waaJ* synthesized a distinct and shorter LPS than that of O157. O157 has OmpC and its amino acid sequence shares 94% homology with that of K-12 OmpC. T4, however, cannot use O157 OmpC as a receptor for adsorption (unpublished data). Therefore, I introduced a plasmid

harboring K-12 *ompC* under the control of inducible promoter into two O157 strains carrying a deletion of *ompC*, TY0750 (O157 Δ*ompC*) and TY0752 (O157 Δ*waaJ* Δ*ompC*). To check expression level of OmpC, outer membrane proteins were purified from cells with and without induction of OmpC and were analyzed by SDS-PAGE and CBB staining. When OmpC was induced from plasmid, its expression level was as high as that of endogenous OmpC (Fig. 6B).

T4 could adsorb to O157 Δ*waaJ* Δ*ompC* expressing K-12 OmpC as efficiently as to K-12 wild-type, but hardly at all to O157 Δ*ompC* expressing K-12 OmpC (Fig. 10A). This result strengthened the idea that T4 has no specificity for the sugar sequence of the outer core in OmpC-dependent adsorption and O-antigen is strongly suggested to function as an inhibitor of T4 adsorption.

Next, I examined the necessity of the inner core for T4 adsorption. WaaF and WaaC are Hep II and Hep I transferases, respectively. Therefore, Δ*waaF* and Δ*waaC* lack two or three heptoses in the inner core (Fig. 3). LPS synthesized by these mutants were checked as described in materials and methods. Δ*waaF* and Δ*waaC* synthesized shorter LPS than LPS of Δ*waaG* (Fig. 5B). T4 formed plaques on these mutants at similar efficiency of plating to that on wild-type, although they were more faint than those on wild-type (Fig. 8A). As shown in Fig. 9A, T4D formed plaques with transparent area in the center of plaque and slightly cloudy area called halo around it (Fig. 9A). However, plaques on Δ*waaF* have only halo and no transparent area (Fig. 9B). plaques on Δ*waaC* were obviously more faint and smaller (Fig. 9C). These results indicate that the growth of T4 on Δ*waaF* and Δ*waaC* was not as efficiently as on BW25113. T4 could not adsorb to these mutants efficiently and only 30% of phages adsorbed within 15 min after phage addition (Fig. 8B). This inefficient adsorption would cause the faintness of plaques on these mutants. This result demonstrates that intact inner core is necessary for efficient adsorption of T4 in the presence of OmpC.

T4 adsorption independent of OmpC

T4 efficiently adsorbs to strain B, using LPS alone for adsorption to this strain (Wilson *et al.*, 1970; Prehm *et al.*, 1976). Although the core oligosaccharide of strain B is the R1 type, the main chain of which is composed of Glc I, Glc II and Gal I, LPS of B strains used for T4 adsorption have only two glucoses, Glc I and Glc II, in the outer core (Fig. 1) because of an IS*1* insertion in *waaT* encoding Gal I transferase (Heinrichs *et al.*, 1998a; Jeong *et al.*, 2009). Mizushima and his colleagues showed that T4 could adsorb to K-12 mutant that has only one glucose in outer core independently of OmpC (Yu and Mizushima, 1982). Therefore, this specific type of LPS functions as a receptor for T4 adsorption without OmpC. However, their mutant was isolated sopntaneously and their analysis was limited in a number of LPS mutants. In addition, their mutant was uncharacteraized for mutation locus. To reevaluate which structure of LPS supports T4 adsorption in the absence of OmpC, *ompC* was deleted from K-12 wild-type and LPS mutants. Expression of OmpC of deletion mutants was checked by the method as described in materials and methods. Clearly, OmpC was not expressed in the double mutants (Fig. 6A). T4 grew and adsorbed to TY0723 (Δ*waaOB* Δ*ompC*) efficiently, but not grew on other LPS mutants deficient in OmpC (Fig. 7C and D). The LPS of Δ*waaOB* Δ*ompC* has an outer core with only Glc I, similarly to the B strain, and this result is consistent with the previous result (Yu and Mizushima, 1982). Interestingly, when only a galactose was branched to Glc I in TY0722 (Δ*waaO* Δ*ompC*), T4 did not adsorb to this strain. Furthermore, when all of the outer core and parts of the inner core were removed, T4 could neither form plaques nor adsorb (Fig. 8C and D). T4 adsorbed to O157 Δ*waaI*, which has only Glc I in the outer core (Fig. 4), as efficiently as to the K-12 strain (Fig. 10B). However, T4 could not adsorb to O157 Δ*waaJ* and O157 Δ*per*. Since the inner core of O157 is slightly different from that of K-12 type (Fig. 1 and 4), these results indicate that T4 can adsorb to a specific LPS with only one (Glc

I) or two glucoses (Glc I and Glc II) in the outer core, regardless of the sugar sequences in the inner core.

When at least three of six long tail fibers of T4 bind to *E. coli* receptors, the conformation of the baseplate changes from hexagonal to star-shaped (Simon and Anderson, 1967; Wilson *et al.*, 1970; Crawford and Goldberg, 1980; Hu *et al.*, 2015). After this conformational baseplate change, T4 is inactivated or can never bind to newly encountered *E. coli* strains. Previous study showed that T4 is inactivated by LPS of B strain, suggesting that T4 LTF directly binds to LPS having two glucoses in the outer core. This fact led me to examine whether or not T4 adsorption in the absence of OmpC strictly correlates to direct binding to LPS. LPS was purified from a variety of *E. coli* mutants. When T4 was mixed with LPS purified from a B strain (B40su1), 95% of input phage particles were inactivated within 10 min (Fig. 11), as expected. On the other hand, the LPS of wild-type, Δ*waaR*, Δ*waaO* or Δ*waaG* strain could not inactivate T4. Remarkably, the LPS of Δ*waaOB*, to which T4 could adsorb without OmpC (Fig. 7D), inactivated more than 90% of input particles. These results strongly suggest that T4 can adsorb directly to specific LPS (Glc I or Glc I-Glc II in outer core) produced by strains to which T4 can adsorb in the absence of OmpC. As mentioned above, Δ*waaR* synthesize two forms of LPS and the shorter has a terminal glucose without branch, raising a possibility that T4 can binds to the shorter LPS but the longer LPS inhibits T4 from accessing the shorter LPS. To examine this possibility, I performed inactivation assay of T4 with a mixture of two forms of LPS, the one is longer LPS, wild-type LPS, to which T4 cannot bind and the other is shorter LPS, Δ *waaOB* or B40su1 LPS, to which T4 can bind. As shown in Fig. 12, even when 40 μM BW25113 LPS was mixed with 10 μM B40su1 or Δ*waaOB*, T4 inactivation was as efficient as that in the absence of BW25113 LPS. This result indicates that the longer LPS to which T4 cannot bind is not inhibitory to T4 from accessing the shorter LPS. Accordingly, it is strongly suggested that a branched Gal in Glc I in Δ*waaR* LPS inhibits T4 from binding to Glc II.

Isolation and characterization of T4 Nik and Nib mutants

As demonstrated above, there are two different modes in T4 adsorption, OmpC-dependent and OmpC-independent and it may be possible that the two modes use different part of DT of long tail fiber. To clarify which part of DT is involved in each mode and identify amino acids in DT essential for each mechanism, I attempted to isolate T4 mutants defective in each mode of adsorption. For this purpose, I used K-12 and B strains because T4 absorbs to strain K-12 dependent on OmpC and to strain B independent of OmpC. I obtained three Nik (No infection to K-12 strain) mutants (1, 2, and 8) that grew on B cells (BB), but not on K-12 cells, and 5 Nib (No infection to B strain) mutants that grew on K-12 cells, but not on B cells (see Experimental Procedures). Sequence analysis around the DT region of these mutants identified amino acid substitutions in the DT region but the sequence of other regions except for the DT was not determined. Instead, marker rescue analysis confirmed that the mutations in the DT region result in the phenotypes of Nik and Nib.

pLTF was a plasmid with gene *37* and gene *38* of T4. pLTF-Nik1, pLTF-Nik2, pLTF-Nik8 and pLTF-Nib have a single-base substitution corresponding to each of Nik1, Nik2, Nik8 and Nib, respectively. BW25113 cells harboring one of these plasmids were inoculated with T4 having double amber mutations in gene *37* and gene *38*. Recombinant phage that lost amber mutations was selected on BB cells or BW25113 cells harboring pBSSupD as an indicator cells and the phenotype of recombinants was examined by spotting them on BB and BW25113. When the double amber mutant of T4 was inoculated into bacteria harboring pLTF-Nik1, pLTF-Nik2 or pLTF-Nik8, emergence rate of recombinants that have the Nik phenotype was 0.42, 0.90 and 0.57%, respectively. In the case of pLTF-Nib, the emergence rate of recombinants that have the Nib phenotype was 4.7%. On the

other hand, when inoculated with bacteria harboring pLTF, the emergence rate of recombinants having the Nik or Nib phenotype was less than 0.015% (table. 2). These results revealed that each single-base substitution in Nik1, -2, -8, or Nib caused the Nik or Nib phenotype.

Nik1, 2 and 8 had single-base substitutions at T2822A, C2864A and G2827A of gene *37*, replacing amino acids at V941E, A955E and G943S, respectively (Fig.18). All of these amino acids were located in the DT region of LTF. None of the Nik mutants could form plaques efficiently on BW25113 (Fig. 13). While Nik2 and Nik8 formed normal plaques on BB, Δ*waaOB*, and Δ*waaOB* Δ*ompC* cells, Nik1 formed smaller and more faint plaques on Δ*waaOB* and Δ*waaOB* Δ*ompC* cells than on BB. Importantly, Nik mutants could not grow on other LPS mutants, even though expressing OmpC, suggesting the loss of OmpC-dependency. To confirm this possibility, the OmpC-requirement for the adsorption of Nik mutants was examined (Fig.14). All Nik mutants adsorbed to BB, Δ*waaOB*, and Δ*waaOB* Δ*ompC* cells, although the efficiencies of Nik1 on these strains were lower than those of other Nik mutants. As expected, Nik mutants did not adsorb to any other strains expressing OmpC. This result indicates that Nik loses ability of adsorption dependent on OmpC and mutation sites of Nik is important for this mode of adsorption. To confirm that Niks adsorb to host cells by direct binding to their LPS, I carried out an inactivation assay of Niks by purified LPS. Nik2 and Nik8 were inactivated by B40su1 and Δ*waaOB* LPS, though the efficiencies were lower than that of T4 wild type (Fig. 15). Nik1 was hardly inactivated by both LPSs. However, when an inactivation assay was carried out under different conditions, about 60% of Nik1 was inactivated by B40su1 LPS (see discussion).

Although I isolated five Nib mutants, all of them had the same base substitution at C2816T of gene *37*, causing the amino acid substitution T939I. As shown in Fig. 16, Nib formed plaques on K-12 wild-type and LPS outer-core mutants having *ompC*, but not on strain B or any K-12 Δ*ompC* strain. Adsorption assays showed that Nib

adsorbed, depending on OmpC, to all LPS outer-core mutants, though more than 60% of phage particles remained unadsorbed at 10 min after phage addition (Fig. 17). On the other hand, Nib hardly adsorbed to the same LPS outer-core mutants in the absence of OmpC. Notably, Nib could not adsorb to Δ*waaOB* Δ*ompC* that T4 wildtype can adsorb in the absence of OmpC in addition to strain B, indicating that Nib loses the ability of OmpC-independent adsorption and that T939 is responsible for the recognition of terminal glucoses (Glc I and Glc I-Glc II) in the outer core.

Discussion

Two different modes of adsorption of T4 phage dependent on and independent of OmpC were proposed decades ago (Prehm *et al.*, 1976; Yu and Mizushima, 1982). However, precise requirements for LPS structure have not been clarified. In addition, information about the mechanism of interaction between DT and host receptors has been entirely lacking. In this study, I first performed a comprehensive analysis of LPS structure in relation to T4 adsorption in each mode using various LPS mutants of K-12 strain. All the tested outer-core mutants allowed T4 adsorption depending on OmpC (Fig. 7B). T4 was also able to adsorb to O157 Δ*waaJ* Δ*ompC* expressing K-12 OmpC (Fig. 10A). These results indicate that T4 has no specificity for outer-core structure in OmpC-dependent adsorption, although the length of the outer core correlated weakly with the efficiency of adsorption (Fig. 7B). Intriguingly, the efficiency of adsorption was significantly decreased when part of the inner core was lacked (Fig. 8B). The previous study showed that OmpC alone could not inactivate T4, but when lipoprotein-bearing peptidoglycan sacculus was mixed with both OmpC and LPS, a hexagonal lattice was formed on the sacculus, to which T4 could adsorb (Furukawa *et al.*, 1979). These observations suggested that OmpC and LPS of K-12 could not function as receptors independently, but the complex of OmpC and LPS could serve as a receptor. In this case, it is unknown whether DT of T4 binds to OmpC or LPS on the sacculus. However, considering the fact that T4 had no specificity for sugar sequence of the outer core, a binding site for DT seems to be offered by OmpC. OmpC is a transmembrane protein that exists as a trimer in the outer membrane. Each OmpC monomer has a pore formed by 16 beta-barrels and 8 loops that connect each β-sheet on the extracellular side (Baslé *et al.*, 2006). Trojet suggested that loops of OmpC were used as binding site by T4-like phage in adsorption (Trojet *et al.*, 2011). Similarly to OmpC, the DT is also composed of a trimer of gp37. From these facts, it is likely that the binding of the DT to OmpC

requires simultaneous interaction between respective loops of monomers of OmpC and the DT. However, the pore geometries in the crystal structure of OmpC correspond to a larger conductance than experimentally measured (Baslé *et al.*, 2006) In addition, LPS affected the conductance property of OmpC (Buehler *et al.*, 1991) and LPS was involved in the assembly of OmpC (Ried *et al.*, 1990). All of these observations suggest that the width of OmpC is compressed by the surrounding LPS in the outer membrane, rendering OmpC in a form favorable to interaction with the DT. A preliminary analysis from our group reveals that a phenylalanine in the extracellular loop 4 of OmpC is essential for T4 adsorption. Although this fact demonstrates that a phenylalanine is a binding site of DT, it is difficult to dock three phenylalanines in loop 4 with three any specific amino acids in DT, because the former is arranged wider than the latter. This consideration also supports a compressed form of OmpC in the outer membrane. The observations that shortening of the inner core, but not the outer core, drastically reduced adsorption (Fig. 8B) and that the difference in inner-core structure among B, K-12 and O157 strains hardly affects adsorption (Fig. 7B and 10A) indicate that the intact inner core of LPS is essential for efficient adsorption depending on OmpC and suggest that the length of the inner core is important for compressing OmpC.

Previous studies showed that T4 adsorbed to LPS which has only a glucose in outer core (Yu and Mizushima, 1982) and T4 adsorption to the B strain was inhibited by glucose (Dawes, 1975). In this study, I reevaluated LPS structure that support T4 adsorption independent of OmpC using not only B and K-12 strains but also O157 strain. T4 efficiently adsorbed only to *E. coli* B and to K-12 Δ*waaOB* in the absence of OmpC (Fig. 7D), and LPS purified from these strains inactivated T4 (Fig. 11). Also, T4 could adsorb to O157 Δ*waaI* in the absence of functional OmpC (Fig. 10B). All of these strains commonly have a terminal glucose(s) without a branch in outer core. Interestingly, when only a galactose was branched to Glc I in Δ*waaO* Δ*ompC*, T4 could not adsorb (Fig. 7D) and the LPS of this strain was unable to inactivate T4 (Fig.

11). Taken together with the previous results showing that T4 adsorption to the B strain was inhibited by glucose (Dawes, 1975), a terminal glucose(s) is an important factor for T4 adsorption independent of OmpC and should be a site of DT interaction. T4 could not adsorb to Δ*waaR* Δ*ompC* (Fig. 7D). As mentioned in the result, Δ*waaR* results in two types of LPS. One of them, the shorter LPS, has a terminal glucose (Glc II). The result that long but inert LPS did not inhibit inactivation of T4 by active LPS (Fig. 12) strongly suggests that T4 cannot bind to the shorter LPS of Δ*waaR*, namely that Gal branched to Glc I inhibits T4 adsorption to the terminal glucose, Glc II. Taken together, all of these results suggest that glucose(s) without branch in outer core is essential for T4 adsorption without ompC.

Amino acid substitutions in the DT changed the host range (Tétart *et al*., 1996). However, whether these substitutions are involved in the binding to LPS or OmpC is not determined. Also, Bartual *et al*. proposed two docking models of the DT and OmpC based on the crystal structure of the DT: The DT binds to OmpC vertically using the DT head domain or transversally using the DT head domain and needle domain (Bartual *et al*., 2010). Isolation and characterization of Nik and Nib mutants indicate that sub-regions of DT required for binding to OmpC and LPS do not completely overlap. Nik1, Nik2 and Nik8 have V941E, A955E and G943S substitutions, respectively. All of these residues are located on the lateral surface of the DT head domain (Fig. 18B and C) and when an interaction by one of these amino acids is lost, adsorption dependent on OmpC is inactivated (Fig. 14). These results suggest that DT binds to the center of an OmpC trimer vertically and the lateral surface of the DT head domain interacts at multiple sites with the extracellular loops of OmpC. Niks were inactivated by LPS purified from B strain and Δ*waaOB*, but efficiencies of inactivation of Niks were lower than that of T4. This means substitution of amino acid affected affinity between T4 and LPS. Especially, Nik1 was seldom inactivated by B and Δ*waaOB* LPSs. However, 60% of Nik1 was inactivated in the presence of Mg^{2+} and phosphate (data not shown). This result

suggests that Nik1 has enough affinity for these LPSs to adsorb but the present conditions for T4 inactivation by LPS was not optimum for Nik1.

Nib lost its ability to adsorb to the B strain having two glucoses in the outer core and to Δ*waaOB* Δ*ompC* having a single glucose (Fig. 17). The observation that originally isolated five Nib mutants had the same amino acid substitution of T939I (Fig. 18A), implicating a narrow interaction site with a terminal glucose in the outer core of LPS. T939 is located at the top of the DT head domain and is flanked with glycines, making the side chain of threonine exposed. In this connection, Krisch and his colleagues reported that a substitution of G938V rendered T4 unable to grow on B strain (Tétart *et al.*, 1996). Valine has a side chain with a branch and is more bulky than glycine. Therefore, it would be reasonable to assume that a valine next to T939 hinders an interaction of T939. Therefore, T939 plays a key role in the recognition of a terminal glucose in the outer core. Supporting this conclusion, Bartual *et al*. lined up some amino acids as candidates to bind to a glucose of LPS based on the crystal structure (Bartual *et al.*, 2010), and one of these candidates, Y949, is mapped in the top of the DT head domain, although other candidates are not mapped in the top. Interestingly, T939 in Nib is not included in these candidates. Further study of whether these candidates are indeed involved in binding to a glucose or other sugars will give a hint towards more understanding of the interaction between the DT and LPS.

Tables

Strains	Genotype	Source/Reference
TY0807	MH1 *araD*$^{+}$	Koga *et al.*, 2011
BW25113	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	NBRP-*E. coli* at NIG
JW3601	BW25113 Δ*waaR*::*kan*	NBRP-*E. coli* at NIG
JW3602	BW25113 Δ*waaO*::*kan*	NBRP-*E. coli* at NIG
JW3606	BW25113 Δ*waaG*::*kan*	NBRP-*E. coli* at NIG
TY0703	BW25113 Δ*waaOB*::*cm*	This study
TY0707	BW25113 Δ*waaF*::*cm*	This study
TY0708	BW25113 Δ*waaC*::*cm*	This study
JW2203	BW25113 Δ*ompC*::*kan*	NBRP-*E. coli* at NIG
TY0721	BW25113 Δ*waaR* Δ*ompC*::*kan*	This study
TY0722	BW25113 Δ*waaO* Δ*ompC*::*kan*	This study
TY0723	BW25113 Δ*waaOB*::*cm* Δ*ompC*::*kan*	This study
TY0726	BW25113 Δ*waaG* Δ*ompC*::*kan*	This study
TY0727	BW25113 Δ*waaF*::*cm* Δ*ompC*::*kan*	This study
TY0728	BW25113 Δ*waaC*::*cm* Δ*ompC*::*kan*	This study
BB	B strain wild-type *sup*^{0}	Kai *et al.*, 1999
B40su1	B strain *supD*	Kai *et al.*, 1999
O157:H7	Wild-type (ATCC43888)	Morita, 2002
TY0731	O157:H7 Δ*waaI*::*cm*	This study
TY0732	O157:H7 Δ*waaJ*::*cm*	This study
TY0750	O157:H7 Δ*ompC*::*kan*	This study
TY0751	O157:H7 Δ*waaI*::*cm* Δ*ompC*::*kan*	This study
TY0752	O157:H7 Δ*waaJ*::*cm* Δ*ompC*::*kan*	This study

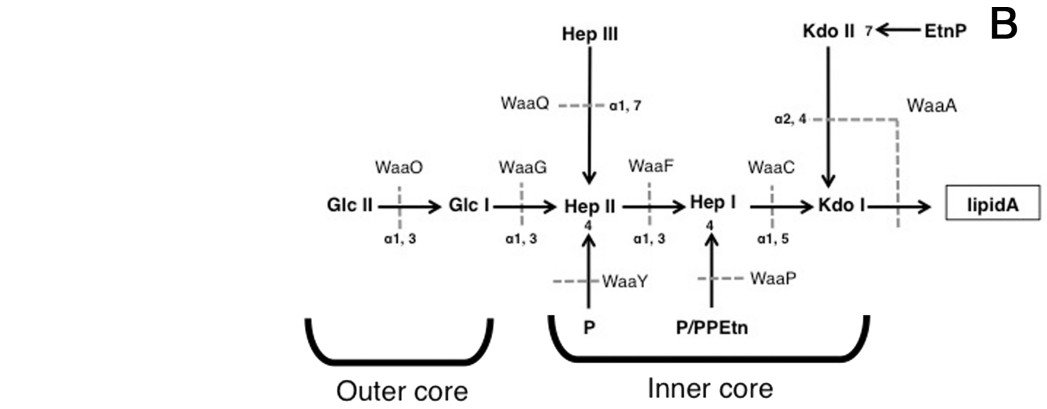
Table 1. Bacterial strains used in this study.

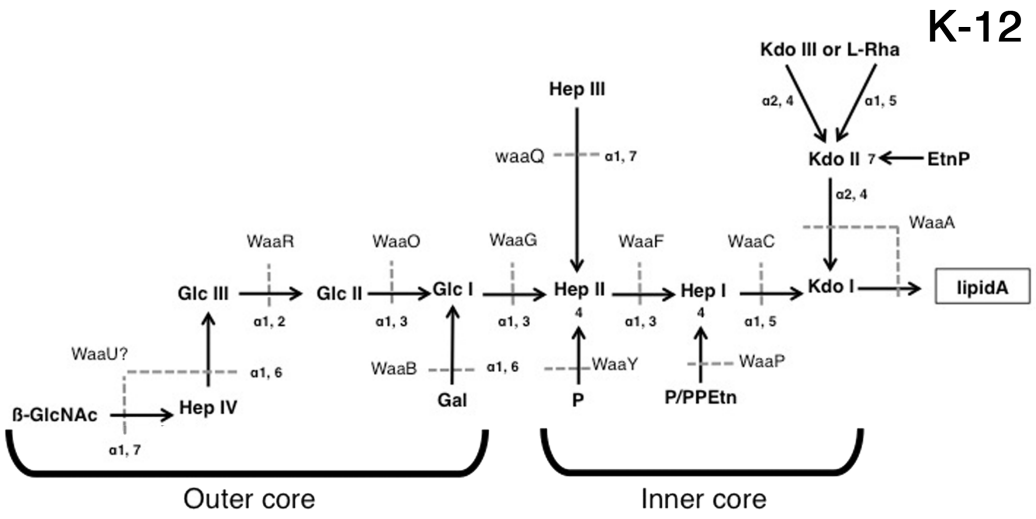
Plasmid used for marker rescue assay	Emergence rate (%)
pLTF	< 0.015
pLTF-Nik1	0.43
pLTF-Nik2	0.90
pLTF-Nik8	0.57
pLTF-Nib	4.7

Table 2. Marker rescue test between am g*37* g*38* and pLTF, pLTF-Nik1, pLTF-Nik2, pLTF-Nik8 and pLTF-Nib.

Marker-rescue test was performed as described in materials and methods. The emergence rate (%) shows the ratio of the number of phage plaques showing same host range as Nik or Nib to the total number of phage plaques. Each value indicates the mean of three independent experiments. The mutations located in genes *37* and *38* in Nik and Nib mutants caused the changes of host range of T4 adsorption.

Figures


B
Hep III
Kdo II 7 ← EtnP
WaaQ α1, 7
α2, 4
WaaA
WaaO
WaaG
WaaF
WaaC
Glc II
Glc I
Hep II
Hep I
Kdo I
lipidA
α1, 3
α1, 3
4
α1, 3
4
α1, 5
WaaY
WaaP
P
P/PPEtn
Outer core
Inner core


K-12
Kdo III or L-Rha
α2, 4
α1, 5
Hep III
waaQ α1, 7
Kdo II 7 ← EtnP
α2, 4
WaaA
WaaR
WaaO
WaaG
WaaF
WaaC
Glc III
Glc II
Glc I
Hep II
Hep I
Kdo I
lipidA
α1, 2
α1, 3
α1, 3
4
α1, 3
4
α1, 5
WaaU?
α1, 6
WaaB
α1, 6
WaaY
WaaP
ß-GlcNAc
Hep IV
α1, 7
Gal
P
P/PPEtn
Outer core
Inner core

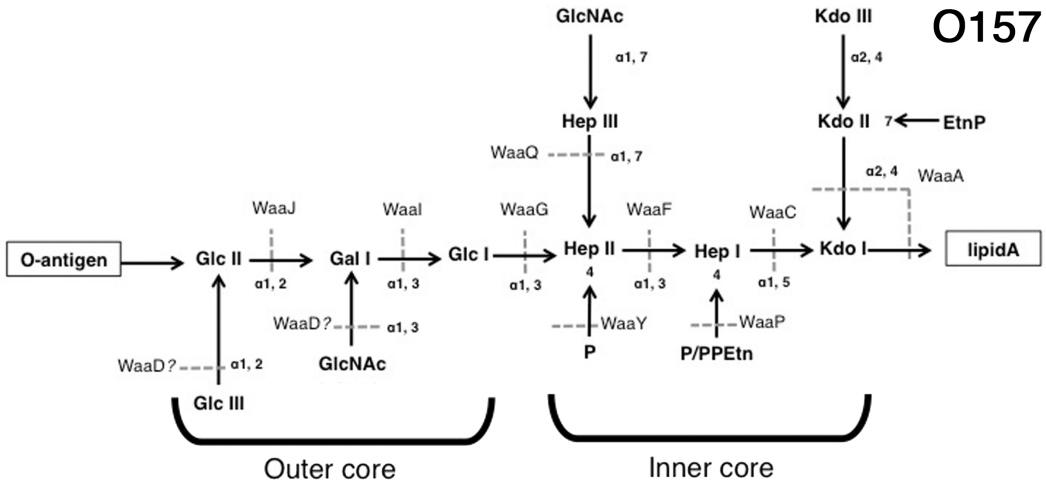

O157
GlcNAc
α1, 7
Kdo III
α2, 4
Hep III
Kdo II 7 ← EtnP
WaaQ α1, 7
α2, 4
WaaA
WaaJ
WaaI
WaaG
WaaF
WaaC
O-antigen
Glc II
Gal I
Glc I
Hep II
Hep I
Kdo I
lipidA
α1, 2
α1, 3
α1, 3
4
α1, 3
4
α1, 5
WaaD?
α1, 3
WaaY
WaaP
WaaD?
α1, 2
GlcNAc
P
P/PPEtn
Glc III
Outer core
Inner core

Figure 1. Structures of LPS in *E. coli* B, K-12 and O157 strains.

Structures of LPS in B (top panel), K-12 (middle panel) and O157 (bottom panel) strains are shown. Horizontal and vertical arrows indicate a main chain and sugar branches, respectively. Dotted lines show the reactions catalyzed by sugar transferases. The abbreviations used in the figure are; Glc Glucose; Gal, Galactose; GlcNAc, N-acetylglucosamin; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Hep, L-glycero-D-manno heptose; Rha, Rhamnose; EtnP, ethanolamine phosphate; PPEtn, 2-aminoethyl diphosphate; and P, Phosphate.

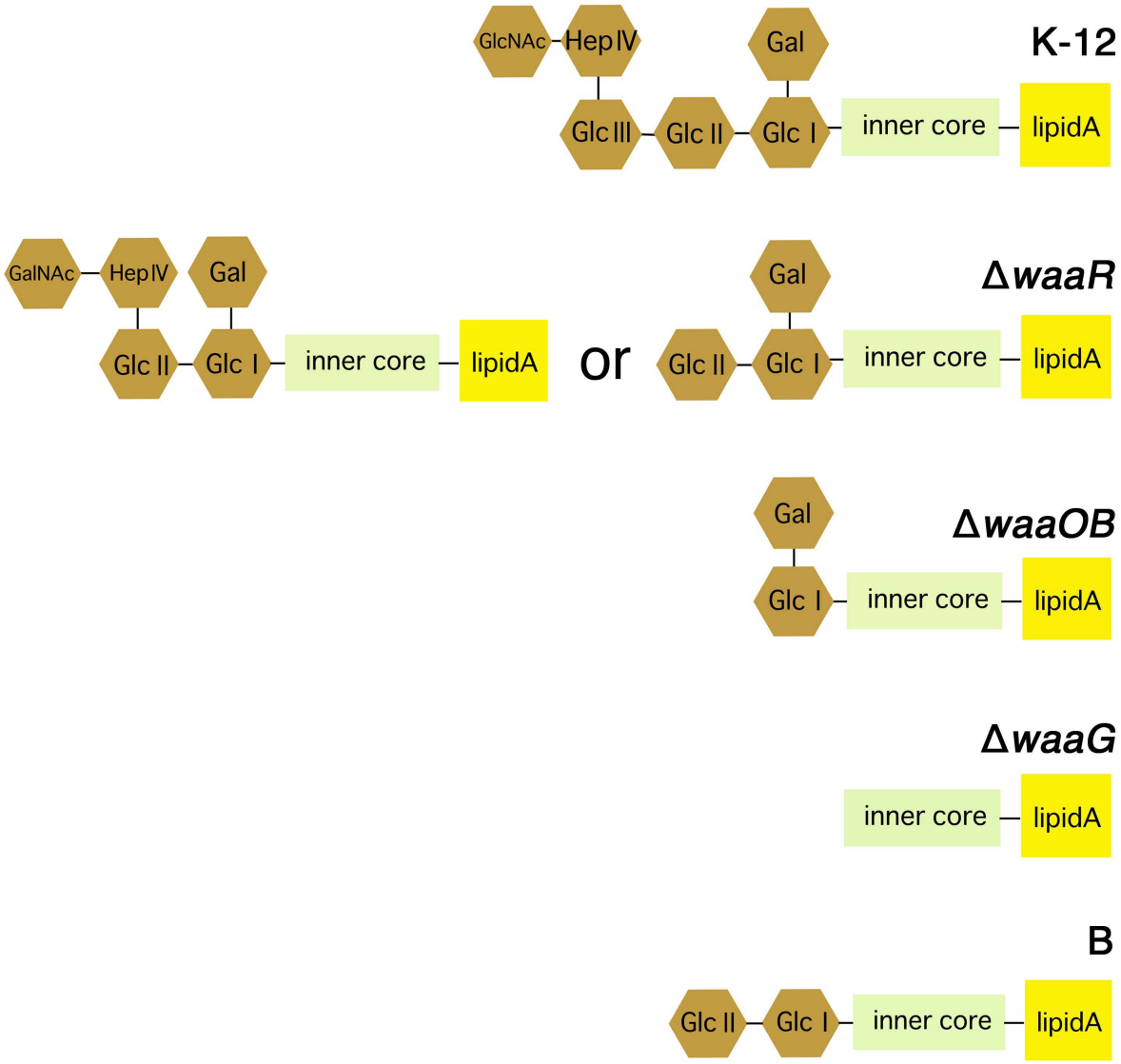


Figure 2. LPS structure of K-12 outer core mutants

LPS structures of K-12 outer core mutants are shown. The abbreviations used in the figure are described in the legend of Fig. 1. Yellow, green and brown parts show lipid A, inner core and outer core respectively.

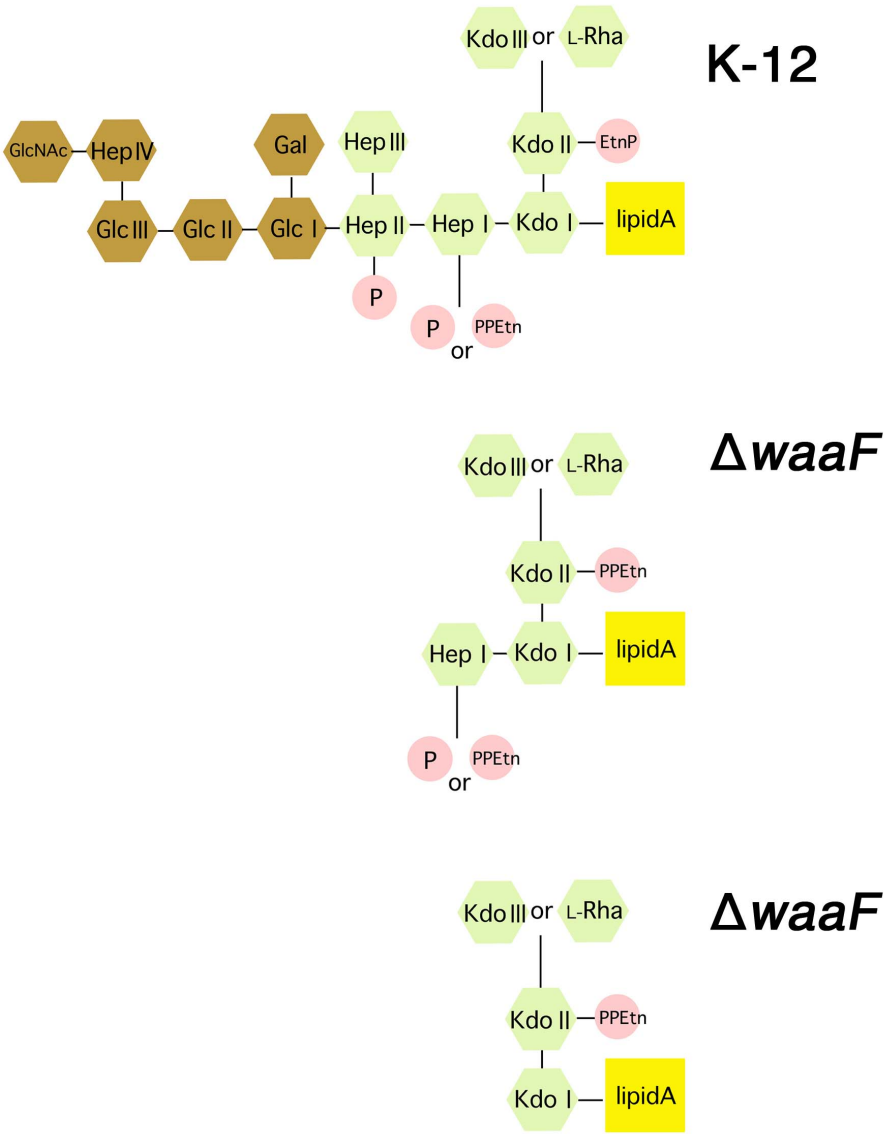


Figure 3. LPS structure of K-12 inner core mutants

LPS structures of K-12 inner core mutants are shown. The abbreviations used in the figure are described in the legend of Fig. 1. Yellow, green and brown parts show lipid A, inner core and outer core respectively.

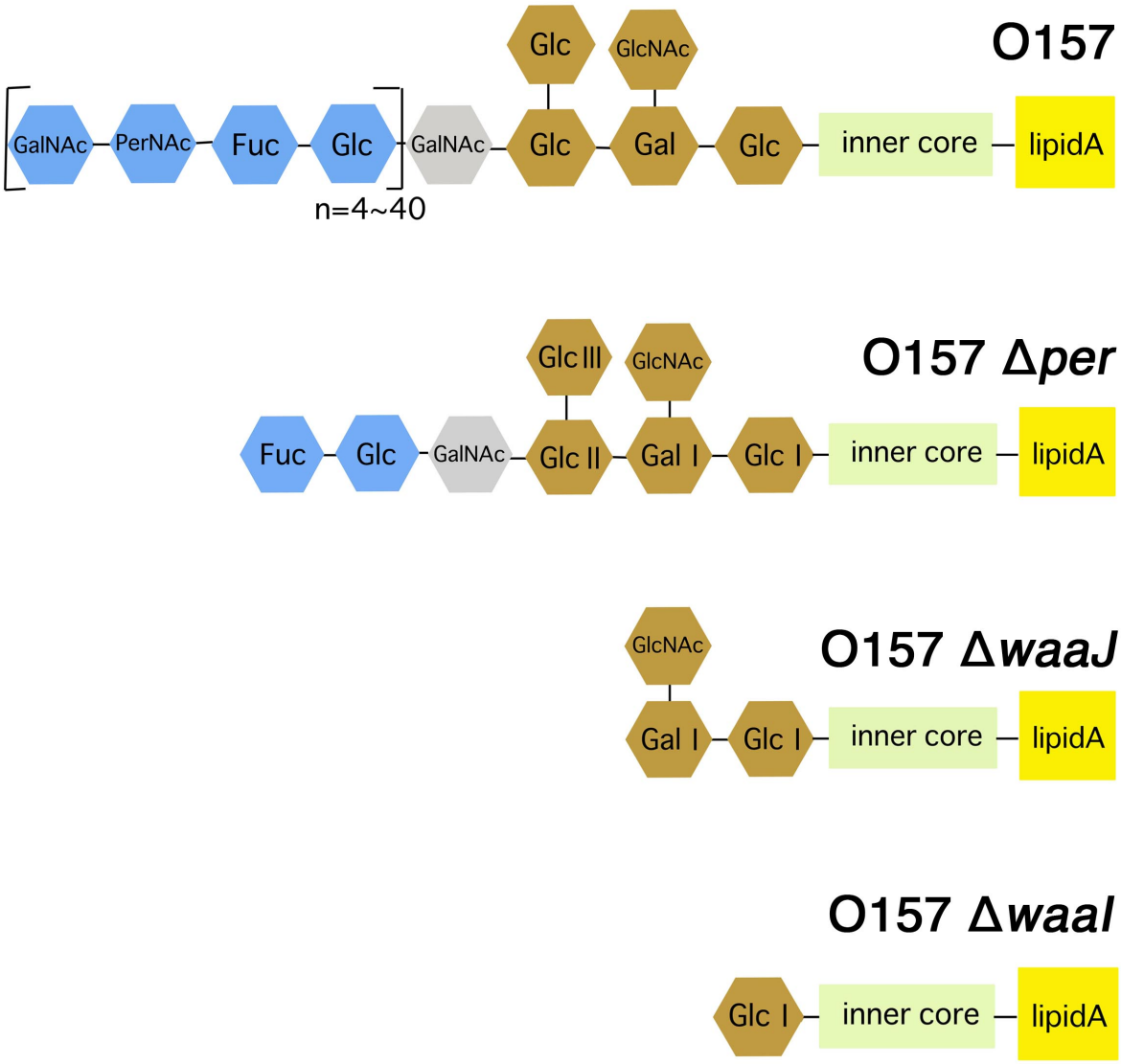


Figure 4. LPS structure of outer core mutants

LPS structures of O157 LPS mutants are shown. The abbreviations used in the figure are described in the legend of Fig. 1. Yellow, green, brown and blue parts show lipid A, inner core, outer core and O-antigen respectively.

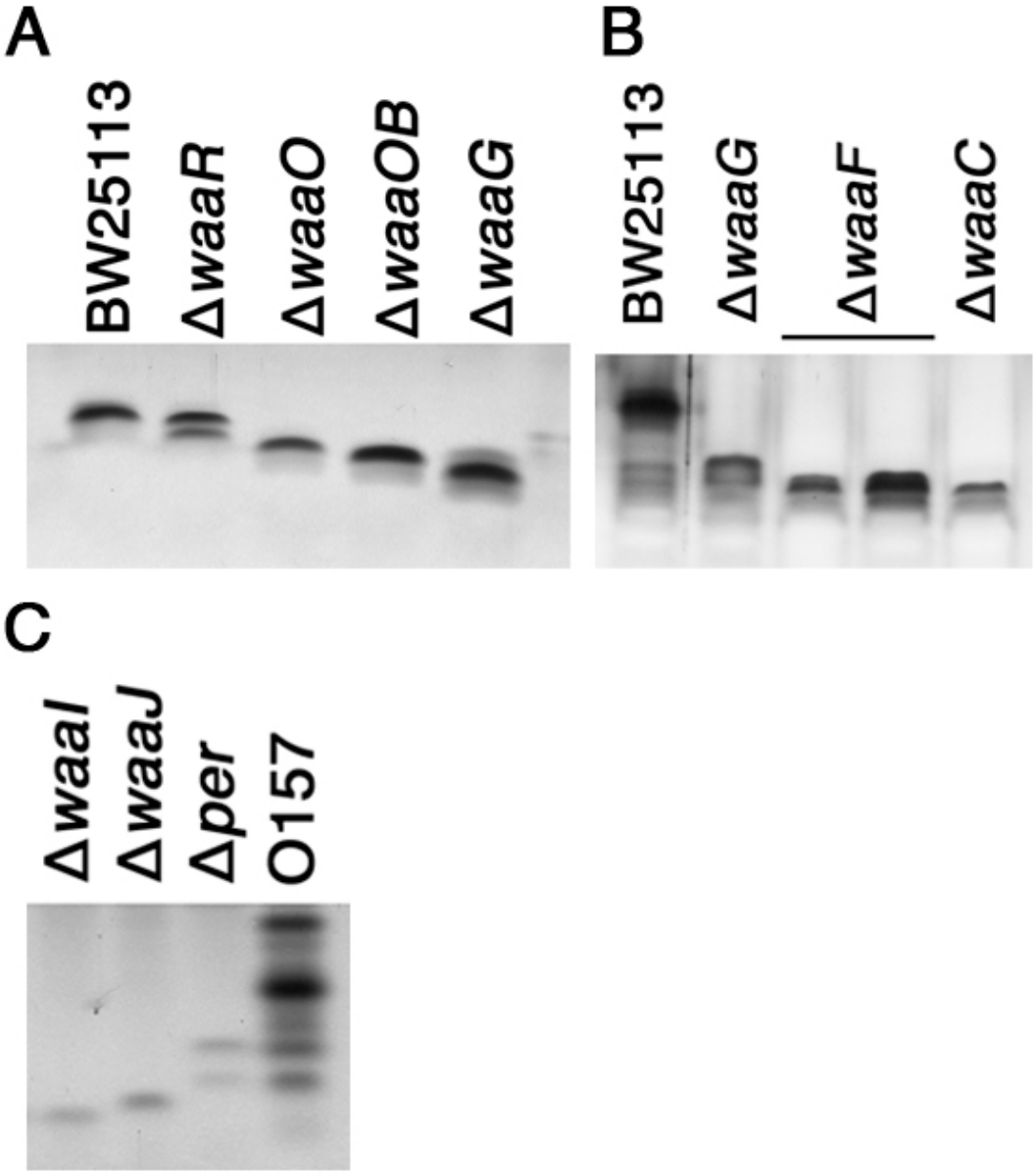


Figure 5. Analysis of LPS purified from *E. coli* strains.

(A) 120 μg of LPS purified from the *E. coli* strain indicated at the top was analyzed by 20% Tricine-SDS gel and silver staining. (B and C) 20 μl of LPS solution purified from *E. coli* strain indicated at the top was analyzed by 20 % Tricine-SDS gel and silver staining.

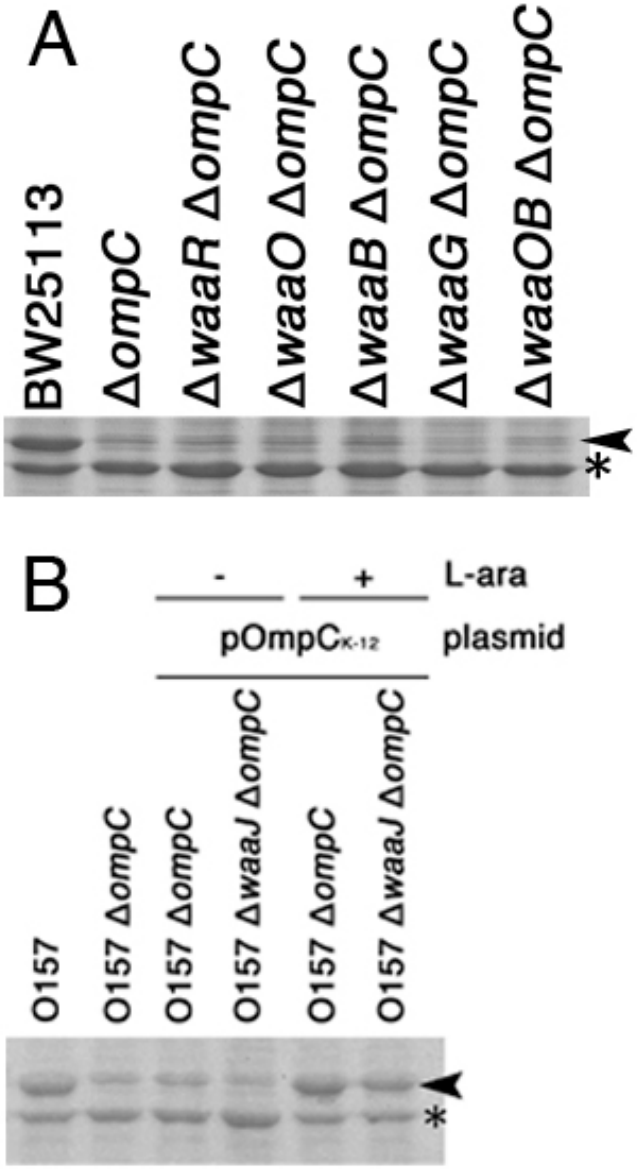


Figure 6. Expression of OmpC in each OmpC deletion mutant and mutant harboring $pOmpC_{K\text{-}12}$.

(A) OmpC was extracted from E. coli cells as described in materials and methods and analyzed by 15 % SDS-PAGE and CBB staining. (B) OmpC was induced from plasmid by L-arabinose as described in materials and methods and analyzed by 15 % SDS-PAGE and CBB staining. Symbols: allow head, OmpC; star, OmpA

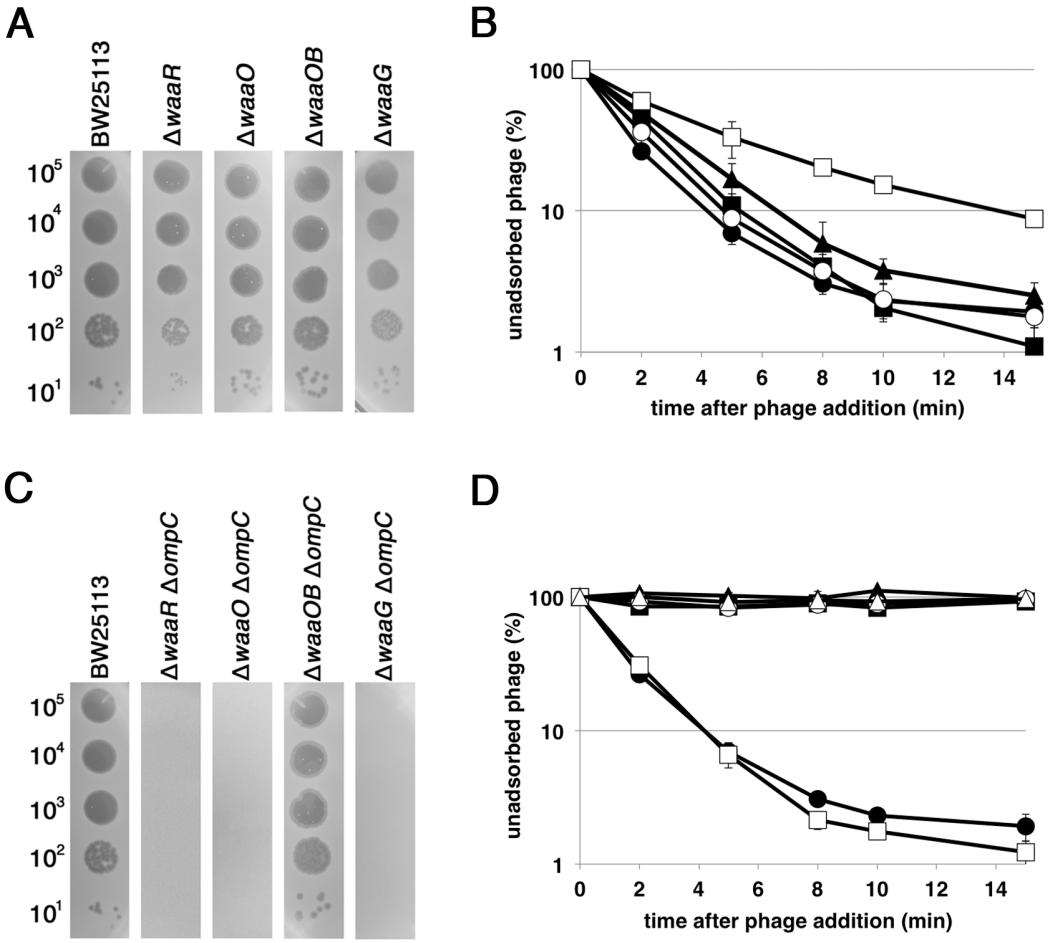


Figure 7. Growth and adsorption of T4 phage on K-12 outer-core mutants.

(A and C) The solution containing the number of T4 phage particles indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37°C overnight. (B and D) Adsorption analyses were performed as described in materials and methods. Symbols for (B): ●, BW25113; ■, Δ*waaR*; ▲, Δ*waaO*; ○, Δ*waaOB*; □, Δ*waaG*. Symbols for (D): ●, BW25113; ■, Δ*ompC*; ▲, Δ*waaR* Δ*ompC*; ○, Δ*waaO* Δ*ompC*; □, Δ*waaOB* Δ*ompC*; △, Δ*waaG* Δ*ompC*.

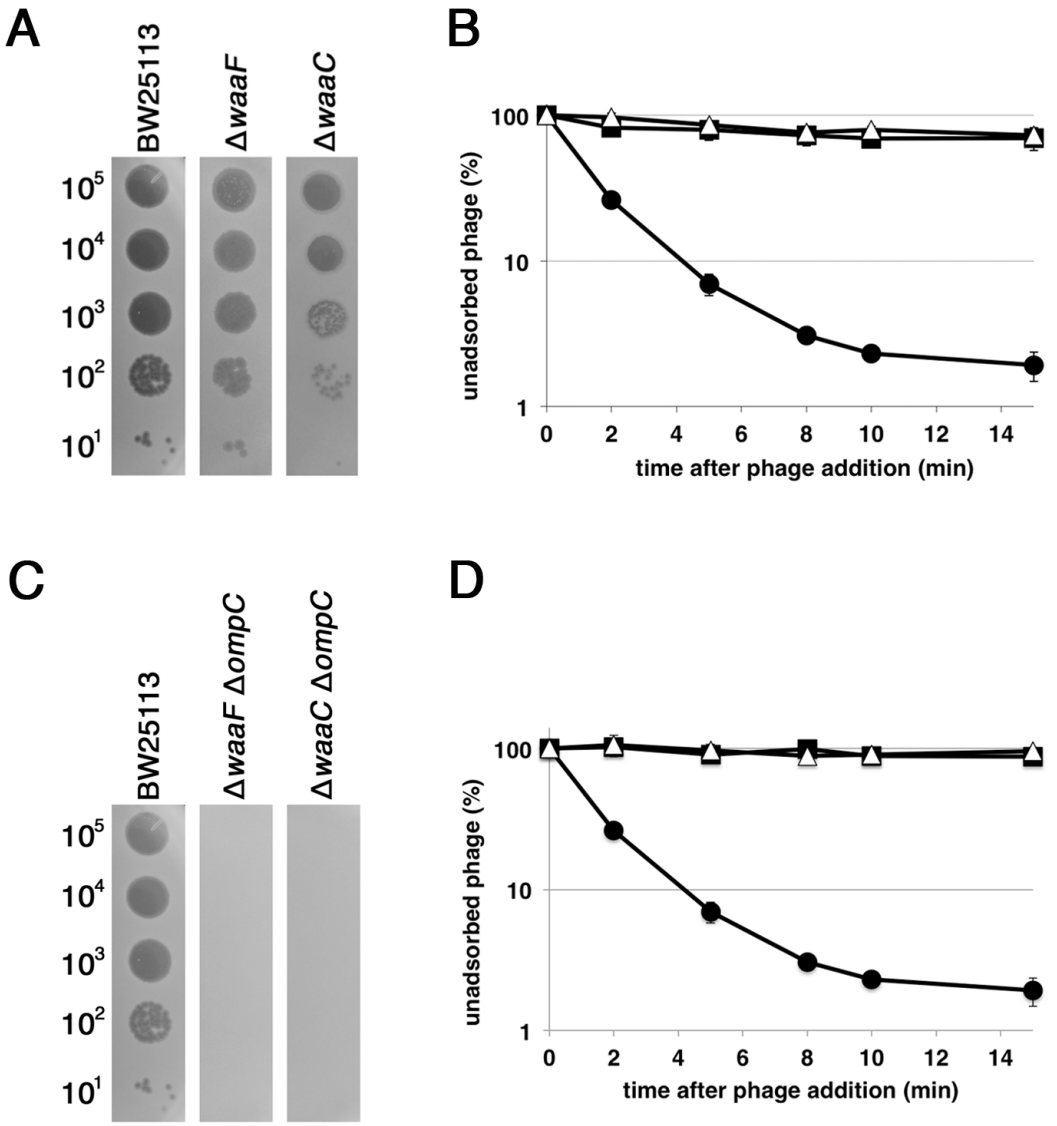


Figure 8. Growth and adsorption of T4 phage on K-12 inner-core mutants.

(A and C) The solution containing the number of phage particles indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37°C overnight. (B and D) Adsorption analyses were performed as described in materials and methods. Symbols for (B): ●, BW25113; ■, Δ*waaF*; △, Δ*waaC*. Symbols for (D): ●, BW25113; ■, Δ*waaF* Δ*ompC*; △, Δ*waaC* Δ*ompC*.

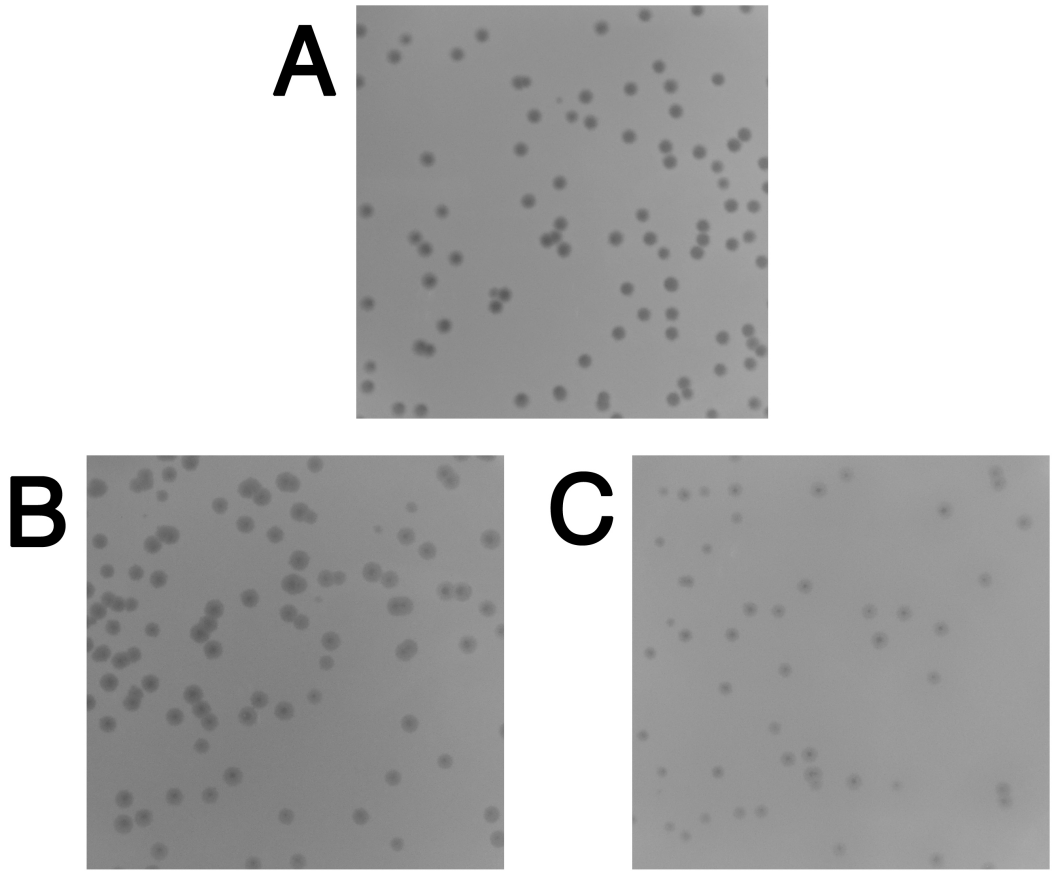


Figure 9. Morphology of plaques of Inner core mutants

About 200 T4 particles were plated on a plate with indicator cells (A, BW2513; B, Δ*waaF*; C, Δ*waaC*) and incubated at 37 °C overnight.

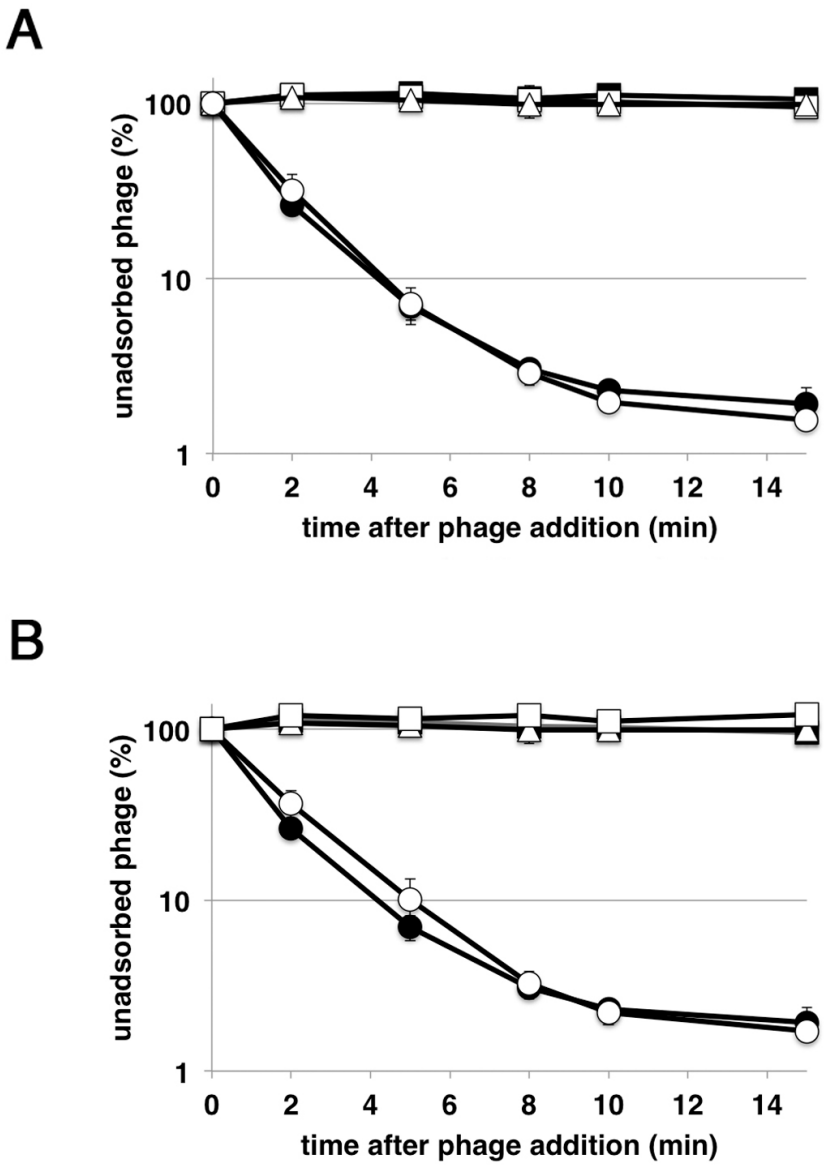


Figure 10. Adsorption of T4 on O157 LPS mutants.

(A) *E. coli* mutants harboring pBAD33-*ompC*$_{K\text{-}12}$ were incubated in LB medium until the OD_{600} reached 0.3, and arabinose was added to a final concentration of 0.2% for 30 min. Adsorption analyses were performed as described in materials and methods. Symbols: ●, BW25113; ■, O157; △, O157 Δ*waaJ*; ○, O157 Δ*waaJ* Δ*ompC* harboring pBAD33-*ompC*$_{k\text{-}12}$; □, O157 Δ*ompC* harboring pBAD33-*ompC*$_{k\text{-}12}$. (B) Adsorption analyses were performed as described in materials and methods. Symbols: ●, BW25113; ■, O157; □, O157 Δper; △, O157 ΔwaaJ; ○, O157 ΔwaaI.

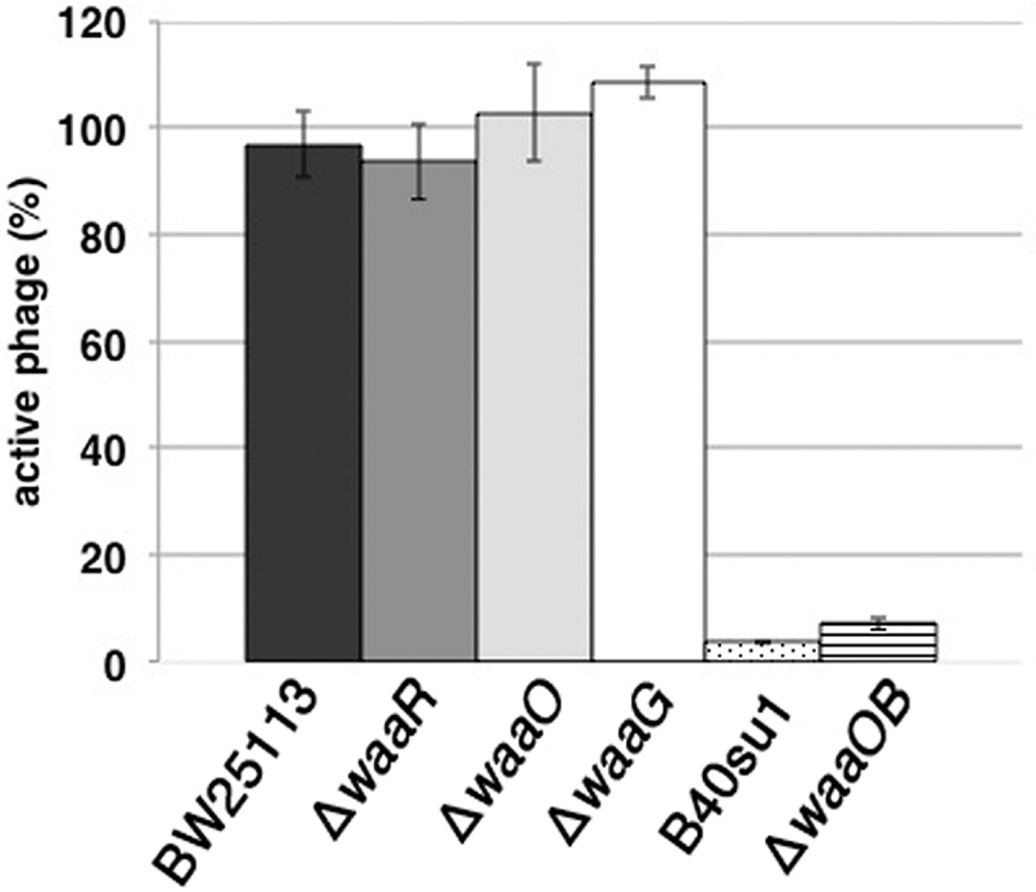


Figure 11. Inactivation assay of T4 phage using purified LPS.

A solution containing 3 x 10^7 T4 phage particles was mixed with 35 μM of LPS purified from the *E. coli* strains indicated at the bottom and then the inactivation assay was performed as described in materials and methods.

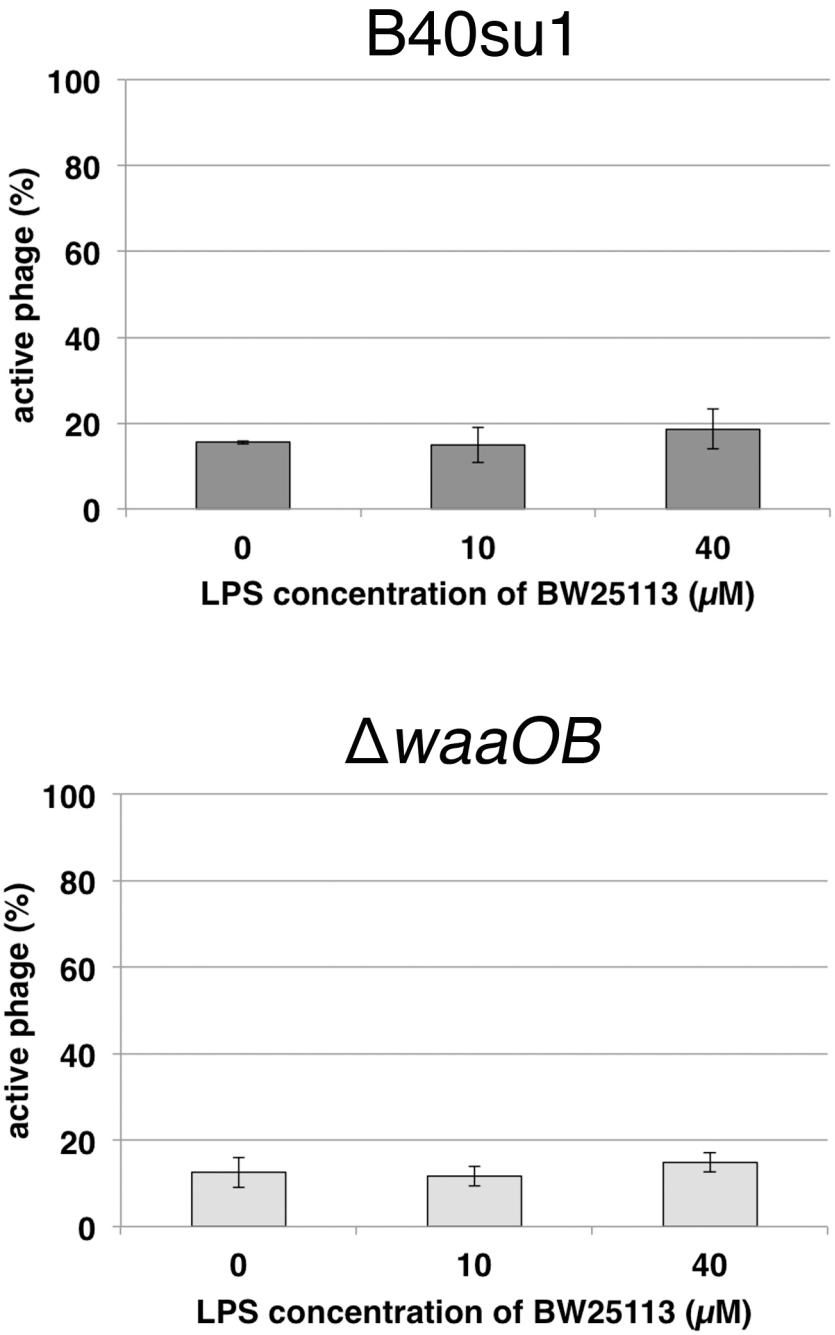


Fig 12. Inactivation assay with LPS from two strains of bacteria

10 μM of LPS purified from the strain indicated top of graph was mixed with BW25113 LPS at the concentration indicated at the bottom. Inactivation assay was performed as described in materials and methods.

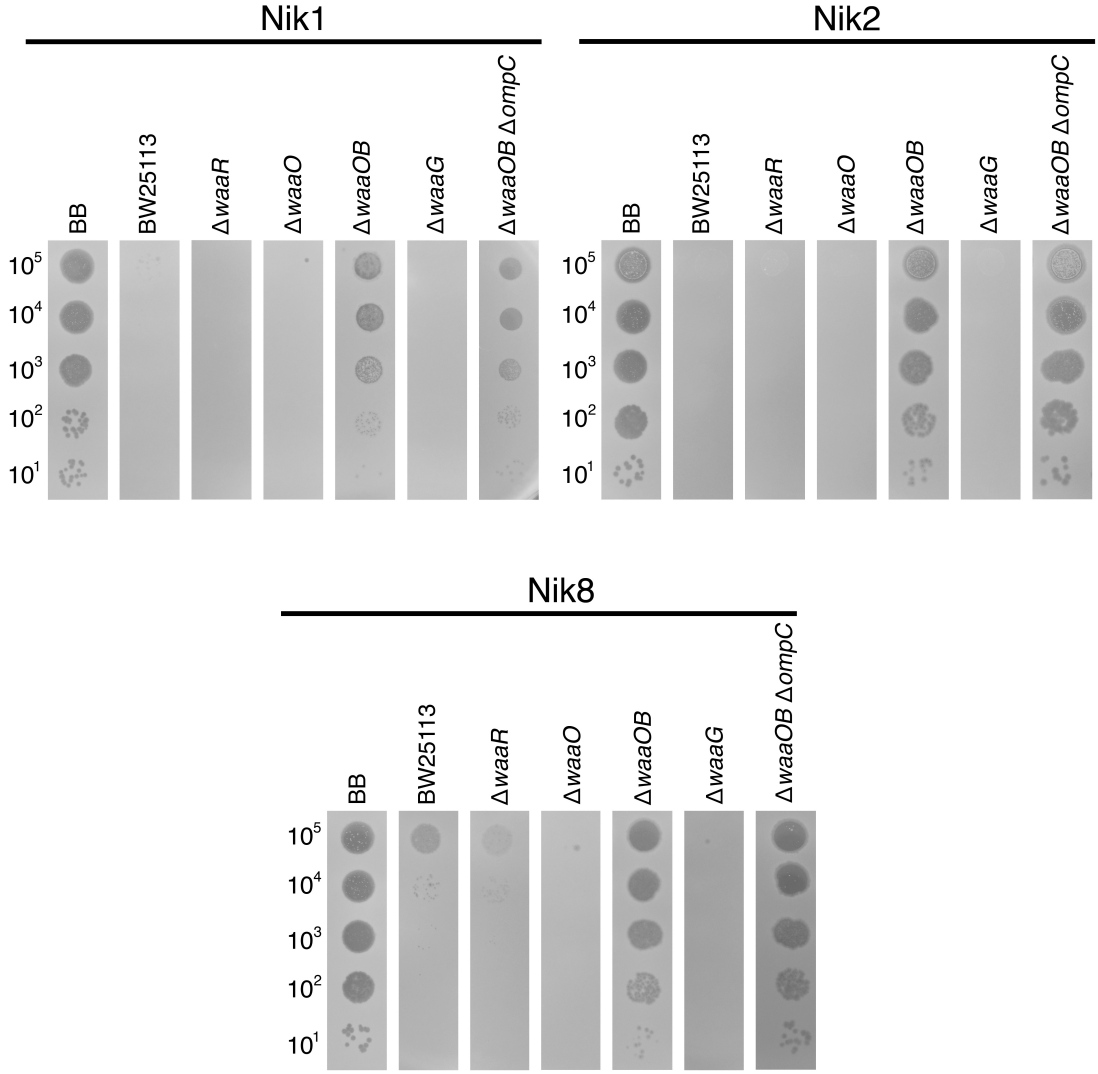


Figure 13. Spottest of T4 Nik mutants.

The solution containing the number of phage particles (Nik1, left panel; Nik2, right panel; Nik8, bottom panel) indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37°C overnight.

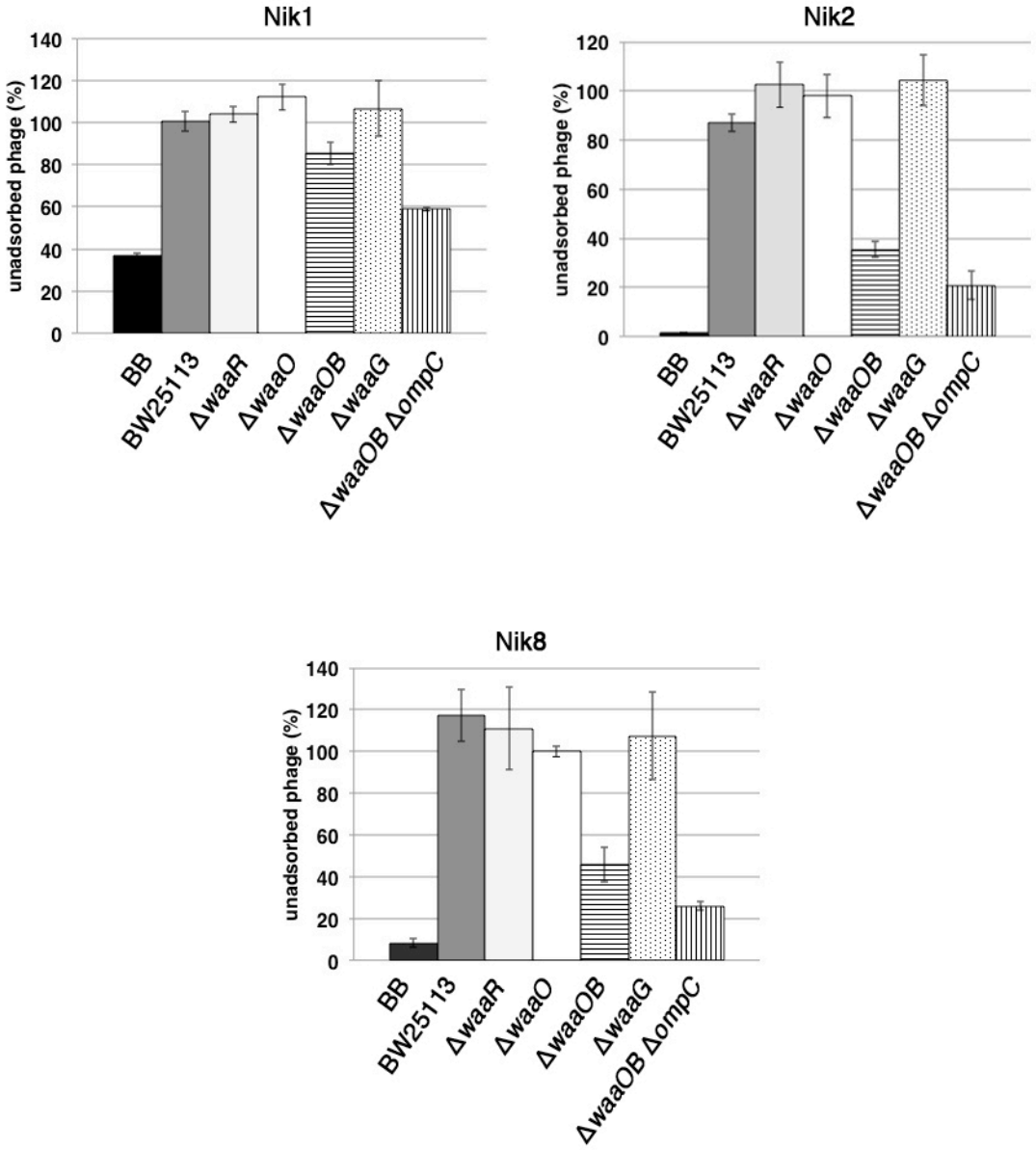


Figure 14. Adsorption assay of T4 Nik mutants.

Adsorption analyses were performed with Nik1 (left graph), Nik2 (right panel) and Nik8 (bottom panel) with the *E. coli* strain indicated at the bottom. The relative numbers of unadsorbed phage particles at 10 min after phage addition were calculated with the numbers of input phage particles set to 100%.

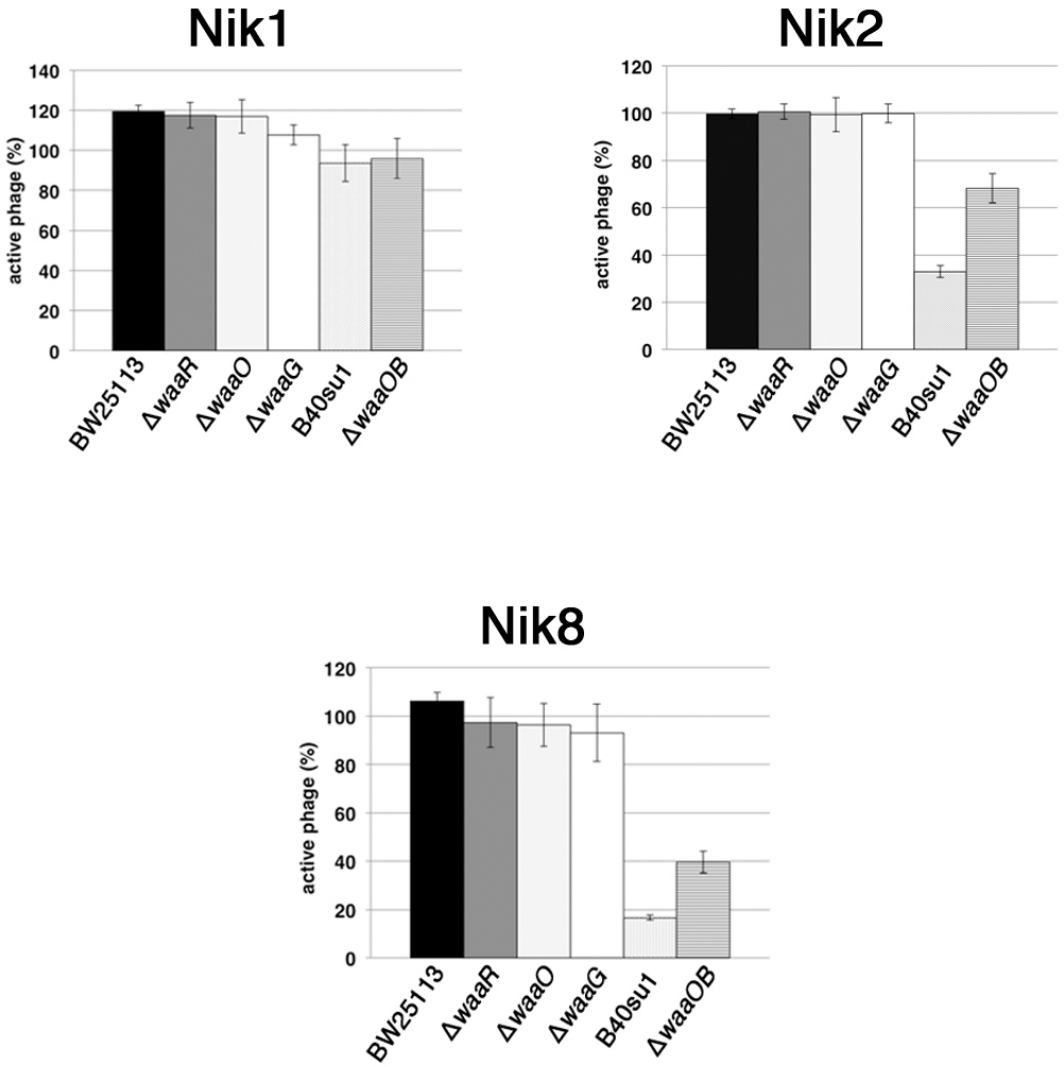


Figure 15. Inactivation assay of Nik mutants.

A solution containing 3 x 10^7 phage particles indicated top of each graph was mixed with 35 μM of LPS purified from the *E. coli* strains indicated at the bottom and then the inactivation assay was performed as described in materials and methods.

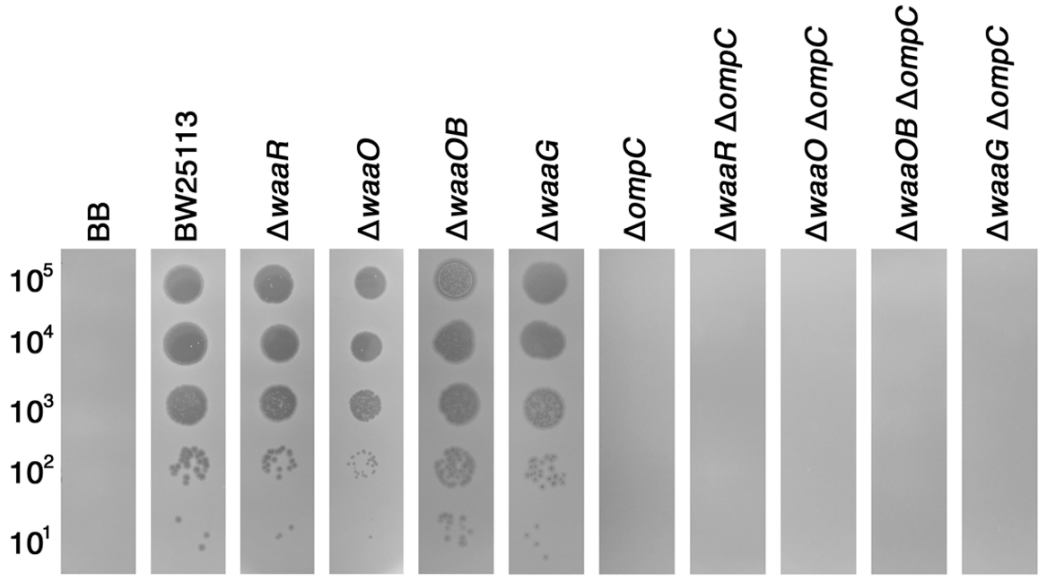


Figure 16. Spot test of Nib

A solution containing the number of Nib phage particles indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37ºC overnight.

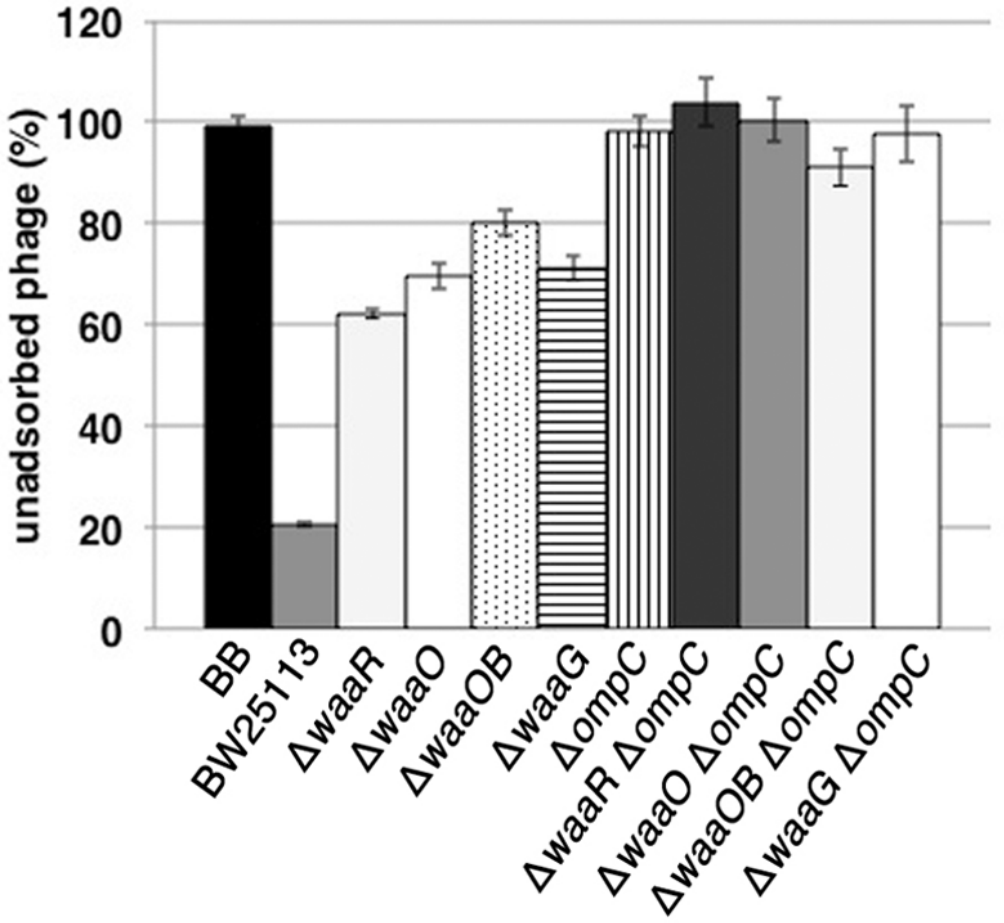


Figure 17. Adsorption assay of Nib

Adsorption analyses with the *E. coli* strains indicated at the bottom were performed as described in materials and methods.

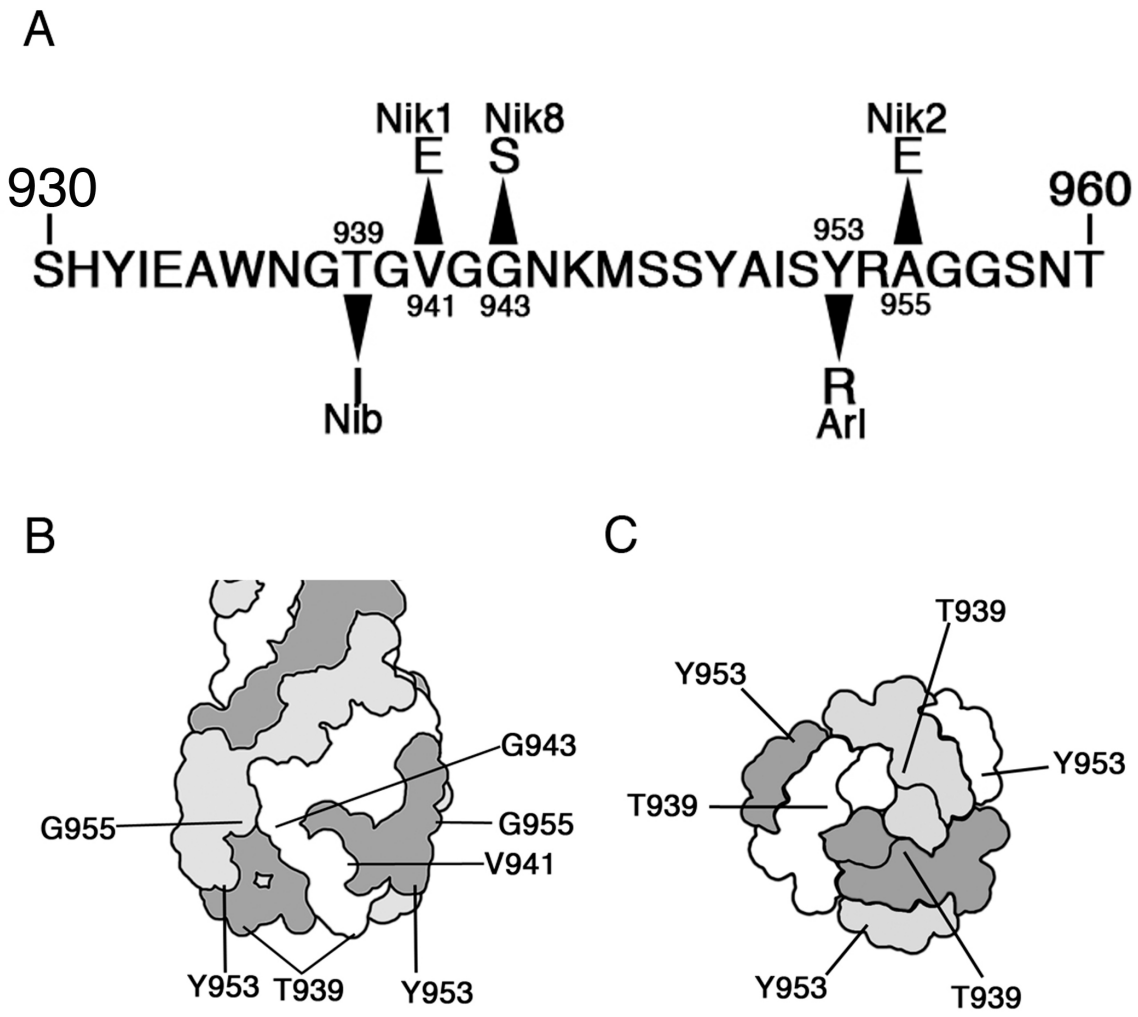


Figure 18. Mutation sites of Niks, Nib and Arl.

(A) Amino acid sequence of the DT region in gp37. Arrowheads indicate substitutions of amino acids in Nik1, 2, 8, and Nib and Arl mutants. Schematic diagrams of the DT region viewed from the lateral surface (B) and the top surface (C) are shown. Lines indicate positions of Niks, Nib and Arl mutations.

Chapter 2.

Challenge for modification of host range of T4 and future prospect of phage therapy by T4 phage.

Abstract

For application of phage therapy, the construction of the method to modify host range is needed. Strict host specificity of T4 is mainly determined by adsorption step. Here we isolated endogenous mutant, Arl-R. Arl-R could adsorb to Δ*waaR*Δ*ompC* which wildtype T4 cannot adsorb. Arl-R has two base substitutions C terminal region of gene *37* and single amino acid were substituted tyrosine for isoleucine. These mutations made Arl-R bind to Δ*waaR*Δ*ompC* LPS. Mutated tyrosine located at the border between lateral and top surface of head domain of long tail fiber and side chain of tyrosine protrudes toward top surface. This shows that host range of T4 can be expanded to become a tool for phage therapy. T4 cannot grow on O157 Δ*waaI* even though T4 can adsorb to it. To analyze which step of life cycle of T4 was aborted, gene expression and DNA replication was examined. T4 neither replicate nor express its genome but DNA injection into bacteria was observed.

Introduction

As mentioned in general introduction, bacteriophage has strict host specificity. This is the one of problems in the application of phage therapy. So far, when new pathogenic bacteria or phage resistant bacteria appear, scientists have tried to isolate new phages that can infect the bacteria from nature. However, isolation of useful phages is not guaranteed. Moreover, even if such a phage is luckily isolated, its safety in the usage for phage therapy must be ensured by various ways at least in today's advanced country. As a minimal condition, whole genome analysis is essential (Brüssow, 2012; Henein, 2013). However, it is time consuming and costs high expenses.

Modifying host range of a phage with established safety artificially to infect pathogenic bacteria beyond its natural hosts is one of practical ways to alleviate this problem (Fig. 19). There are 3 key points to achieve this; to pick up appropriate candidate phages for therapy, construction of the method to modify host range of given phage and to understand the host range of the modified phage to know whether we need more candidate phages.

Previous works reveal that the adsorption of phage to host cells is one of the major factors for host range determinant. In addition, there are a variety of bacterial resistant mechanisms against phages to determine host ranges of phages other than adsorption step, such as restriction-modification system, abortive infection (Abi) system, clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas system (Samson *et al.*, 2013) and toxin-antitoxin system (Otsuka and Yonesaki, 2012). Thus we can modify host range of phage by making T4 overcome these host range determinants. Modification in adsorption machinery will have lower risk of changing safety of phage than modification in growth mechanism of phage. Accordingly, an attempt to establish a method to modify adsorption step is important.

Bacteriophage T4 is one of the most appropriate candidates for phage therapy because it has been well studied in molecular biology for decades, the whole genome is sequenced and it is recognized safe. T4 infects only limited strains of *Escherichia* and *Shigella* (Miller, 1949; Jesaitis and Goebel, 1953). However, T4 can produce progeny in some bacteria other than *Escherichia* and *Shigella*, such as *Salmonella*, *Aerobacter*, *Proteus*, *Serratia* and *Yersinia*, by injecting T4 genomic DNA into spheroplasts of these bacteria (Wais and Goldberg, 1969; Dawes, 1975). This fact indicates that T4 phage can infect a variety of bacteria beyond its natural hosts when T4 can absorb to them. From these facts, T4 may be a candidate for phage therapy when a method to modify adsorption machinery is introduced.

As described in Chapter I, DT region of Gp37 is important for adsorption and determination in host range and can bind to different types of molecules, protein (K-12 OmpC) and LPS that has only glucose(s) without branch. Binding to OmpC requires multiple amino acids in the lateral surface of the DT head domain. In contrast, binding to LPS may require a narrow interaction site in the top surface of the DT head domain and the top surface is consisted only by 8 amino acids. Thus it would be reasonable to assume that modification of affinity between DT and LPS is easier than that between DT and protein.

In this study, as an initial step for manipulating T4 host specificity toward phage therapy, I isolated various T4 mutants, Arl-R (altered recognition of LPS) that can bind to LPS for which wild-type T4 does not have affinity. An isolated mutant, Arl-R, which has affinity for LPS of ΔwaaR has an amino acid change from Threonine at 953 to Arginine.

During the work described in Chapter 1, I found that T4 did not produce progeny in O157 Δ*waaI*, even if T4 could adsorb to it efficiently, suggesting that O157 has a mechanism to block the growth of T4 other than inhibition of adsorption by O-antigen. Since O157 is pathogenic and a challenging target of T4 in phage therapy, it would be valuable to clarify the reason. To understand which step inhibits

T4 growth on O157, I analyzed the specific step of infection cycle of T4 that did not work in O157. T4 could inject its DNA into O157 after adsorption but neither DNA replication nor gene expression was observed at all.

Materials and Methods

E. coli strains and phages

Escherichia coli strains used in this Chapter are listed in Table 1. Wild-type phage is T4D (Doermann and Hill, 1953).

Isolation of Arl mutant phages

When 1.7 x 10^7 pfu of T4 phage were plated with TY0721 (Δ*waaR* Δ*ompC*) as an indicator, 147 faint, small plaques were formed. We randomly picked up 4 plaques, and those phages were plated with Δ*waaR* Δ*ompC* cells again. Next, a single bigger and clearer plaque from each clone was picked up and inoculated into Δ*waaR* Δ*ompC* to prepare a high-titer stock by polyethylene glycol as described previously (Yamamoto *et al.*, 1970) and prepared phage were named Arl-R1, Arl-R2 and Arl-R3. Other Arl mutant phages were isolated by the similar method as for Arl-R1, Arl-R2 and Arl-R3. Briefly, 4 x 10^9 pfu of T4 were plated with TY0722 (Δ*waaO* Δ*ompC*) and TY0726 (Δ*waaG* Δ*ompC*) as indicator cells and two and one very faint and small plaque(s) were isolated on respectively. Mutant phages were purified from the plaque and plated again. After this plating, we were able to obtain more than 1,000 plaques on each plates and purified phage from softagar of each plate. Purified phage are plated and two clearer plaques were picked up. Two mutant phages from clearer plaques were plated again and purified from soft agar as described above. Mutant phages that can grow on Δ*waaO* Δ*ompC* were named Arl-O1 and Arl-O2 and phages that are able to grow on Δ*waaG* Δ*ompC* were named Arl-G1 and Arl-G2. For

isolation of Arl mutants that are able to grow on JW2203 (Δ*ompC*), 250 pfu of T4 were plated with Δ*ompC*. Three clear plaques were found. High-titer stocks of three mutant phages were prepared in M9C medium. These mutant phages that are able to grow on Δ*ompC* were named Arl-Wt1, Arl-Wt2 and Arl-Wt3. The sequence of the DT region was determined as described above. Sequence analysis for DT region of Arl-R mutants were performed using two primers, 5′-AAGTCCGCATATCCAAAGTTAGCTGTTGC and 5′-TATATTTTCATATTTAGAAGGGCCGAAGC.

Recombination assay between Arls and pLTF

Arl-Wt1, Arl-Wt2, Arl-Wt3, Arl-R1, Arl-R2 and Arl-R3 were spotted on plate BW25113 harboring pLTF was seeded. About 20 plaques were randomly picked up and diluted with dil2. Phages in dil2 was plated with TY0807 (K-12 Wild type) as indicator cell and formed single plaques. Plaques obtained in this step were randomly selected and spotted on two plates with appropriate indicator cells to check the percentage of phage gained phenotype of Wt by recombination.

Adsorption assay

Adsorption assay of Arl was carried out as described in materials and methods of Chapter 1.

Inactivation assay of Arl by purified LPS

Inactivation assay of Arl was carried out as described in materials and methods of Chapter 1.

Analysis of T4 DNA expression by pulse label of [^{35}S] methionine

Cells were incubated at 37°C until OD_{600} reach 0.6 in M9 with 0.3% of casamino acids. Centrifuged 3 ml of culture at 5,000 rpm for 5 min at 4°C and resusbended pellet with 1.5 ml of M9 with 0.1% of casamino acids. Before inoculation of phage, took 100 μl of culture and added 1 μl of [^{35}S]methionine. This is the 0 min sample. Incubated at 37°C for 3 min then added 2 μl of 20% of casamino acids to stop labeling. Remained culture was inoculated by T4 at m.o.i is 20 at time 0. At 2, 4, 7, 10, 15 and 20 min, took an aliquot and labeled for 3 min as described above. All samples were centrifuged at 7,500 rpm for 5 min at 4°C and pellets were resuspended with 40 μl of milliQ. Labeled proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) through 15% gels and visualized by Bio-Image analyzer (Fuji BAS-1800).

Dot southern blotting

Incubated bacteria until OD_{600} reached 0.4 ~ 0.5. T4 phage was inoculated at m.o.i 20 at 0 min. At each time point, took 100 μl of culture and centrifuged at 15,000 rpm for 1 min at 4°C. The pellet was resuspended with 10 μl of BS and 3 μl of them was spotted onto Hybond N+ nylon membrane (GE Healthcare). The membrane was treated sequentially with 10% SDS for 3 min, buffer B [0.5 N NaOH and 1.5 M NaCl] for 5 min, buffer C [0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl] for 5 min, and 1× SSC for 5 min. After drying membrane for 30 min at room temperature, the membrane was hybridized with labeled probe at 42°C overnight. Radioactive probes for T4 phage DNA were prepared by PCR using one primer 5' end-labeled with T4

kinase and [γ-^{32}P]ATP, another unlabeled primer and phage genomic DNA as a template. Primers used for PCR were as follows: 5'-32P-CCTGCAGTAACAAGTTCGGCTC and 5'-CCTGCAGTACTCTCCTCTATA.

DNA degradation analysis

Cells were incubated at 37°C till mid log (4 x 10^8/ ml) and T4D was inoculated at m.o.i = 5 at 0 min. At 0, 0.5, 5, 10 and 20 min, 1.5 ml of culture was taken and centrifuged at 1,000 rpm for 2 min at 4°C. The pellet was resuspended with 300 *μ*l of lysis buffer (0.25 M Tris-HCl (pH 6.8), 10 mM EDTA (pH 8.0), 5% SDS). Degraded protein by adding proteinase K at final concentration 0.5 *μ*g/*μ*l and incubate for 2 h at 65°C. Protein was removed by phenol-chloroform (1:1) solution and stored at 4°C. Sample at 0 min was taken just before inoculation of phage. A part of sample was digested by *Hae* III at 37°C overnight. Samples were separated by 0.8% agarose electrophoresis.

Results

Isolation and host range of T4 Arl mutants

As an initial step for manipulating T4 host specificity toward phage therapy, I attempted to isolate Arl (altered recognition of LPS) mutants that can recognize LPS, which wild-type T4 cannot do. For this purpose, I looked for T4 mutants that could form plaques on Δ*ompC*, Δ*waaR* Δ*ompC,* Δ*waaO* Δ*ompC* and Δ*waaG* Δ*ompC*. As a result, Arl-Wt1, Arl-Wt2, Arl-Wt3, Arl-R1, Arl-R2, Arl-R3, Arl-O1, Arl-O2, Arl-G1 and Arl-G2 were isolated.

Arl-Wt1~3 were mutants that can grow on Δ *ompC*. They grew and formed as clear plaques as T4 wild-type formed on BW25113 (K-12 wild-type) (Fig. 20). Arl-R1~3 could grow on Δ*waaR* Δ*ompC* and Δ*ompC* though their plaques on Δ*waaR* Δ*ompC* were faint but the efficiency of plating (EOP) was similar to that on BW25113 and Δ*ompC* (Fig. 21). For further study, high titer preparation of phage stock was prepared using a liquid culture in large scale as described in materials and methods. Arl-O1 and Arl-O2 could grow on Δ*waaO* Δ*ompC*, and Arl-G1 and Arl-G2 did on Δ*waaG* Δ*ompC*. Plaques of Arl-O1 and Arl-O2 on Δ*waaO* Δ*ompC* were very faint and pinholes (Fig. 22A). These plaques were hardly seen unless holding plates towards the light. EOP of Arl-O1 on Δ*ompC* decreased by 10-fold compared to that on BW25113, but Arl-O2 showed the same e.o.p on BW25113 and Δ*ompC*. Plaque of Arl-G1 and Arl-G2 on Δ*waaG* Δ*ompC* were barely seen. Arl-G1 can grow only on Δ*waaG* Δ*ompC*, on the other hand, Arl-G2 has weak growth ability on all LPS mutants of K-12. Arl-G1 formed clearer plaques on Δ*waaG* Δ*ompC* than Arl-G2 (Fig. 22B). These results suggest that Arl-G1 is specific to Δ*waaG* Δ*ompC* and Arl-G2 acquires a broad host range. I tried to prepare high titer stocks of these phages, however, growth ability of these phages in a liquid culture were too low to obtain a

sufficient concentration of phage. Therefore, I characterized Arl-Wts and Arl-Rs hereafter.

Analysis of mutation site of Arl-Rs and Arl-Wts

To identify a mutation site, I performed a recombination assay between pLTF and Arl mutants. Table 3 shows that about 20% of Arl-R1, Arl-R2 and Arl-R3 lost the ability to grow on Δ*waaR* Δ*ompC* result from recombination between phage DNA and plasmid, suggesting that Arl-R1, Arl-R2 and Arl-R3 have a mutation in gene *37*. On the other hand, Arl-Wt1, Arl-Wt2 and Arl-Wt3 did not yield a significant fraction of recombinant that cannot grow on Δ*ompC*. This result strongly suggests that the mutation sites of Arl-Wts are outside gene *37*. Hence, I further studied about Arl-R1, Arl-R2 and Arl-R3.

Characterization of Arl-R

Sequence analyses of Arl-R1, Arl-R2 and Arl-R3 demonstrated that all three had the same two base substitutions at T2857C and A2858G of gene *37*, causing one amino acid substitution, Y953R (Fig. 18). Thus I named these three mutants Arl-R. The two base changes would probably have arisen by successive mutational events, because the originally isolated three mutants formed small, faint plaques while finally obtained Arl-R1~3 formed normally sized clear plaques. Host range of Arl-R was analyzed more precisely by spot test. Arl-R could grow on BB, BW25113, Δ*ompC*, Δ*waaR*, Δ*waaOB*, Δ*waaR* Δ*ompC* and Δ*waOB* Δ*ompC*, but not on Δ*waaO*, Δ*waaG*, Δ*waaO* Δ*ompC* and Δ*waaG* Δ*ompC* (Fig. 23A). Arl-R adsorbed to BB, Δ*waaOB* and Δ*waOB* Δ*ompC* more efficient than T4. Arl-R also could adsorb to BW25113 and Δ*ompC* efficiently. Though adsorption efficiency was lower than those 4 strains, about 60% of Arl-R was adsorbed to Δ*waaR* and Δ*waaR* Δ*ompC* at

10 min after phage addition (Fig. 23B). These results strongly suggest that Arl-R is a gain-of-function mutant to recognize the outer cores of K-12 wild-type and Δ*waaR* in addition to those of strain B and Δ*waaOB*. To confirm this suggestion Arl-R inactivation assay by purified LPS was performed. Arl-R was quickly inactivated by B40su1 and Δ*waaOB* LPS. About 40% and 25% of Arl-R was inactivated by BW25113 and Δ*waaR* Δ*ompC* LPS respectively (Fig. 23C). These results indicate that Arl-R is a gain-of-function mutant to recognize the outer cores of K-12 wild-type and Δ*waaR* in addition to those of strain B and Δ*waaOB*. Also this indicates that the specificity of T4 adsorption to LPS can be changed by a mutation in the DT region.

Growth ability of T4 on O157

As described in Chapter 1, T4 efficiently adsorbed to O157 Δ*waaI*, O157 Δ*waaI* Δ*ompC* and O157 Δ*waaJ* Δ*ompC* harboring pOmpC (Fig. 10). The ability of T4 growth on these strains was examined by spot test. Surprisingly T4 did not grow to form plaques on these strains (Fig. 24). A spot containing 10^8 phages resulted in a killing zon, because a considerable fraction of indicator cells were killed. This result indicates that T4 cannot produce progenies on O157 and it is impossible to make T4 able to grow on O157 by introducing mutations in DT.

The step of infection cycle that inhibits T4 growth

T4 can adsorb to O157 Δ*waaI* but not produce progenies. Therefore, I chose O157Δ*waaI* to study what mechanism inhibits T4 growth on O157. To identify which step of T4 infection cycle is impaired in O157, I first checked protein synthesis of T4 after infection to O157Δ*waaI*. Bacteria was infected with T4 at 0 min and at appropriate time points, aliquots from T4-infected culture were taken and proteins synthesized for 3 min were labeled by [^{35}S]methionine. When T4 infects, gene

expression of the host bacteria is shut off immediately after T4 DNA injection, and in turn, T4 gene expression begins. T4 genes are classified into early, middle and late genes, depending on T4 growth phase when they are expressed. When T4 infected BW25113, bacterial gene expression was observed at 0 min, just before phage addition, then T4 early gene expression started at 2 min after infection (Fig. 25). Next, expression of middle genes was observed at 7 min and late genes were mainly expressed from 10 min. On the other hand, when T4 infects O157, the O157 gene expression was unchanged till 20 min after phage addition because T4 cannot adsorb to it. In the case of O157 Δ*waaI*, bacterial gene expression was slightly decreased after phage infection but expression pattern was unchanged till 20 min. Even T4 early gene expression was not observed (Fig. 25). These results indicate that T4 gene expression was not allowed in O157.

To confirm whether T4 can replicate its DNA in O157, dot southern blotting was performed. Bacteria was infected with T4 at 0 min. At appropriate times, cells were collected and lysed as described in materials and methods. After the lysate was spotted onto a membrane, T4 DNA was detected by *soc* probe. Weak but significant signal was detected at 0.5 min for BW25113 and the signal strength increased along the time after T4 infection. This indicates that T4 DNA was injected into BW25113 cells by 0.5 min and phage DNA was increased along the time. On the other hand, no signal was detected for wild-type O157 even at 15 min. When T4 infected O157 Δ*waaI*, a weak signal was detected at 0.5 min, but the strength of signals was not increased until 20 min after T4 infection (Fig. 26). This result suggested that T4 DNA was injected into O157 Δ*waaI* but T4 could not replicate its DNA.

T4 DNA replication was also monitored by agarose gel electrophoresis. After T4 infection, cells were collected by centrifugation and DNA was extracted as described in materials and methods. Extracted DNA contained host DNA and T4 DNA, the former of which was susceptible to digestion by *Hae*III. *Hae*III cannot degrade T4 DNA because cytosine of T4 DNA is modified by hydroxymethylation. Thus only T4

DNA remains intact after the treatment of *Hae*III, while host DNA is digested by the four base cutter into fragments mainly shorter than 1 kb. In accordance with the result of dot southern assay, T4 DNA was detected at 0.5 min and increased along time in T4-infected BW25113. On the other hand, a weak but undoubted band appeared at 0.5 min but remained unchanged until 20 min in T4-infected O157 Δ*waaI* (Fig. 27). These results suggest that T4 DNA might not be injected into O157 Δ*waaI* as efficiently as to BW25113 or part of T4 DNA injected into cells was immediately degraded in O157 Δ*waaI*. T4 degrades host DNA after infection and uses resulting nucleotides for T4 DNA replication. In accordance with this notion, the total amount of DNA did not change dramatically for 20 min in T4-infected BW25113 despite the fact that T4 DNA was increased during 20 min after phage addition (Fig. 27). On the other hand, the total amount of DNA also did not change for 20 min in T4-infected O157 Δ*waaI*, while T4 DNA was not increased (Fig. 27). This result might suggest that host DNA is not degraded in T4-infected O157 Δ*waaI*.

Discussion

Phage therapy is an attractive strategy against disease caused by drug resistant pathogenic bacteria, but the strict host range of phage and the safety of phage are major concerns. To clear the concerns, establishment of the method to modify host range of safe phages are useful. In this study, I isolated T4 Arls which adsorb to a LPS that wild-type T4 cannot do as the initial step to modify host range of T4.

Arl-Wt1,ArlWt2 and Arl-Wt3 are T4 mutants which infect Δ*ompC*. Surprisingly, Arl-Wt1,ArlWt2 and Arl-Wt3 does not have a mutation in gene *37* (table. 3). After the interaction between DT and receptors, T4 baseplate binds to its receptors. Therefore Arl-Wts might have a mutation in genes encoding the base plate.

The growth abilities of Arl-O1, Arl-O2, Arl-G1 and Arl-G2 were very weak and I could not get a stock solution with a high titer required for further experiment (Fig. 22). These mutants were raised at a frequency of 3 X 10^{-9}. A single-base substitution occurs at a frequency of 10^{-6} to 10^{-5}. Taking into account this, it is assumed that Arl-O1, Arl-O2, Arl-G1 and Arl-G2 had two base change mutations or a deletion/insertion, but such spontaneous mutations may not cause a high growth ability on Δ*waaO* Δ*ompC* or Δ*waaG* Δ*ompC*. Previous study reveals that T4 mutant which has a duplication of His-box in DT can infect *Yersinia pseudotuberculosis*, an evolutionary distant bacterium from *E. coli* (Tétart *et al.*, 1996). Therefore, to isolate T4 mutants that grow on Δ*waaO* Δ*ompC* or Δ*waaG* Δ*ompC* efficiently, it would be required to mutate artificially and more dynamically like duplication, deletion or insertion.

Arl-R, which has an amino acid substitution at 953 from tyrosine to arginine, could adsorb to K-12 Δ*waaOB* and to the B strain having a terminal glucose in the outer core, like wild-type T4. In addition, the Arl-R mutant was able to adsorb to strains having LPSs of K-12 wild-type and Δ*waaR* Δ*ompC* (Fig. 23B). As mentioned in Chapter 1, Δ*waaR* synthesizes two types of LPS and it is suggested that Arl-R

recognizes the longer LPS when adsorb to Δ*waaR* cells because GlcNAc and/or Hep at the terminus of outer core are common sugars between the longer LPS of Δ*waaR* and K-12 wild-type LPS. The Arl-R mutant conserves threonine at 939 that is important for recognizing a terminal glucose in the outer core, and it is located at the top of the DT head domain. On the other hand, Y953 is located at the border between the lateral and top surfaces of the head domain. The side chain of Y953 protrudes towards the top surface (Bartual *et al.*, 2010) and the side chain of arginine replacing tyrosine is also expected to protrude towards the top surface of the DT head domain. In addition, the side chain of arginine is longer than that of tyrosine. This change may enable T4 long tail fibers to interact with the LPSs of K-12 wild-type and Δ*waaR* Δ*ompC*. Taking into account the mutational site of Nib, this result also support the suggestion that the top surface of DT would play an important role in binding to LPS.

Isolation of Arl mutants implies that it is not difficult to change host range by manipulating the structure of the DT head domain, especially the top surface, to recognize various types of LPSs because the top surface of the head domain is composed of only eight amino acids and current technology can replace any amino acid, even all, with designated one(s). As described above, point mutation is not enough to make DT enable to bind some kind of LPS such as Δ*waaO* or Δ*waaG*. Thus, combination point mutation of 8 amino acids at top surface of DT and dynamic mutation like duplication or insertion make the host range of T4 could expanded largely and make T4 be a good tool for phage therapy.

T4 could not grow on O157 Δ*waaI* Δ*ompC* despite T4 could adsorb to it (Fig. 24). On this strain, T4 could neither replicate its DNA nor express its genes (Fig. 25, 26 and 27). In addition, host DNA was not degraded significantly after T4 infection to O157 Δ*waaI* (Fig. 27). These results indicate T4 infection was aborted after DNA injection, although T4 DNA may not be degraded, without both DNA replication and gene expression.

Phage and host have coevolved in long history of continuing battle. In this battle, host bacteria have evolved many strategies to abolish progenies production (Labrie *et al.*, 2010; Samson *et al.*, 2013). In terms of application of phage therapy, genes required to overcome a phage-resistant mechanism should be incorporated into susceptible phage genome or another phage that is resistant to the mechanism should be added to candidates for phage therapy. My study shows that T4 currently cannot be an agent against O157 strain. Previous research showed that T-even phage CEV1 could infect K-12, B and O157. CEV1 has a similar shape to T4 and its genome size is almost same to that of T4 (Raya *et al.*, 2006). This makes me assume that CEV1 should be added to a candidate phage against O157 if its safety is ensured. Also it is possible to transfer the gene that is essential for growth on O157 from CEV1 into T4. Attempts to make an ideal set of candidate phages and modification of host range by mutation in adsorption machinery may contribute to application of phage therapy.

Tables

A

Tested strain	Δ*waaR* Δ*ompC*		
Mutant phage	Arl-R1	Arl-R2	Arl-R3
The number of tested plaque	110	100	100
The number of defective plaque on tested strain	21	17	22
Ratio of recombinants to total	0.19	0.17	0.22

B

Tested strain	ΔompC		
Mutant phage	Arl-Wt1	Arl-Wt2	Arl-Wt3
The number of tested plaque	100	100	100
The number of defective plaque on tested strain	0	0	0
Ratio of recombinants to total	0	0	0

Table. 3 Recombination assay between Arl-R1, Arl-R2, Arl-R3, Arl-Wt1, Arl-Wt2 and Arl-Wt3 and pLTF.

Recombination assay was carried out and emergence rate was calculated as described in materials and methods. (A) 100 or 110 recombinants between Arl-R1, Arl-R2 and Arl-R3 and pLTF were picked up and tested phenotype on Δ*waaR* Δ*ompC*. (B) 100 recombinants between Arl-Wt1, Arl-Wt2 and Arl-Wt3 and pLTF were picked up and tested phenotype on Δ*ompC*.

Figures

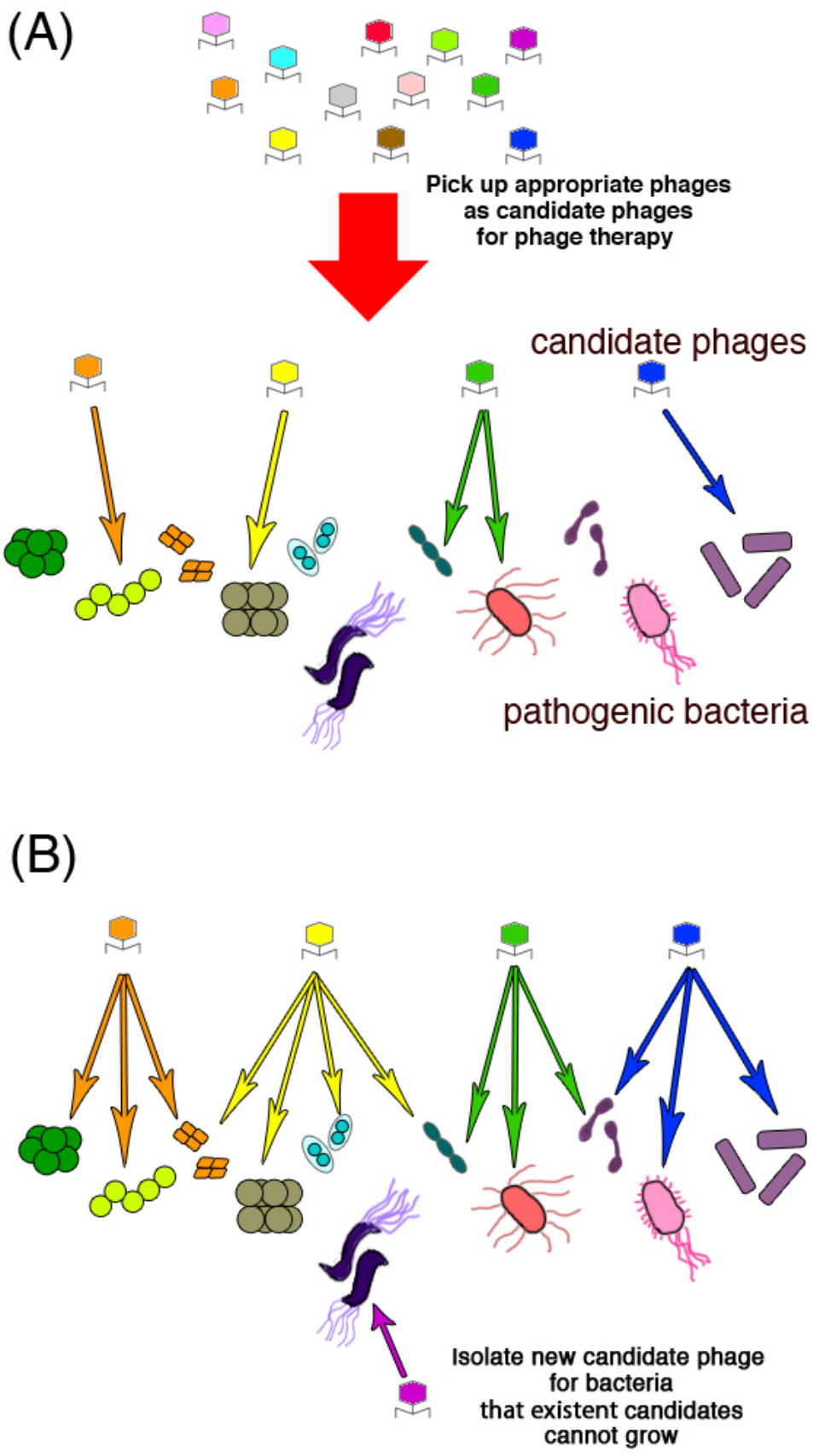

(A)
Pick up appropriate phages
as candidate phages
for phage therapy
candidate phages
pathogenic bacteria
(B)
Isolate new candidate phage
for bacteria
that existent candidates
cannot grow

Figure. 19 strategy of phage therapy

(A) Several phages, which are ensured safety, are lined up as candidates for phage therapy. Allow indicate natural host range of each phage. Generally, each phage has strict host specificity. (B) Host range of candidate phages are modified by mutation. This step expands host range of each phage. When a bacterium, which candidate phage cannot grow even if mutation was introduced appears, isolate new candidate that can infect the bacterium and is ensured safety.

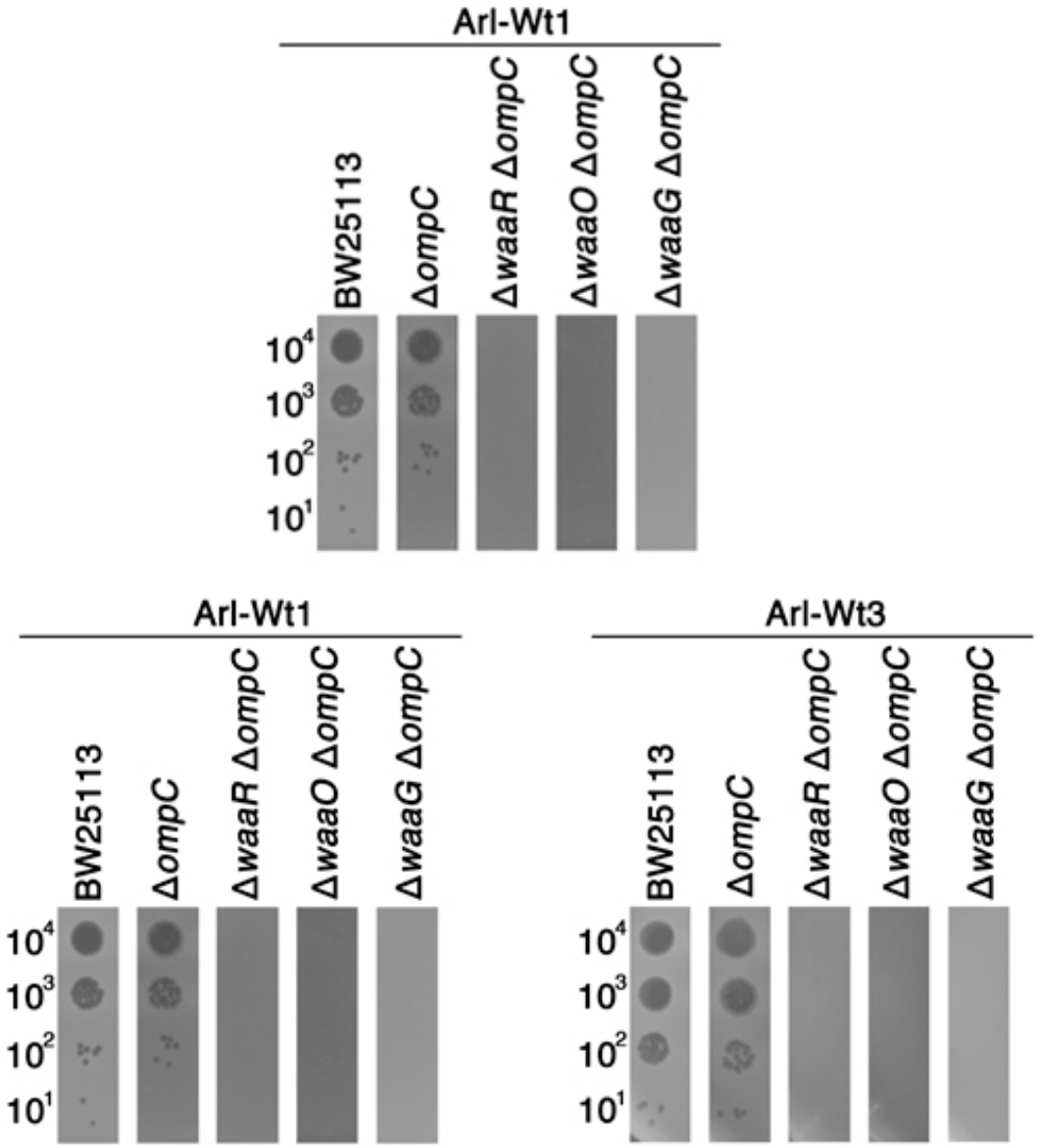


Figure 20. Spot test of Arl-Wt1, Arl-Wt2 and Arl-Wt3 on LPS mutants in the absence of OmpC.

The solution containing the number of phage particles (Arl-Wt1, top; Arl-Wt2, left; Arl-Wt3, right panel) indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37°C overnight.

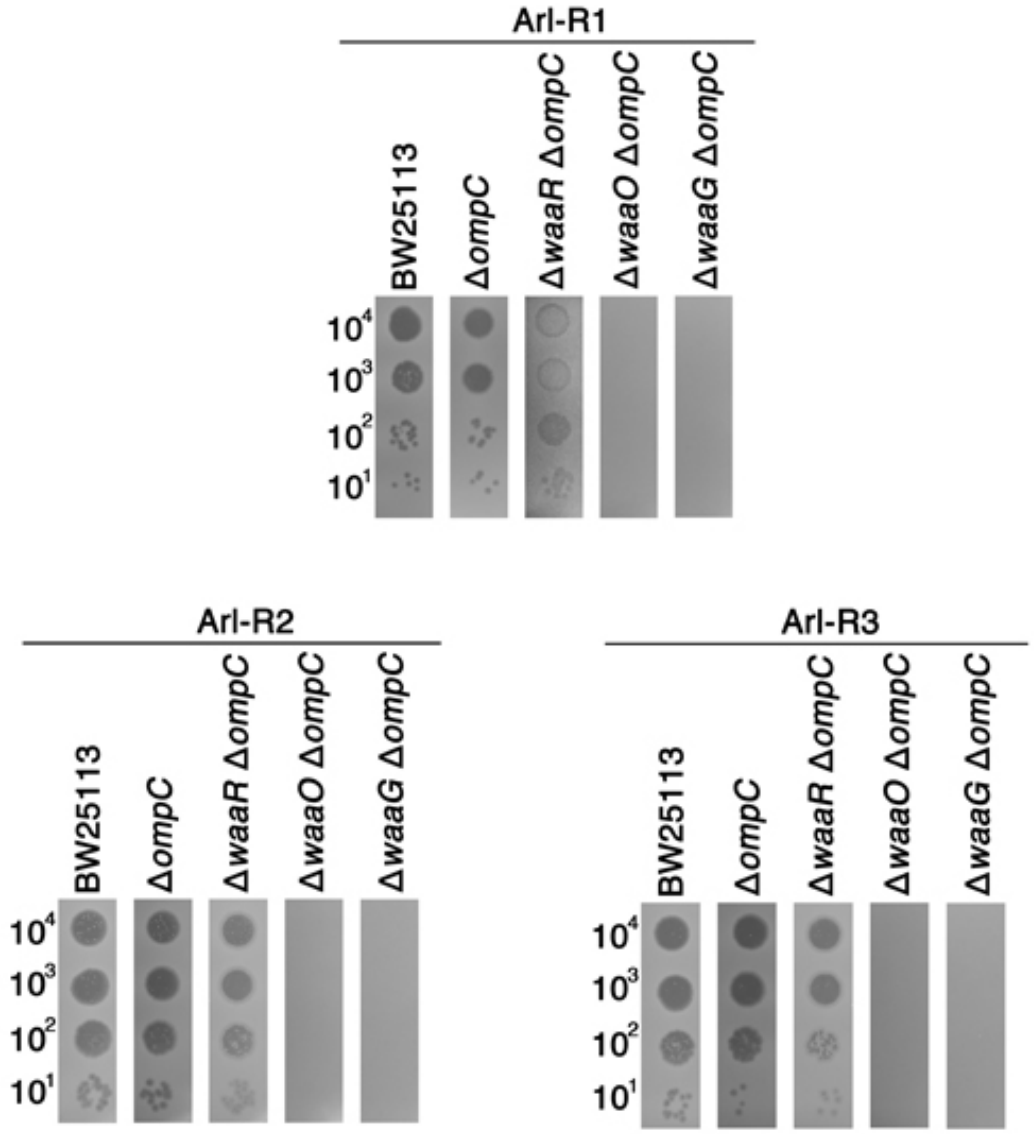


Figure 21. Spot test of Arl-R1, Arl-R2 and Arl-R3 on LPS mutants in the absence of OmpC.

The solution containing the number of phage particles (Arl-R1, top; Arl-R2, left; Arl-R3, right panel) indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37°C overnight.

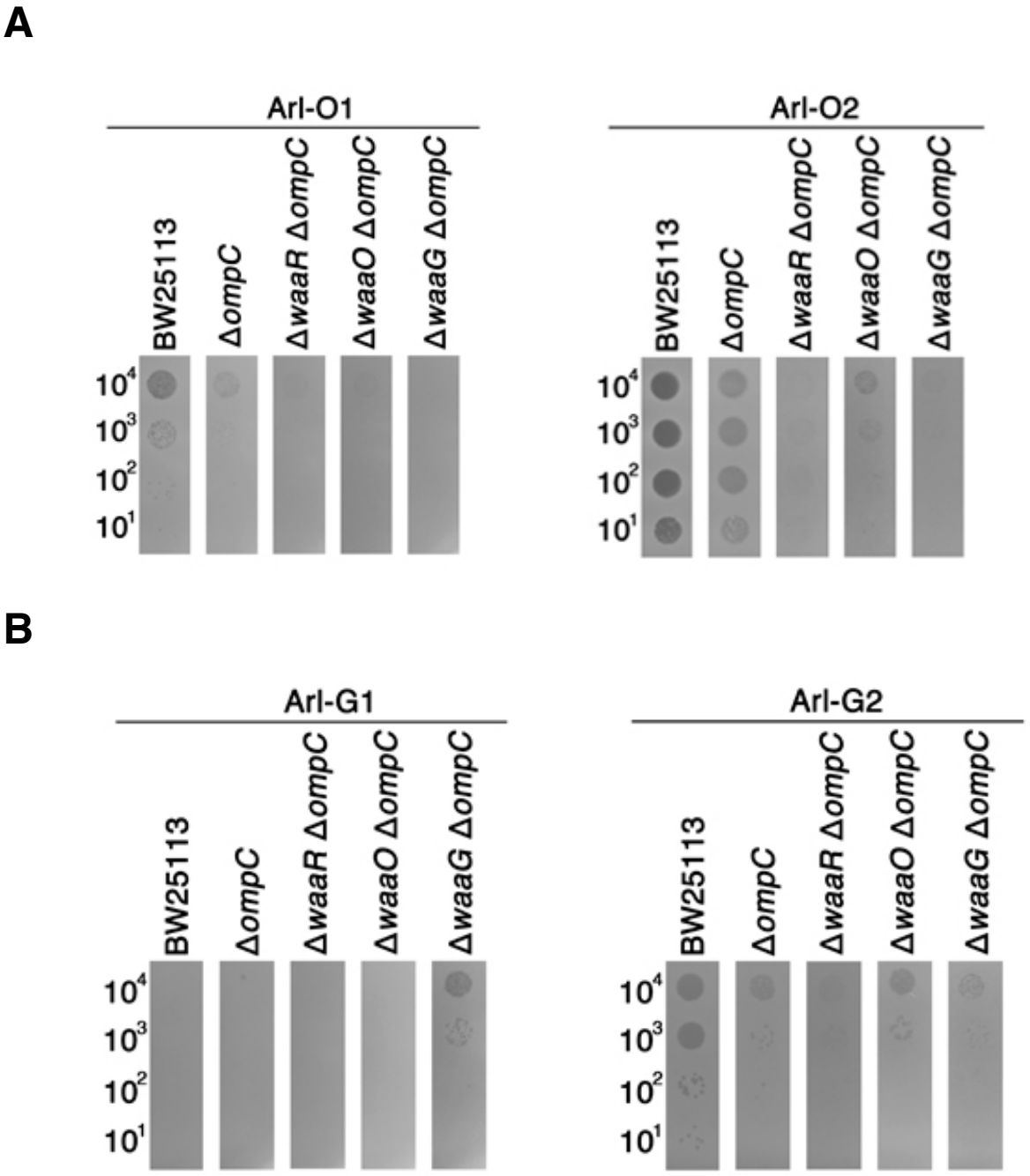


Figure 22. Spot test of Arl-O1, Arl-O2, Arl-G1 and Arl-G2 on LPS mutants in the absence of OmpC.

(A) The solution containing the number of phage particles (Arl-O1, left; Arl-O2, right panel) indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37°C overnight.

(B) The solution containing the number of phage particles (Arl-G1, left; Arl-G2, right panel) indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37°C overnight.

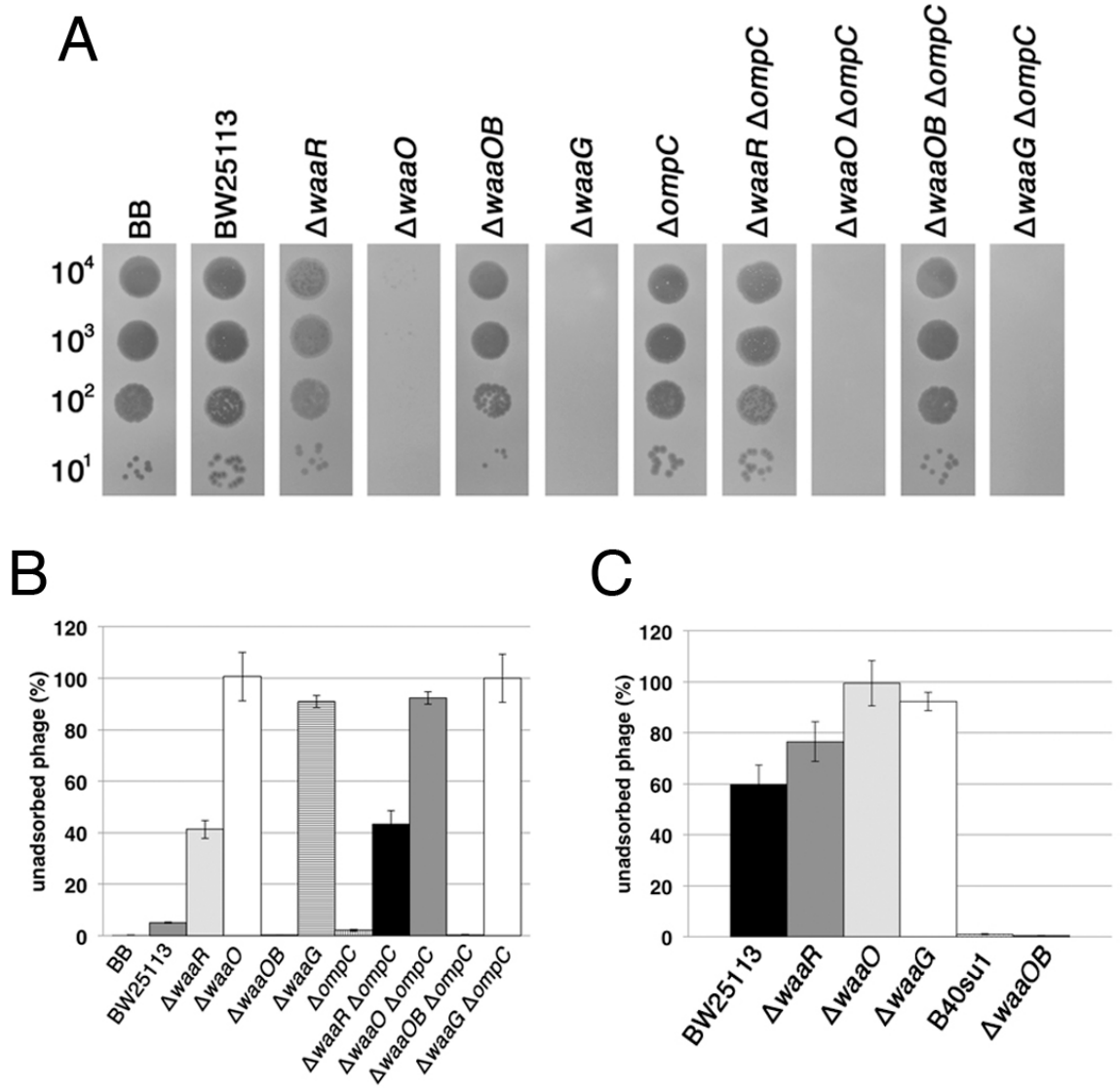


Figure 23. Characterization of T4 Arl-R mutant.

(A) A solution containing the number of Arl phage particles indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37ºC overnight. (B) Adsorption analyses with the *E. coli* strains indicated at the bottom were performed as described in materials and methods. (C) A solution containing 3 x 10^7 T4 phage particles was mixed with 35 μM of LPS purified from the *E. coli* strains indicated at the bottom and then the inactivation assay was performed as described in materials and methods.

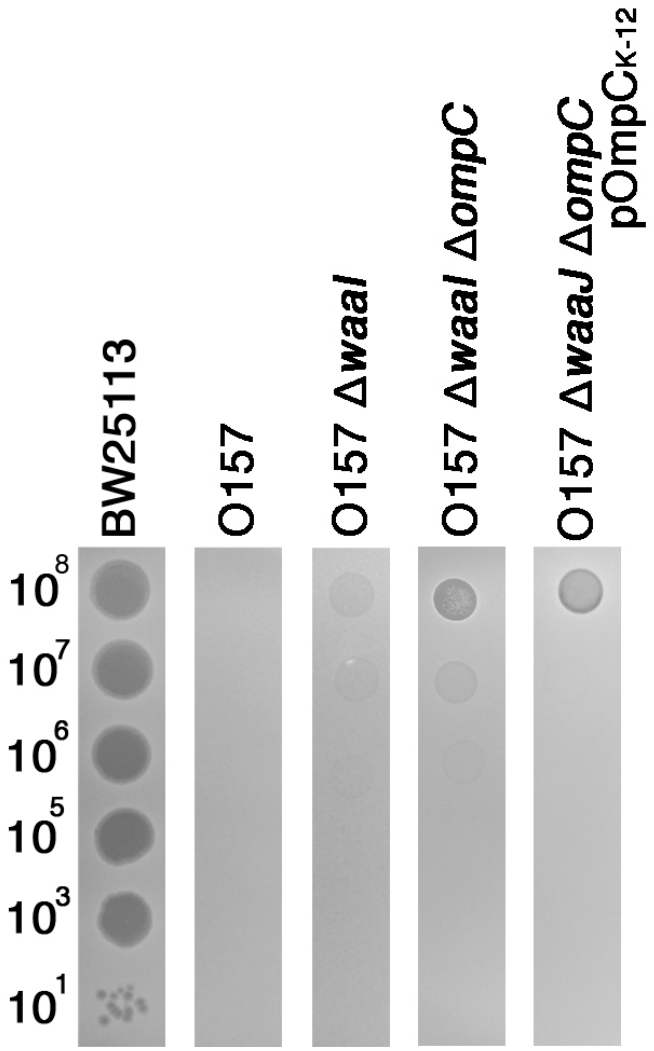


Figure 24. Spot test of T4 on O157 LPS mutants.

A solution containing the number of T4 particles indicated on the left was spotted on a lawn of the *E. coli* strain indicated at the top and the plates were incubated at 37ºC overnight.

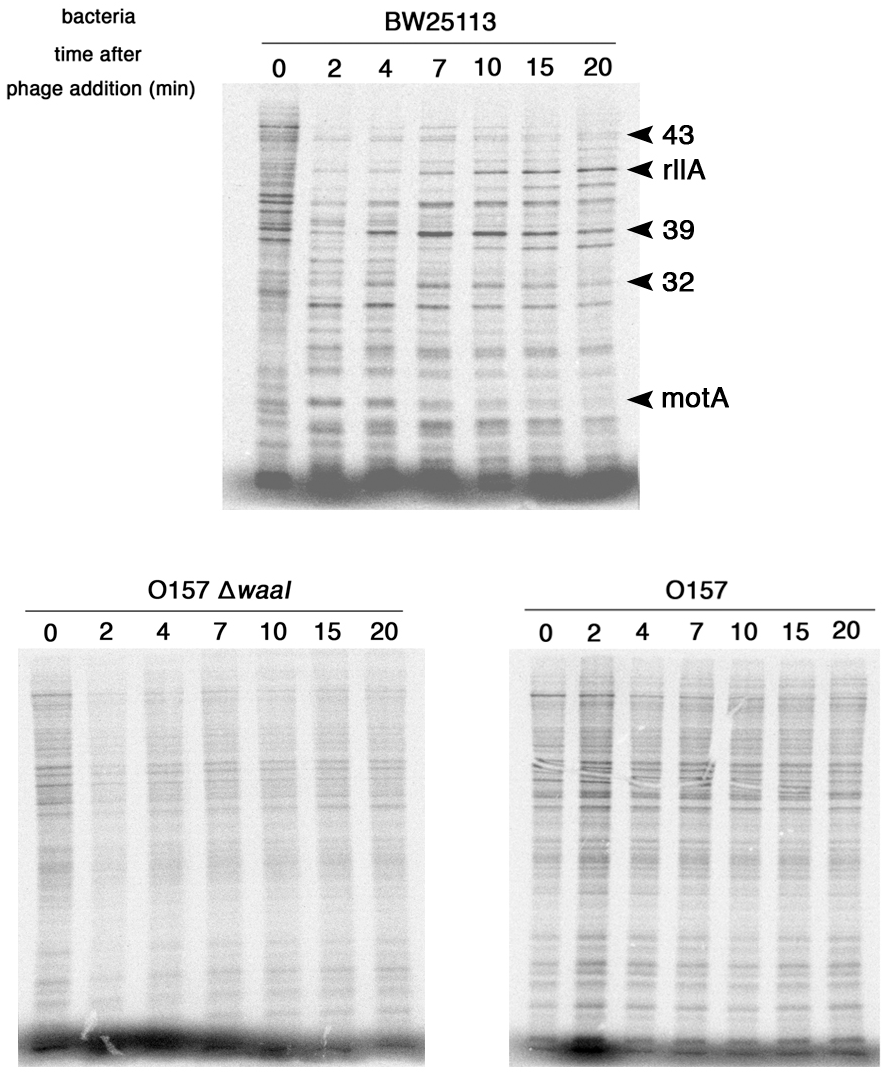


Figure 25. S35 protein synthesize analysis of T4 on O157 Δ*waaI*

Proteins synthesized in *E. coli* strains indicated at the top of figure were infected with T4 and protein synthesized for 3min at the time indicated at the top of figure were labeled with [^{35}S]methionine as described in materials and methods. In the right margin, representative genes were showed.

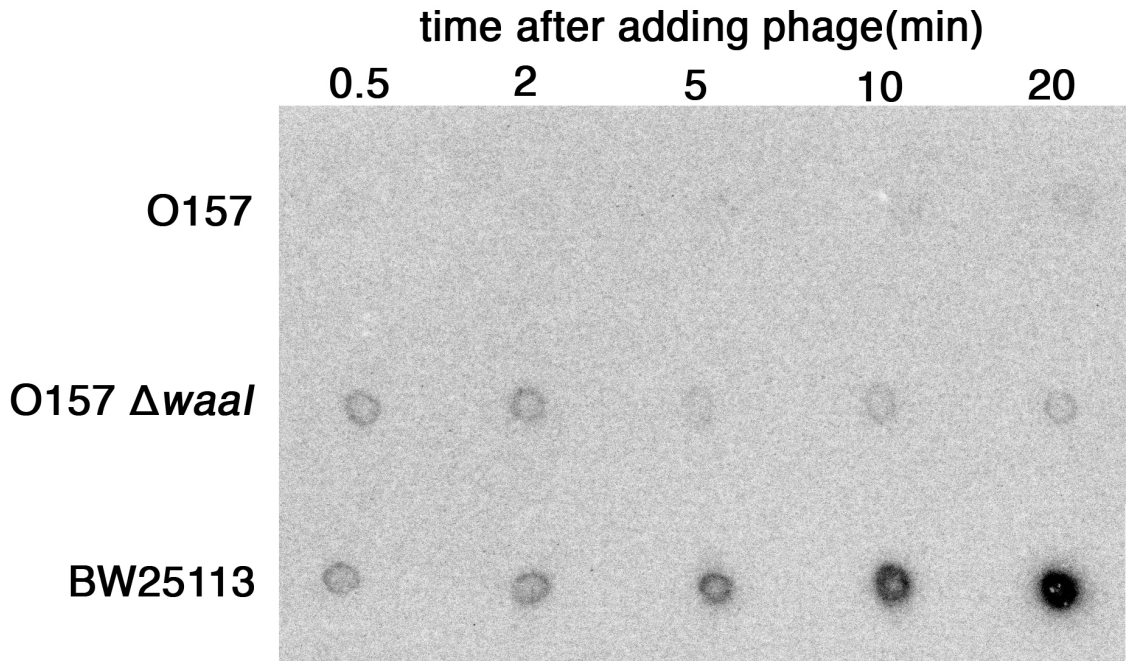


Figure 26. Dot southern of T4 D+

E. coli strain indicated left were infected with T4. Total DNA was extracted at each times after phage addition indicated at the top and analyzed by Southern dot blotting as described in materials and methods.

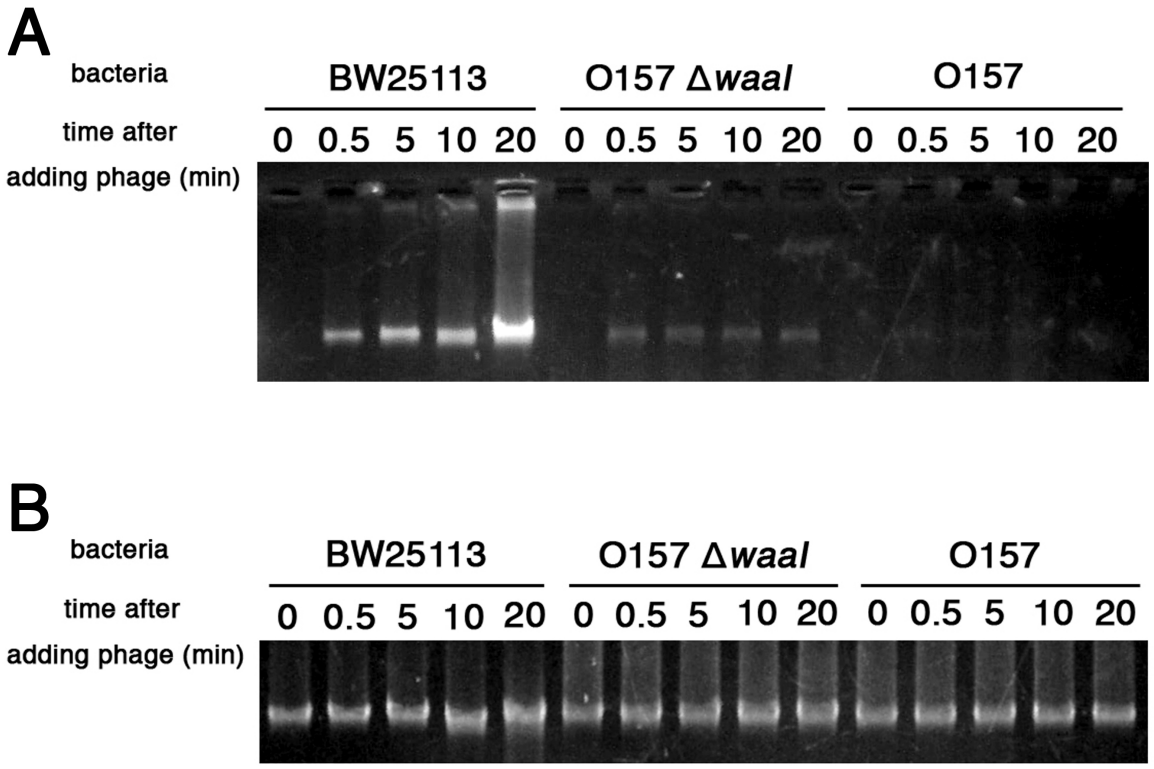


Figure 27. DNA replication analysis of T4 on O157

E. coli strains indicated at the top of figures were infected with T4. At each time indicated in figures whole DNA was extracted and analyzed as described in materials and methods. 0 min means just before phage addition to bacteria. (A) Extracted DNA was digested by *Hae*III before electrophoresis. (B) Extracted DNA was analyzed by electrophoresis without digestion by *Hae*III.

Reference

Amor, K., Heinrichs, D.E., Frirdich, E., Ziebell, K., Johnson, R.P., and Whitfield, C. (2000) Distribution of core oligosaccharide types in lipopolysaccharides from Escherichia coli. *Infect Immun* **68**: 1116–1124.

Bartual, S.G., Otero, J.M., Garcia-Doval, C., Llamas-Saiz, A.L., Kahn, R., Fox, G.C., and Raaij, M.J. van (2010) Structure of the bacteriophage T4 long tail fiber receptor-binding tip. *Proc Natl Acad Sci U S A* **107**: 20287–20292.

Baslé, A., Rummel, G., Storici, P., Rosenbusch, J.P., and Schirmer, T. (2006) Crystal structure of osmoporin OmpC from E. coli at 2.0 A. *J Mol Biol* **362**: 933–942.

Brüssow, H. (2012) What is needed for phage therapy to become a reality in Western medicine? *Virology* **434**: 138–42.

Buehler, L.K., Kusumoto, S., Zhang, H., and Rosenbusch, J.P. (1991) Plasticity of Escherichia coli porin channels. Dependence of their conductance on strain and lipid environment. *J Biol Chem* **266**: 24446–24450.

Cherepanov, P.P., and Wackernagel, W. (1995) Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**: 9–14.

Crawford, J.T., and Goldberg, E.B. (1980) The function of tail fibers in triggering baseplate expansion of bacteriophage T4. *J Mol Biol* **139**: 679–690.

Crowlesmith, I., Gamon, K., and Henning, U. (1981) Precursor proteins are intermediates in vivo in the synthesis of two major outer membrane proteins, the OmpA and OmpF proteins, of Escherichia coli K12. *Eur J Biochem* **113**: 375–380.

Currie, C.G., and Poxton, I.R. (1999) The lipopolysaccharide core type of Escherichia coli O157:H7 and other non-O157 verotoxin-producing E. coli. *FEMS Immunol Med Microbiol* **24**: 57–62.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640–6645.

Dawes, J. (1975) Characterisation of the bacteriophage T4 receptor site. *Nature* **256**: 127–128.

Demerec, M., and Fano, U. (1945) Bacteriophage-Resistant Mutants in Escherichia Coli. *Genetics* **30**: 119–36.

Doermann, A.H., and Hill, M.B. (1953) Genetic Structure of Bacteriophage T4 as Described by Recombination Studies of Factors Influencing Plaque Morphology. *Genetics* **38**: 79–90.

Furukawa, H., Yamada, H., and Mizushima, S. (1979) Interaction of bacteriophage T4 with reconstituted cell envelopes of Escherichia coli K-12. *J Bacteriol* **140**: 1071–1080.

Galanos, C., Lüderitz, O., and Westphal, O. (1969) A new method for the extraction of R lipopolysaccharides. *Eur J Biochem* **9**: 245–249.

Goldberg, E., Grinius, L., and Letellier, L. (1994) Recognition, attachment, and infection. In Molecular Biology of Bacteriophage T4 Karam, J.D., Drake, J.W., Kreuzer, K.N., Mosig, G., Hall, D.H., Eisering, F.A., et al. (eds). Washington, D.C.: American Society for Microbiolgy Press, pp. 347-356

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177**: 4121–4130.

Heinrichs, D.E., Yethon, J.A., Amor, P.A., and Whitfield, C. (1998a) The assembly system for the outer core portion of R1- and R4-type lipopolysaccharides of Escherichia coli. The R1 core-specific beta-glucosyltransferase provides a novel attachment site for O-polysaccharides. *J Biol Chem* **273**: 29497–29505.

Heinrichs, D.E., Yethon, J.A., and Whitfield, C. (1998b) Molecular basis for structural diversity in the core regions of the lipopolysaccharides of Escherichia coli and Salmonella enterica. *Mol Microbiol* **30**: 221–232.

Henein, A. (2013) What are the limitations on the wider therapeutic use of phage? *Bacteriophage* **3**: e24872.

Henning, U., and Jann, K. (1979) Two-component nature of bacteriophage T4 receptor activity in Escherichia coli K-12. *J Bacteriol* **137**: 664–666.

Hu, B., Margolin, W., Molineux, I.J., and Liu, J. (2015) Structural remodeling of bacteriophage T4 and host membranes during infection initiation. *Proc Natl Acad Sci U S A* **112**: E4919–28.

Inagaki, M., Kato, M., Ohsumi, Y., Kaitani, K., Nishikawa, S., and Kashimura, N. (1995) Simple Preparation of Large Amount of Lipopolysaccharide with Receptor Activity for Bacteriophage ϕXl74 from Escherichia coli C Strain. *Bull Fac Bioresour Mie Univ* **15**: 33–40.

Jeong, H., Barbe, V., Lee, C.H., Vallenet, D., Yu, D.S., Choi, S.-H., *et al.* (2009) Genome sequences of Escherichia coli B strains REL606 and BL21(DE3). *J Mol Biol* **394**: 644–652.

Jesaitis, M.A., and Goebel, W.F. (1953) The interaction between T4 phage and the specific lipocarbohydrate of phase II Sh. sonnei. *Cold Spring Harb Symp Quant Biol* **18**: 205–208.

Kai, T., Ueno, H., Otsuka, Y., Morimoto, W., and Yonesaki, T. (1999) Gene 61.3 of bacteriophage T4 is the spackle gene. *Virology* **260**: 254–259.

Koga, M., Otsuka, Y., Lemire, S., and Yonesaki, T. (2011) Escherichia coli rnlA and rnlB compose a novel toxin-antitoxin system. *Genetics* **187**: 123–30.

Labrie, S.J., Samson, J.E., and Moineau, S. (2010) Bacteriophage resistance mechanisms. *Nat Rev Microbiol* **8**: 317–27.

Miller, E.M. (1949) Studies on bacteriophage: I. The relationship between the somatic antigens of Shigella sonnei and their susceptibility to bacterial viruses. *J Exp Med* **90**: 255–265.

Mizoguchi, K., Morita, M., Fischer, C.R., Yoichi, M., Tanji, Y., and Unno, H. (2003) Coevolution of Bacteriophage PP01 and Escherichia coli O157:H7 in Continuous Culture. *Appl Environ Microbiol* **69**: 170–176.

Moller, A.K., Leatham, M.P., Conway, T., Nuijten, P.J.M., Haan, L.A.M. de, Krogfelt, K.A., and Cohen, P.S. (2003) An Escherichia coli MG1655 Lipopolysaccharide Deep-Rough Core Mutant Grows and Survives in Mouse Cecal Mucus but Fails To Colonize the Mouse Large Intestine. *Infect Immun* **71**: 2142–2152.

Montag, D., Hashemolhosseini, S., and Henning, U. (1990) Receptor-recognizing proteins of T-even type bacteriophages. The receptor-recognizing area of proteins 37 of phages T4 TuIa and TuIb. *J Mol Biol* **216**: 327–334.

Morita, M., Tanji, Y., Mizoguchi, K., Akitsu, T., Kijima, N., and Unno, H. (2002) Characterization of a virulent bacteriophage specific for Escherichia coli O157:H7 and analysis of its cellular receptor and two tail fiber genes. *FEMS Microbiol Lett* **211**: 77–83.

Otsuka, Y., and Yonesaki, T. (2012) Dmd of bacteriophage T4 functions as an antitoxin against Escherichia coli LsoA and RnlA toxins. *Mol Microbiol* **83**: 669–681.

Pradel, E., Parker, C.T., and Schnaitman, C.A. (1992) Structures of the rfaB, rfaI, rfaJ, and rfaS genes of Escherichia coli K-12 and their roles in assembly of the lipopolysaccharide core. *J Bacteriol* **174**: 4736–4745.

Prehm, P., Jann, B., Jann, K., Schmidt, G., and Stirm, S. (1976) On a bacteriophage T3 and T4 receptor region within the cell wall lipopolysaccharide of Escherichia coli B. *J Mol Biol* **101**: 277–281.

Raya, R.R., Varey, P., Oot, R. a., Dyen, M.R., Callaway, T.R., Edrington, T.S., *et al.* (2006) Isolation and characterization of a new T-even bacteriophage, CEV1, and determination of its potential to reduce Escherichia coli O157:H7 levels in sheep. *Appl Environ Microbiol* **72**: 6405–6410.

Ried, G., Hindennach, I., and Henning, U. (1990) Role of lipopolysaccharide in assembly of Escherichia coli outer membrane proteins OmpA, OmpC, and OmpF. *J Bacteriol* **172**: 6048–6053.

Samson, J.E., Magadán, A.H., Sabri, M., and Moineau, S. (2013) Revenge of the phages: defeating bacterial defences. *Nat Rev Microbiol* **11**: 675–87.

Schägger, H. (2006) Tricine-SDS-PAGE. *Nat Protoc* **1**: 16–22.

Schnaitman, C.A., and Klena, J.D. (1993) Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol Rev* **57**: 655–682.

Sheng, H., Lim, J.Y., Watkins, M.K., Minnich, S. a, and Hovde, C.J. (2008) Characterization of an Escherichia coli O157:H7 O-antigen deletion mutant and effect of the deletion on bacterial persistence in the mouse intestine and colonization at the bovine terminal rectal mucosa. *Appl Environ Microbiol* **74**: 5015–22.

Shimizu, T., Yamasaki, S., Tsukamoto, T., and Takeda, Y. (1999) Analysis of the genes responsible for the O-antigen synthesis in enterohaemorrhagic Escherichia coli O157. *Microb Pathog* **26**: 235–247.

Simon, L.D., and Anderson, T.F. (1967) The infection of Escherichia coli by T2 and T4 bacteriophages as seen in the electron microscope I. Attachment and penetration. *Virology* **32**: 279–297.

Sutcliffe, J.G. (1979) Complete nucleotide sequence of the Escherichia coli plasmid pBR322. *Cold Spring Harb Symp Quant Biol* **43**: 77–90.

Tétart, F., Desplats, C., and Krisch, H.M. (1998) Genome plasticity in the distal tail fiber locus of the T-even bacteriophage: recombination between conserved motifs swaps adhesin specificity. *J Mol Biol* **282**: 543–556.

Tétart, F., Repoila, F., Monod, C., and Krisch, H.M. (1996) Bacteriophage T4 host range is expanded by duplications of a small domain of the tail fiber adhesin. *J Mol Biol* **258**: 726–731.

Trojet, S.N., Caumont-Sarcos, A., Perrody, E., Comeau, A.M., and Krisch, H.M. (2011) The gp38 adhesins of the T4 superfamily: a complex modular determinant of the phage's host specificity. *Genome Biol Evol* **3**: 674–86.

Wais, A.C., and Goldberg, E.B. (1969) Growth and transformation of phage T4 in Escherichia coli , Salmonella, Aerobacter, Proteus, and Serratia. *Virology* **39**: 153–161.

Wilson, G.G., Young, K.K.Y., Edlin, G.J., and Konigsberg, W. (1979) High-frequency generalised transduction by bacteriophage T4. *Nature* **280**: 80–82.

Wilson, J.H., Luftig, R.B., and Wood, W.B. (1970) Interaction of bacteriophage T4 tail fiber components with a lipopolysaccharide fraction from Escherichia coli. *J Mol Biol* **51**: 423–434.

Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L., and Treiber, G. (1970) Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40**: 734–744.

Yu, F., and Mizushima, S. (1982) Roles of lipopolysaccharide and outer membrane protein OmpC of Escherichia coli K-12 in the receptor function for bacteriophage T4. *J Bacteriol* **151**: 718–722.

Publication

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