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Variability in the tail fiber gene locus of T-even phage causing host recognition shift

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Contents

Chapter 1
Introduction

1.1 General introduction

In 1915 F. W. Twort found an infectious agent which lysed the colony of a kind of micrococcus [53]. On the other hand, quite independently, in 1917 F. d'Hérelle isolated invisible microorganisms from the stool of a dysentery patient which killed *Shigella dysenteriae* specifically and furthermore, propagated only when it was incubated with *S. dysenteriae* [13]. These were the first discoveries of bacteriophage.

Bacteriophage can be divided into two groups according to their life cycles. After the infection, temperate phages integrate their genome into that of host bacteria and multiply the genome along with the propagation of the host bacteria. This is called lysogenization. Temperate phage shift their metabolism to the so-called lytic cycle under specific conditions such as UV irradiation and newly generated phages are released with the lysis of host bacteria. On the other hand, virulent phage always cause lysis of host bacteria during its life cycle.

M. Demerec and U. Fano isolated seven kinds of bacteriophage in 1945 and named them serially from T1 to T7, all virulent phages [33]. Among them, T2, T4 and T6 are very similar in their morphology and have more than 85% homology in their DNA sequences. Because of these similarities, they are grouped together as T-even phage. On the other hand, T1, T3, T5 and T7 are sometimes called T-odd phage. T3 and T7 are very similar to each other but they are very different from T1, T5 and T-even phages.

The essential components of T-even phage consist of icosahedral head (capsid), six whiskers, a helical contractile tail sheath, a tail tube, a hexagonal baseplate and six long tail fibers (Fig. 1-1). T-even phages recognize the host bacteria with the tips of the long tail fibers. The structure of the long tail fiber is shown in Figure 1-1 B. The long

tail fibers of the T2-type phage consist of Gp34, 35, 36, 37 and 38 in order starting from the proximal part. The long tail fiber of T4 phage doesn't contain Gp38, so the last protein at the end of the fiber is Gp37. Gp38 (Gp37 for T4) play the role of host recognition. More than three of six tail fiber tips are needed to interact with the receptors to start the following infection process. Interaction between the proximal part of Gp34 and Gp9 transmits the signal from the long tail fiber to the baseplate (Fig. 1-1 B, C). Then, the signal causes the conformational change of the baseplate from the hexagonal shape (Fig. 1-1 C-1) to the star shape (Fig. 1-1 C-2). Gp12 in the baseplate is protruded due to this conformational change (Fig. 1-1 C-2) and binds irreversibly to the lipopolysaccharide (LPS) on the surface of the host bacterial cell. The conformational change also causes the contraction of the tail sheath and phage DNA is introduced into the host bacterial cell (Fig. 1-2). Host bacteria recognition by bacteriophage is highly specific which makes them attractive for practical applications, such as phage therapy and bacteria identification.

In 1919, large outbreaks of lethal fowl typhoid in chickens occurred in the Acris-sur-Aube region of France. d'Herelle analyzed several dead animals from the outbreaks, and he isolated and identified "*Salmonella gallinarum*" as the etiologic agent of the disease. d'Herelle also isolated bacteriophages from the chickens, and he examined their efficacy in preventing and treating *S. gallinarum* infections in six experimentally infected chickens [14, 15]. The results of the study were promising: phage administration prevented the birds from succumbing to the bacterial infection, whereas the two control chickens not treated with phages died after a single dose of the challenge strain. It was the first application of phage as therapeutic agents. After some subsequent successful experiments with animals including rabbits and buffalo, d'Herelle

used other phages in his collection to treat bacterial dysentery of humans. His most potent anti-dysentery phage preparation was orally administered to a 12-year-old boy hospitalized with severe dysentery (10 to 12 bloody stools per day). The patient's condition rapidly improved after phage ingestion: he passed three more bloody stools the same afternoon and one non-bloody stool during the night, and all symptoms had disappeared by the next morning [48].

In this way, the first human therapy had succeeded. Thereafter, a number of trials were performed from 1920s to 1950s. However, there were many therapeutic failures as well as successes. In the early studies, little preparatory work was done to select phages with high lytic activity *in vitro* before they were used *in vivo*. Some of phages may have had low or no activity on the field strain or may have lysogenized the bacteria. Moreover, there were inadequate scientific methodologies, which included the failure to remove endotoxins from the preparations. Such oversights produced poor and uncontrolled results and the use of phage in bacterial disease therapy largely lapsed with the success of chemotherapy [1, 4, 6, 47]. Penicillin was discovered in 1929 and Chain *et al.* documented its therapeutic properties in 1940, which was the initiation of the widespread use of antibiotics. Additionally images of bacteriophage from electron microscopy became available, and it had a strong negative impact on the acceptance of phage therapy ideas.

In general, the antibiotics are used to treat infectious disease caused by pathogenic bacteria. However, the emergence of pathogenic bacteria resistant to most, if not all, currently available antibiotics has prompted interest in alternatives to conventional agents. These crises have brought up renewed interest in phage therapy in recent years [1, 4, 6, 47]. Potential advantages of phages over antibiotics result from their infectious specificity and their replication. Even though bacterial resistance is a

concern, unlike antibiotics, phage can mutate in step with evolving bacteria. The complex microflora of the intestine tract provides protection against colonization by many pathogens. However, increased susceptibility to pathogenic bacteria infection due to the treatment with antibiotics can be explained by disruption of the normal intestinal microflora [8, 55]. Phages only infect specific host bacteria, thus patients can escape from the side effects caused by destruction of natural microflora.

The specificity of the interaction of a virus with its host cell immediately lends itself to methods for the detection of bacteria, in particular the pathogens. While many other procedures based upon antibodies (ELISA) or nucleic acid amplification (PCR) have been artificially developed to allow the differentiation of bacterial cell structures, here we have a naturally evolved system in which the bacteriophage specifically recognizes and binds only to its own host cells. This interaction has been exploited in a number of different methods for the specific detection and differentiation of the individual host bacteria. One of the first uses of phage was in typing schemes, where a panel of phages with different lytic spectra is used to discriminate between different isolates of a bacterial species or genus, according to their ability to infect the isolate and form plaques. Differences in infectivity reflect differences in a number of cellular characteristics, such as cell surface receptors, the presence of restriction modification systems, presence of related prophage etc.

Other detection techniques have been developed to directly apply the specific binding ability of bacteriophage. These include the production of fluorescently-labeled antibodies directed against specifically adsorbing phage particles [58], or fluorescently-labeled phage prepared by display of the fluorescent reagent on the phage surface [3, 19, 25, 42]. In both of these cases the target cells are detected following

adsorption of phage to the host cells and then identification of this interaction by detecting the bound fluorescent signal.

1.2 Recent research

T-even phages are morphologically and genetically related very closely [11, 50]. However, heteroduplex analysis of the tail fiber loci in the T-even genome has indicated substantial sequence diversity [27] though the loci encode functionally analogous proteins in T2, T4 and T6 [43]. There is a significant difference between T4 and other T-even phages in the distal tail fiber locus. In T4, gene *37* encoding the large subunit of the distal tail fiber determines the host specificity and gene *38* is involved in the assembly of Gp37. However, gene *38* of other T-even phages, such as T2, encodes a separate protein that binds to the distal end of Gp37 and specifies the host range. Despite all this heterogeneity, Tétart *et al.* demonstrated the interchangeability of the distal tail fiber locus among T-even phages [49]. T6 gene *38* was cloned into a vector and partially recombined with the genome of T2. The resulting recombinant phage showed the same host specificity as T6. The tail fiber gene locus of T4 could be even exchanged with cloned gene *37* to *38* region of T2-type phage SV76.3 and T6, though the genes responsible for the host recognition are different. Exchange in the distal tail fiber genes caused host-recognition specificity swapping. Exchange of the genes responsible for the host recognition between unrelated phages was also presented [34]. An area of the gene encoding a COOH-terminal region of the *stf* gene and subsequent *tfa* gene of phage λ was recombined into the genome of T4, thus replacing gene*38* and a part of gene *37* that codes for a COOH-terminal part of protein 37. Such T4-λ hybrids,

unlike T4, required the presence of outer membrane protein OmpC for infection of *E.coli* B. An ompC missense mutant of *E. coli* K-12, which was still sensitive to T4, was resistant to these hybrids. These observation suggested that the host recognition genes were recombined between T4 and λ resulting in host range swapping.

Evidence of the independency of the genes responsible for host recognition and their horizontal gene transfer was observed by Scholl *et al.* [45, 46]. Coliphage K1-5 possesses two tail fiber genes, which enable it to infect both K1 and K5 strains of *Escherichia coli*. Each tail fiber gene of K1-5 showed significant homology to that of bacteriophage ΦK1E or ΦK5, suggesting that the bacteriophage ΦK1E or ΦK5 acquired other tail fiber genes, resulting in K1-5 phage generation. On the other hand, the complete genome sequence of bacteriophage K1F, another K1-specific phage, revealed a contrasting genome arrangement. K1F is closely related to bacteriphage T7 in both genome organization and sequence similarity. And the most striking difference between two phage is that K1F encodes endosialidase in the analogous position to the T7 tail fiber gene. Host range expansion of T4 phage was also observed by duplication of a small domain in the tail fiber gene [51]. These investigations suggest that recombination events sometimes cause host range expansion.

1.3 Outline of this dissertation

The objective of this study is the evaluation of the variability of phage host recognition. A general introduction to bacteriophage and the applicability of bacteriophage specificity in terms of phage therapy and bacteria identification are described in chapter 1. In chapter 2, the possibility of rendering a phage infective to a

previously non-infective pathogen by the recombination of genes responsible for the host recognition was assessed. The investigation was performed using a well-known phage T2 and *E. coli* O157:H7 specific phage PP01. Chapter 3 discusses the occurrence of the phage recognition shift during superinfection by two virulent phages. Experiments imitated the direct phage genome recombination occurring in nature. Chapter 4 briefly summarizes the conclusions of this research. The outline of this dissertation is shown in Figure 1-3.

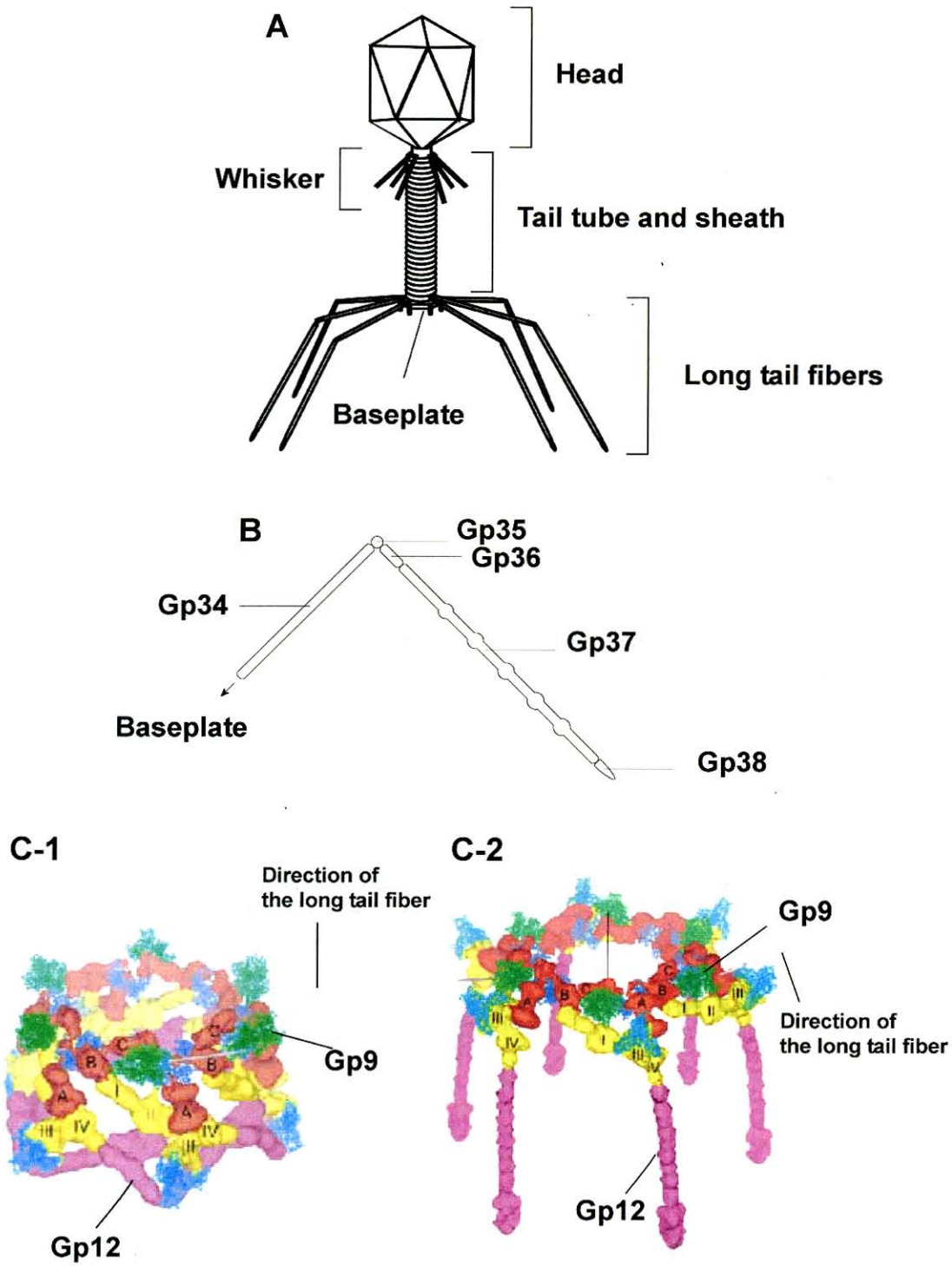

Figure 1-1. Structure of the T-even phage

A: whole virion, B: T2-type long tail fiber, C: baseplate (1. hexagonal shape, 2. star shape, reproduced from reference No. 29)

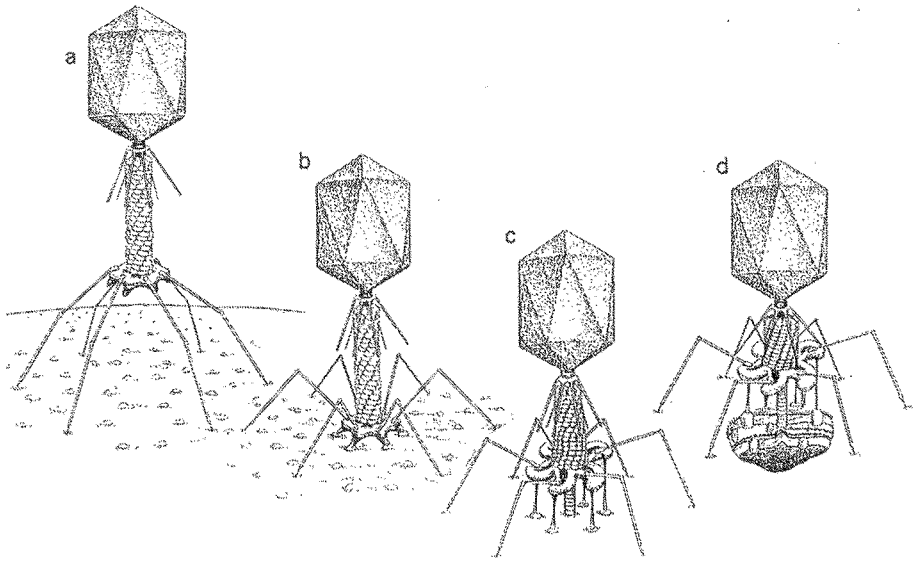

Figure 1-2. Infection process of the T-even phage

a: Initial adsorption by Gp37 for T4 phage (Gp38 for T2pahge). b: Second adsoption by Gp12. c, d: Penetration and phage genome injection into the cell. (Reproduced from reference No. 33)

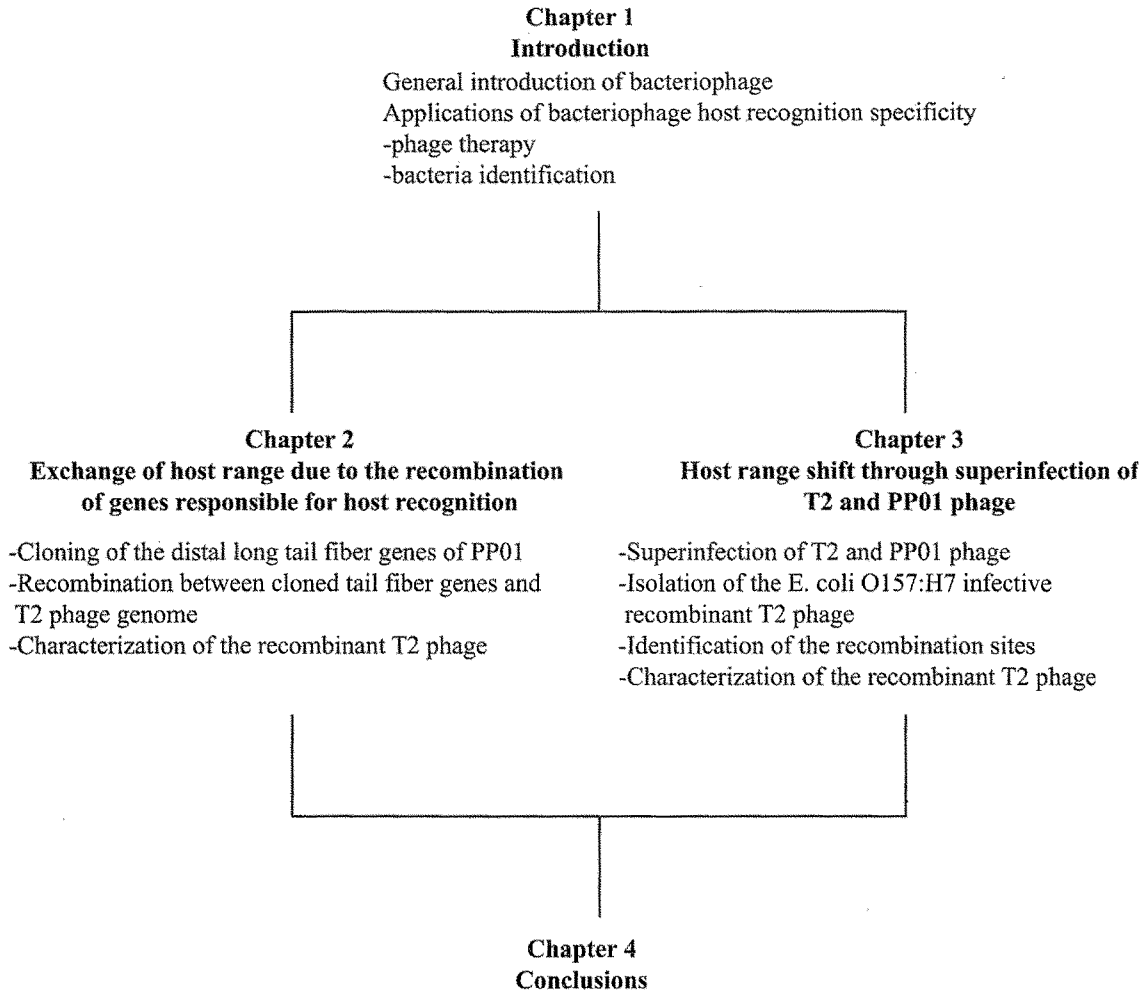

Figure 1-3. Overview of this thesis

Chapter 2
Exchange of host range due to the recombination of genes responsible for host recognition

2.1 Introduction

Escherichia coli is a normal inhabitant of the gastrointestinal tract of humans and other warm-blooded animals. While most *E.coli* strains do not cause human disease, some strains are pathogenic and possess virulence factors which can cause life-threatening conditions. Three general clinical syndromes result from infection with inherently pathogenic *E.coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrheal disease.

Enterohemorrhagic *E. coli* strains are relatively scarce, but they can cause various types of disease. O157:H7 is the principal serotype of enterohaemorragic *E. coli*, which is known to cause watery diarrhea, hemorrhagic colitis and hemolytic uremic syndrome in humans. Production of Stx1 and Stx2 in *E. coli* is conferred by toxin-converting lysogenic phages, which carry genes encoding Stxs (stx) [39, 41, 57, 59]. The presence of phages carrying *stx2* is also common in sewage from developed countries [20, 37, 38]. The high levels of stx2 in sewage indicate an abundance of such phages circulating among the human population. It has been suggested that most *E. coli* O157:H7 infectious to humans are foodborne and that domestic animals are reservoirs of *E. coli* O157:H7 [22, 56]. In addition, *E. coli* O157:H7 seems to persist in food processing because of its acid and heat tolerance [32]. Contamination with *E. coli* O157:H7 may frequently occur at various stages of food processing and drive up the potential for human infection [10].

Because combination of the optimum lytic activity and host cell specificity are essential to realize the use of phage for controlling pathogenic bacteria, a virulent phage (named PP01) was previously isolated from swine stool samples [35]. PP01 was found to infect *Escherichia coli* O157:H7 strains with high specificity by using the outer

membrane protein OmpC as a receptor. Analysis of the deduced amino acid alignment of the tail fiber proteins, Gp37 and Gp38, revealed that PP01 is related to T2. The specific recognition of the *E. coli* O157:H7 OmpC protein by Gp38 determines PP01's host range.

In this study, the transferability of the PP01 specificity to an other virulent phage by the exchange of the genes including the host recognition gene (gene *38*) was investigated.

2.2 Materials and methods

2.2.1 Bacterial strains and bacteriophages

E. coli K12 W3110 was used for propagation of T2 phage and *E. coli* O157:H7 (ATCC43888) was used for propagation of PP01 phage which was isolated from swine stool and was infectious towards *E. coli* O157:H7 strains with high specificity and lytic activity [35]. *E. coli* O157:H7 (ATCC43888) does not produce Shiga-like toxins 1 or 2 because of a lack of genes for these toxins, but possesses a similar envelope structure to enterohemorrhagic *E. coli* O157:H7. Other *E. coli* strains used for phage propagation and examination of host range of recombinant phages are listed in Table 2-1. For the dilution and preservation of phage, SM buffer (10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin, and 50 mM Tris-HCl [pH = 7.5]) [44] was used. Phosphate-buffered saline (PBS) was used for the phage binding assay.

2.2.2 Construction of the plasmid for the recombination of the distal tail fiber genes

A fragment encoding from 100bp-upstream of *gene 37* to 80bp-downstream of

gene38 was amplified by PCR (polymerase chain reaction) using the primers 5'-cgggatccctttttctcgcagaatcctg-3'(S0202) and 5'-cgggatccacaccaaatagaatat-3' (A0202) and PP01 phage genomic DNA as a template. Underlined nucleotides indicate sequences of *Bam*HI. Two primers, S0202 and A0202, anneal to the PP01 and T2 phage DNA. The PCR fragment was digested by *Bam*HI and inserted into pUC118 to produce pPP37-38.

2.2.3 Homologous recombination and isolation of the recombinant phage

E. coli K12 W3110 was transformed by pPP37-38. Transformant cells were used for recombination of the T2 phage tail fiber genes. Transformant cells were incubated in 2 ml of Luria-Bertani (LB) broth with 50 mg l^{-1} ampicillin at 37°C with shaking (120 rpm). The optical density of the medium at 600 nm (OD_{600}) was measured using a Klett spectrophotometer (BACT-550; Nissho-denki Corp., Tokyo, Japan) to estimate the cell concentration. T2 phage infection with a multiplicity of infection (MOI) of 0.01 was performed at an OD_{600} of 0.1. After 24 h incubation, chloroform was added to lyse the cells, and the culture was centrifuged to remove cell debris. The cell lysate was mixed with *E. coli* O157:H7 ATCC43888 and *E. coli* RK4784 pOMPC1 $T2^r$, which does not produce the OmpC protein of *E. coli* K12, but does produce OmpC of *E. coli* O157:H7 [35], in 0.5% agar and overlaid on a LB plate. A single plaque was transferred into SM buffer and used for the plaque assay. The same procedure was repeated three times to purify the recombinant phage.

2.2.4 Sequencing and identification of the recombination sites

Recombinant phage DNA was extracted with phenol and chloroform and precipitated with ethanol. The phage DNA was diluted with distilled water and used as a

template for PCR. The DNA fragment encoding the region around *genes 37-38* was amplified by PCR using the primer set S0202 and A0202 and inserted into the pUC118 vector. Sequencing of the cloned DNA was performed using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) and a DSQ-2000L sequencer (Shimadzu, Kyoto, Japan).

2.2.5 Phage purification

A plaque was isolated and purified twice. The isolated phage was added to *E. coli* O157:H7 culture in 250 ml of LB at MOI=0.01 and incubated for 6 h at 37°C. 2.5 ml chloroform was added to the suspension, which was further incubated for 1 h at 4°C. Cell debris was separated by centrifugation (9,500x*g*, 10 min, 4°C). The phage was precipitated with 25 g of polyethylene glycol (PEG) 6000 and 10g of NaCl, and the culture was allowed to stand overnight at 4°C. The phage was separated by centrifugation (16,000x*g*, 60 min, 4°C) and resuspended in 10 ml of SM buffer. The phage solution was mixed with 20 ml of chloroform, allowed to stand for 6 h at 4°C, and centrifuged (10,000x*g*, 20 min, 4°C) to remove cell debris. The phage was then separated by cesium chloride (CsCl) density gradient (1.45, 1.5 and 1.7 g ml^{-1}) centrifugation (11,000x*g*, 2 h, 4°C). CsCl was removed by dialysis in SM buffer.

2.2.6 Phage binding assay

E. coli cells in the logarithmic growth phase were diluted in LB medium to a final cell concentration of $1x10^7$ CFU ml^{-1}. The cell cultures (10 ml) were prewarmed at 37°C for 10 min and mixed with phage solution to a final concentration of $1x10^5$ PFU ml^{-1} in SM buffer. The mixture was incubated at 37°C. After infection, 110 µl of the mixture was sampled periodically, and the samples were centrifuged (17,400x*g*, 1 min,

4°C). The phage titer of the supernatant was determined by plaque assay using *E. coli* O157:H7 (ATCC43888), and the phage titer at time 0 was defined as 100%.

2.3 Results

2.3.1 Comparison of the structure of the distal tail fiber loci in T4, T2, and PP01

Figure 2-1 (a) shows a comparison of the loci involved in distal tail fiber formation in the T-even phages T2 and PP01. Information regarding DNA alignment around *genes 37-38* of T2 and PP01 was obtained from the GeneBank nucleotide sequence database (Accession numbers: X01755 for T2-*g36*, X04442 for T2-*g37*, X05312 for T2-*g38*, AF349974 for PP01-*g37*, and AF349975 for PP01-*g38*). The putative *g37* of PP01 encoded 3327 base pairs (bp) (1109 amino acids (aa)), which was different from that of T2, which encoded 4026 bp (1341 aa). High identity (91%) of DNA alignment in the 5' first 150 bp of *g37* between T2 and PP01 was observed. On the other hand, only trace homology was observed in the other regions of *g37*. The *g38* of PP01 consisted of 777 nucleotides. The size was closer to that of T2 (789 bp). Homology of DNA alignment between *g38* of PP01 and that of T2 was 61%. However, relatively high homology (73%) of DNA alignment in the 3' last 70 bp was observed. Therefore, homologous recombination of the *g37-38* region between T2 and PP01 was expected.

2.3.2 Homologous recombination of *g37-38* of T2 and PP01

E. coli K12 cells transformed by pPP37-38 were infected with T2. Cell lysate was obtained after 24 h of incubation and used for the plaque assay with *E. coli* RK4784

pOMPC1 $T2^r$. Two clear plaques were found among the many turbid plaques. Clear plaques were picked up, suspended in SM buffer, and used for the plaque assay with *E. coli* O157:H7 ATCC43888. One of the two clear plaques produced plaque on a lawn of *E. coli* O157:H7 cells. The phage was expected to be a recombinant T2 phage infectious towards *E. coli* O157:H7, and named T2ppD1.

To show that T2ppD1 was a derivative of T2, carrying the PP01 distal tail fiber loci, phage genomes of T2, PP01, and T2ppD1 were extracted and used to analyze restriction fragment length polymorphism (RFLP) by *Taq*I digestion (Fig. 2-1 (B)). The RFLP pattern of T2ppD1 was almost identical to that of T2, indicating that the DNA alignment of T2ppD1 was almost identical to that of T2. To confirm that the distal tail fiber loci of T2ppD1 were derived from PP01, the DNA fragment encoding the region of *g37-38* was amplified by PCR using the primer set S0202 and A0202, and digested with *Xho*I and *Eco*RV (Fig. 2-1 (B)). The T2ppD1 restriction fragment was the same as that of PP01, indicating that the region around *g37-38* was derived from PP01.

The PCR fragment of T2ppD1 *g37-38* was digested with *Kpn*I/*Bam*HI or *Sal*I/*Bam*HI and cloned into pUC118 to produce pD1-BK and pD1-SB (Fig. 2-2). Two restriction fragments encoding the junctions of *g36/37* and *g38/gt*, respectively, were used for sequencing (Fig. 2-2). Sequencing analysis revealed that recombination occurred at positions 104-bp downstream from the *g36/37* junction and 15-bp downstream from the *g38* stop codon. Thus, recombinant phage T2ppD1 carried the T2 genome except for the distal tail fiber loci of *g37-38*.

2.3.3 Characterization of recombinant T2 phage

PP01 formed relatively large (0.5-1.0 mm) and clear plaques on a lawn of *E. coli* O157:H7 but did not form plaques on a lawn of *E. coli* K-12 strains or other related

bacteria [35]. The host range of three phages, T2ppD1, PP01, and T2, was examined using eleven *E. coli* strains (Table 2-2). *E. coli* K12 RK4784 cells, which lose OmpC production, became susceptible to PP01 by expressing OmpC of *E. coli* O157:H7. No difference in the host range of T2ppD1 and PP01 was observed. Exchange of Gp37-38 enabled T2 to infect the foreign host cell *E. coli* O157:H7. T2ppD1 used OmpC of *E. coli* O157:H7 as a receptor.

Since the T2ppD1 phage formed relatively small (<0.5 mm) plaque on a lawn of *E. coli* O157:H7 (Fig. 2-3), the efficiency of infection was thought to be low. Figure 2-4 shows *E. coli* O157:H7 cell lysis by phage infection. The increase in OD_{600} came to a halt 2 h after the addition of PP01 and T2ppD1 at a MOI of 0.1, and subsequently, the value decreased. The OD_{600} fell below 0.1 when PP01 was added. On the other hand, the decrease in OD_{600} was small when T2ppD1 was used. After 7 h incubation, OD_{600} increased in both cases due to the appearance of phage resistant cells.

To investigate the effect of Gp37-38 exchange on phage binding to the host cell, a phage adsorption assay was conducted (Fig. 2-4). The time course of the free phage concentration (P_{free}: PFU ml^{-1}) in the culture provided an adsorption rate constant (k_a, ml CFU^{-1} min^{-1}), which represents phage adsorption affinity toward the host cell. *E. coli* O157:H7 cell culture (10^7 CFU ml^{-1}) in the early logarithmic growth phase was mixed with the same amount of phage solution (10^5 PFU ml^{-1}) at 37°C. Free phage in the mixture was analyzed and plotted against the incubation time to estimate k_a values. Free phage adsorption on the host cell (B_{free}: CFU ml^{-1}) surface proceed. Overall adsorption reaction and its kinetics can be described as follows.

$$P_{free} + B_{free} \rightarrow PB_{bound} \quad (1)$$

$$-\frac{d(P_{free})}{dt} = k_a(P_{free})(B_{free}) \quad (2)$$

Under the low MOI (<0.01) condition, B_{free} can be assumed to be constant, that is B_0, until lysis of the host cells. Therefore, integration of equation (2) is as follows.

$$\ln(P_{free}) = -k_a(B_0)t + \ln(P_0) \quad (3)$$

According to equation (3), the time course of P_{free} in the culture provided k_a, which represents phage adsorption affinity on the host cell. 99% of added PP01 phage was adsorbed to *E. coli* O157:H7 cell after 9 min incubation. On the other hand, the value for T2ppD1 was around 50%. The k_a value of T2ppD1 (0.17×10^{-9} ml CFU^{-1} min^{-1}) was almost 1/6 that of PP01 (1.10×10^{-9} ml CFU^{-1} min^{-1}).

2.4 Discussion

T2 and PP01 phage adsorption to the host cell surface initiates a specific but reversible interaction between Gp38 and phage receptor expressed on the host cell surface, followed by an irreversible interaction between Gp12 and LPS. Since T2ppD1 possessed T2-Gp12, an irreversible second interaction formed between T2-Gp12 and LPS of *E. coli* K12 or *E. coli* O157:H7. LPS consists of lipid A, core oligosaccharide (core OS), and O side chain (O antigen). The core OS of *E. coli* can be classified into R1-R4 and K12 [23]. The core OS of *E. coli* K12 is K12-type, while that of *E. coli* O157:H7 is R3-type [2]. The heptose residues of *E. coli* K12 LPS were found to be necessary for efficient binding of Gp12 of K3 phage, which is a T-even phage. Whenever the heptose was missing in the LPS of *E. coli* K12, the binding of Gp12 to

the bacterial surface decreased drastically [43]. The binding of Gp12 to the LPS of K12 could be suppressed with a monoclonal antibody directed against the inner core region. These data indicate that heptose residues of *E. coli* K12 LPS are indispensable for the interaction of Gp12 of K3 phage. These heptose residues are well conserved among the various core OS. Compatibility of Gp 12 and LPS is required for the irreversible binding of phage to the host cell. Low adsorption ability of T2ppD1 to *E. coli* O157:H7 (Fig. 2-5) may be due to the unnatural interaction between phage receptor protein and core OS. The amino acid sequence identity between Gp12 of PP01 and that of T2 was 60%. The slight difference in the PP01 Gp12 may ease the interaction of phage with LPS. To examine this possibility, the simultaneous recombination of gene *12* of T2ppD1 with PP01 gene *12* was investigated. Three kinds of plasmid were constructed for the homologous recombination. One of them contained only gene *12* of PP01. An other contained expanded region around gene *12*, i.e. gene *10*, *11*, *12* and *wac*. The last contained a 450bp fragment of the gene *12* 3'end where the domain interacting with LPS was supposed to be contained [52]. *E. coli* O157:H7 strain ATCC4388 was transformed by these plasmids and used for homologous recombination. T2ppD1 infection of the transformed *E. coli* O157:H7 followed by screening by plaque hybridization was carried out. However no recombinant T2ppD1 phage could be isolated. Baseplate proteins are complexly assembled (Fig. 1-1 C in chapter 1) and interaction among them must be strictly regulated to cause the conformational change. Therefore, it may be difficult to recombine gene *12* region with that of foreign phage.

The second irreversible binding between Gp12 and LPS following the conformational change in the baseplate from the hexagonal shape to the star shape (refer to Fig. 1-1 C in chapter 1) is caused by the signal transferred from the distal tail fibers and transmitted via the interaction between Gp9 in the baseplate and the proximal part

of Gp34 in the long tail fiber. Because T2ppD1 has the chimeric Gp37, another possible reason of the low binding ability of T2ppD1 is that the signal transfer to the baseplate may become inefficient, in spite of the high homology of the DNA sequence in the first 150bp of the 5' end of gene 37 (91%) where the recombination site was contained.

Table 2-1. Bacteria and Bacteriophages

Strain name	
Bacteria	
E. coli O157:H7 (ATCC43888)	
E. coli K12 (W3110)	
E. coli RK4784	*E. coli* K12 ΔOmpC
E. coli RK4784 pOMPC1	RK4784 possessing pOMPC1
E. coli RK4784 pOMPC1 $T2^r$	RK4784 pOMPC1 T2-resistant mutant
E. coli XL-1 blue	
E. coli O157:H7 (CR-3)	for testing phage-host range
E. coli O157:H19 (A2)	for testing phage-host range
E. coli O157:H37 (CE273)	for testing phage-host range
E. coli K12 (Hfr H)	for testing phage-host range
E. coli B^E	for testing phage-host range
E. coli C600	for testing phage-host range
Bacteriophages	
T2	wild-type
PP01	O157:H7 bacteriophage, (isolated from swine feces in our laboratory)
T2ppD1	T2 phage recombinant with PP01 distal tail fiber locus

Table 2-2. Host-Range of T2ppD1

Strain name	Plaque formation[a]		
	T2ppD1	PP01	T2
E. coli O157:H7 (ATCC43888)	+	+	-
E. coli K12 (W3110)	-	-	+
E. coli O157:H7 (CR-3)	+	+	-
E. coli O157:H19 (A2)	+	+	-
E. coli O157:H37 (CE273)	-	-	-
E. coli K12 (Hfr H)	-	-	+
E. coli B^E	-	-	+
E. coli C600	-	-	+
E. coli K12 RK4784	-	-	+
E. coli K12 RK4784 pOMPC1	+	+	+
E. coli K12 RK4784 pOMPC1 $T2^r$	+	+	-

[a] +: forming plaque, -: not forming plaque

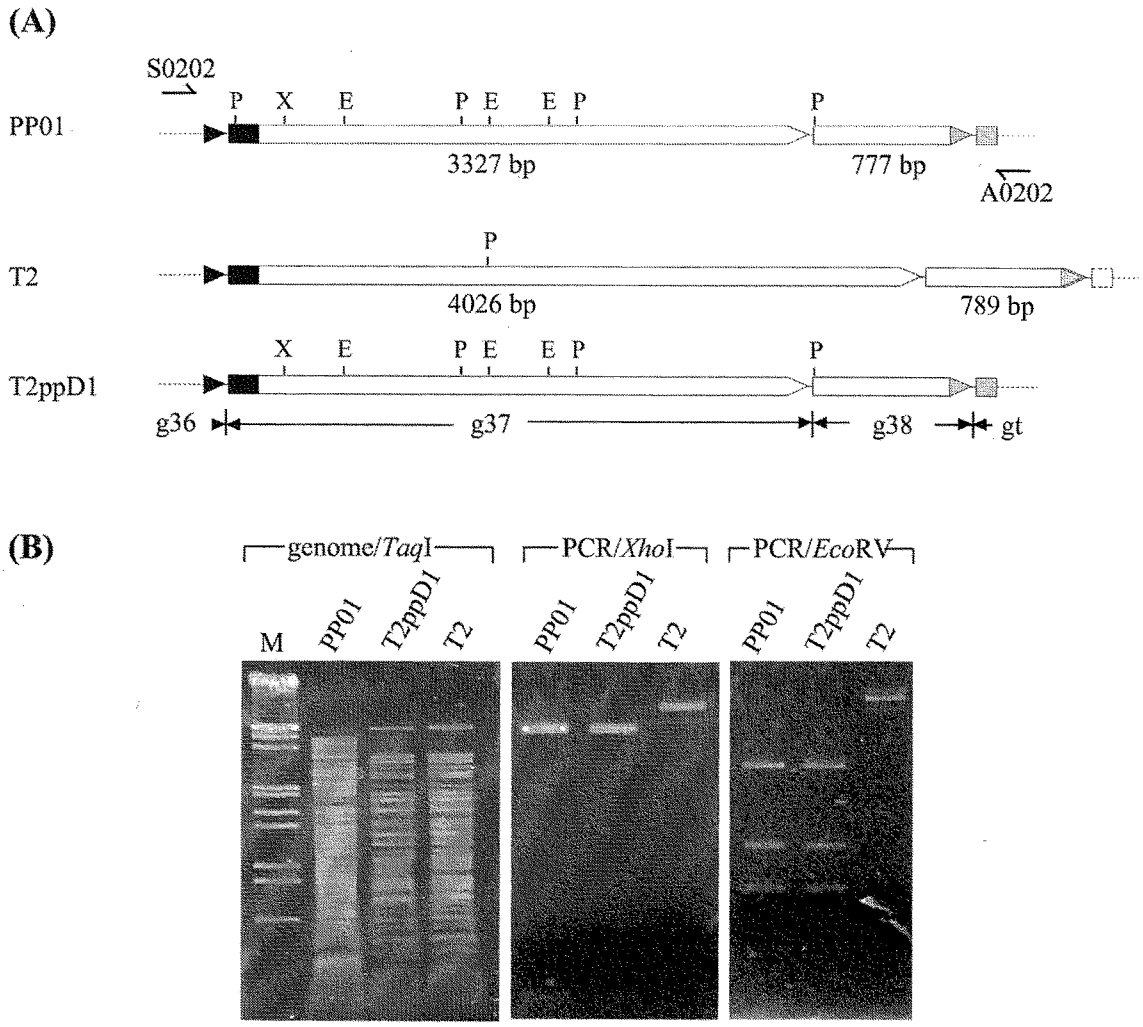

Figure 2-1. (A): Restriction sites in the distal tail fiber locus (from *g36*-3' to *gt*-5'). Each letter indicates a restriction site, P: *Pst*I, X: *Xho*I, E: *Eco*RV. (B): RFLP of the distal tail fiber locus. PCR products were obtained using Primers S0202 and A0202 from PP01 (Lane 1), T2ppD1 (Lane 2), T2 (Lane3) genome, and digested by *Xho*I and *EcoR*V.

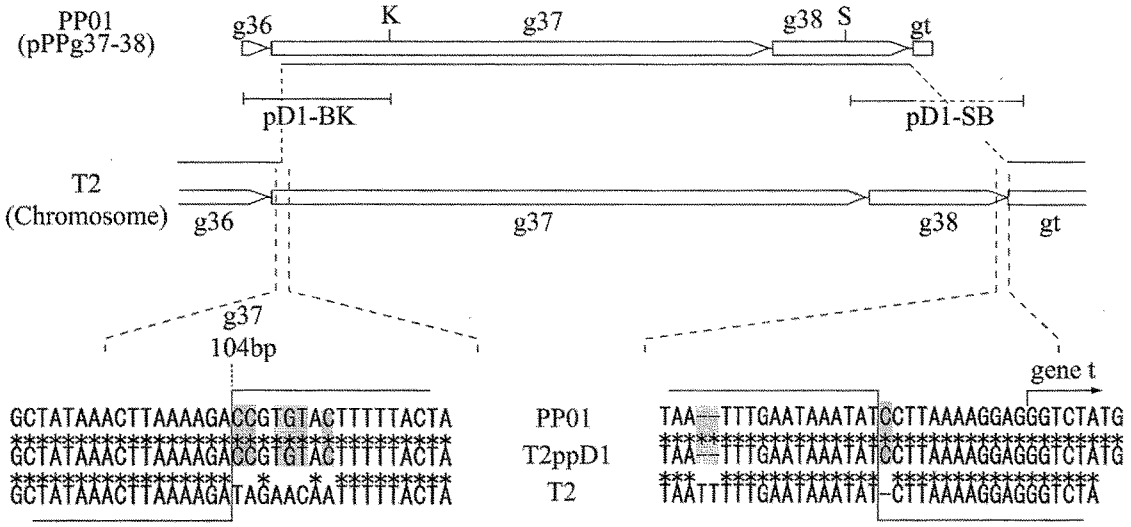

Figure 2-2. The position of recombination between PP01 and T2 phage on the distal tail fiber locus, including details of the cloned PP01 sequence (pPPg37-38) and T2 sequence. Switches between the PP01 and T2 sequences that occurred in each of the recombinants analyzed are shown between the diagrams of two parental segments. The sequences around the point of exchange are shown under the diagrams. The nucleotide coordinates refer to the initiation codon of gene *37*. Two restriction sites, K: *Kpn*I, S: *Sal*I, are indicated.

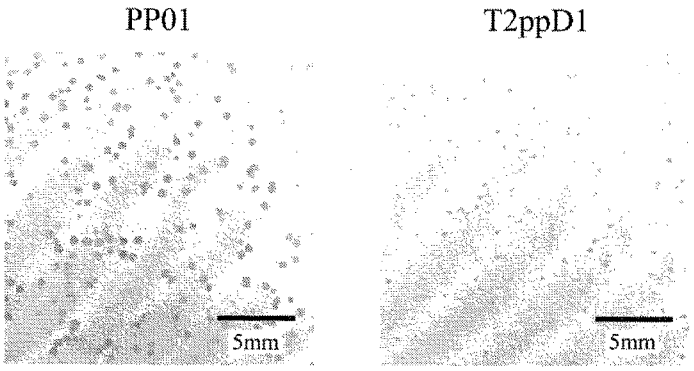

Figure 2-3. Comparison of plaque size on *E. coli* O157:H7 ATCC43888 cells. The bar indicates 5mm.

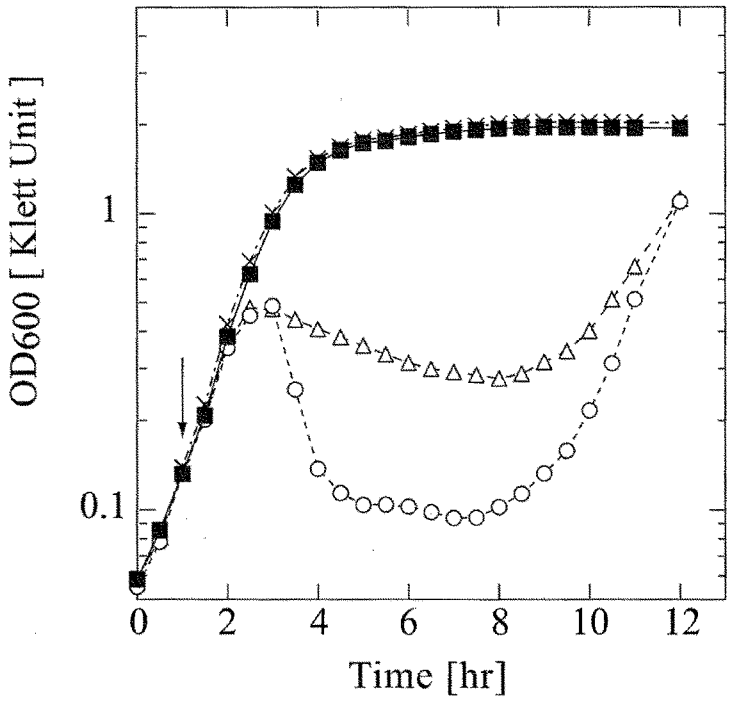

Figure 2-4. *E. coli* O157:H7 ATCC43888 cell lysis due to infection of phages. At 1 h (indicated by the arrow), PP01 phage (open circle), T2ppD1 (open triangle), and T2 phage (cross) were added to the cell culture at an MOI of 0.1. The closed square is a control without phage infection.

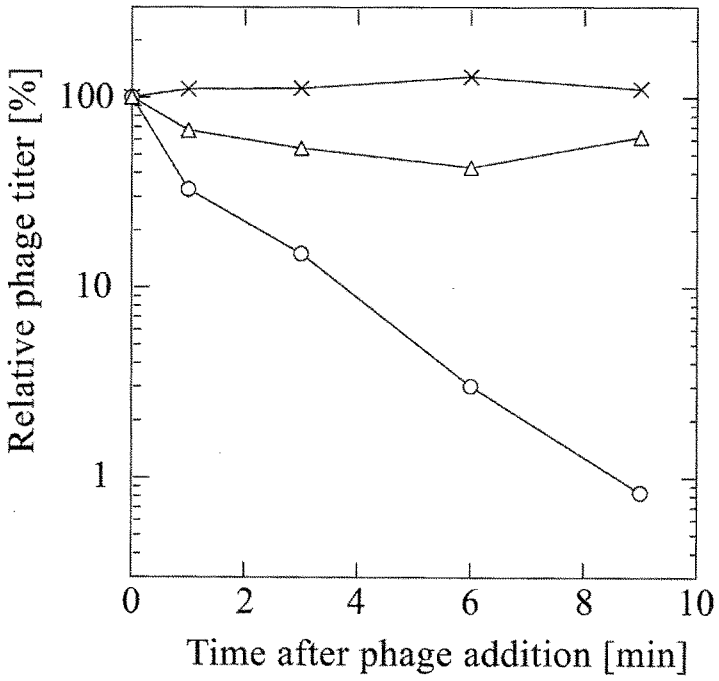

Figure 2-5. Phage adsorption assay. *E. coli* O157:H7 was mixed with PP01 phage (open circle), T2ppD1 (open triangle), and T2 phage (cross). Initial cell concentrations were about 10^7 CFU ml-1. The MOI in each condition was about 0.01.

Chapter 3
Host range shift through superinfection of T2 and PP01 phage

3.1 Introduction

Double-stranded DNA (dsDNA) tailed bacteriophage genomes exhibit mosaic structures, appearing as a patchwork of interchangeable genetic units (modules) [5]. Mosaic structures of phage genomes indicate that bacteriophage evolution progresses by the horizontal gene transfer of modules derived from a common gene pool [12, 24, 26, 31, 40].

The best-characterized group of bacteriophage regarding horizontal gene transfer is the lambdoid phages of *Siphoviridae*. Such mosaic structures have also been observed in the genomes of T4-like phages [7, 11, 28]. Occurrence of intron homing endonuclease in the DNA replication module in T4-type phage genome provides direct evidence of the horizontal gene transfer among these phages [7, 28]. However, the size of the modularly exchanged DNA segments seems to be smaller in T4-like phages than lambdoid coliphages. Also the number of distinct alleles seems to be lower and the structural protein sequence conservation is higher. According to these differences from lambdoid coliphages, it has been suggested that diversity of T4-like phages was caused more by point mutations and occasional gene duplication events than by modular exchanges [7, 11].

Nevertheless, a number of regions including tail fiber genes where modular exchanges had most likely occurred were also identified. Genetic mosaicism is most prominent in the regions encoding receptor-recognizing fibers, and these regions were previously identified as a recombination hot spot [21, 49]. In fact, parts of tail fiber genes closely related with those of bacteriophage Mu, P1, P2 and lambda were found to be contained in the distal tail fiber regions of various T-even phages [28]. Conservation of tail fiber proteins is relatively low among the structural proteins. The degree of

conservation decrease gradually from the proximal part (Gp34) to the distal tail fiber subunit (Gp37 for T4, Gp38 for T2 and T6). However, comparison of the sequences from the end of the gene *36* to gene *t* revealed discrete homologous regions, appearing as a mosaic design (Fig. 3-1) [11, 49].

3.2 Materials and methods

3.2.1 Bacteria strains and bacteriophages

Eleven *Escherichia coli* strains and two bacteriophages T2 and PP01 used in the study presented in Chapter 2 (Table 2-1. except for *E. coli* RK4784 pOMPC1 $T2^r$) were also used in this study. Bacteriophage PP01 e^-/GFP is a derivative of PP01 phage that possesses a gene *e* inactivated by an amber stop codon insertion and gene encoding green fluorescent protein (GFP) in gene *soc* [3]. *E. coli* CR63, a suppressor mutant of *E. coli* K12, was transformed withpOMPC1. Thereafter, *E. coli* CR63 harboring pOMPC1 was used for the propagation of PP01 e^-/GFP. Both T2 and PP01 are infective to *E. coli* C and this strain was used as a host for the superinfection experiment.

3.2.2 Superinfection of T2 phage and PP01 e^-/GFP phage

E. coli C was cultured overnight in LB medium and 1 % of the culture inoculated in 5 mL of LB medium in 30 mL test tube. The bacteria was grown with aeration at 37°C to 1×10^7 CFU/mL. T2 phage solution was added to the culture at a MOI of 10 and incubated at 37°C for 5 min to allow the phage to infect the cells. Then a PP01 e^-/GFP phage solution was added to the culture at a MOI of 10. After another 5 min incubation, the culture was transferred to microtubes and centrifuged at 2,800×g for

3 min and supernatant was discarded to eliminate unbound phage. The cells were resuspended in total volume of 2 mL LB medium and incubated with aeration at 37°C for 1 hour. The culture was transferred to microtubes and centrifuged at 11,100×g for 5 min. One milliliter of supernatant was taken and adequately diluted in SM buffer before used for plaque assay. Sample was stored at 4°C after addition of 50 μL chloroform.

3.2.2 Screening of the recombinant T2 phage

Screening for recombinant T2 phage consisted of two steps. First, a plaque assay using superinfection experiment sample was performed on *E. coli* O157:H7 (ATCC43888) to isolate phage infective to this strain. Plaques were taken and suspended in 300 μL of SM buffer. The second step of screening was selection by PCR in which two sets of primers were used. Sequences of the primers used for the screening are listed in Table 3-1. Each of them was specific for gene *12* of T2 or PP01. Gene *12* was supposed to locate to the furthest region of the genome from gene *38*. Therefore it was assumed that recombinant T2 phage possessing PP01 e⁻/GFP-gene *38* would not have acquired PP01 e-/GFP-gene *12* simultaneously. The sequences of gene *12* for both T2 and PP01 are available in the Genbank nucleotide sequence database (accession numbers: X56555 for T2-gene *12*, AB180231 for PP01-gene *12*). Plaque suspensions were directly used as template for the PCR reaction.

3.2.3 Identification of the recombination sites

Identification of recombination site was performed by PCR-Restriction fragment length polymorphism (RFLP) and DNA sequencing. Respective primers for each gene examined are listed in Table 3-1. The sequenced regions of the genome are fairly limited both for T2 and PP01. Both of them are closely related to bacteriophage

T4 whose whole genome sequence is available in Genbank nucleotide sequence database (accession number: NC_000866). Therefore, primers used for the identification of recombination sites were designed based on the T4 genome sequence. RFLP of the PCR fragments of recombinant phage were compared to those from T2 and PP01 to examine the origin or estimate the recombination site. Some resulting fragments were also cloned and sequenced.

3.3 Results

3.3.1 Superinfection of bacteriophage T2 and PP01 e⁻/GFP to *E. coli* C

E. coli C strain was selected as host bacteria for superinfection experiment because this strain naturally serves as a common host to T2 and PP01. T2 phage is not infective to *E. coli* O157:H7 (ATCC43888) and PP01 e⁻/GFP lysozyme gene was inactivated by amber stop insertion [3]. Therefore neither phage can form plaques on *E. coli* O157:H7 wild type, which makes the selection of recombinant phage much more efficient. PP01 e⁻/GFP revertant for lysozyme gene and recombinant T2 phage that acquired PP01 e⁻/GFP-gene *38* responsible for host recognition were expected to give plaques on a lawn of *E. coli* O157:H7. The resulting lysate contained about 10^4 PFU/mL of phage capable of forming plaques on the lawn of *E. coli* O157:H7. The lysate was diluted in SM buffer before the plaque assay to produce a number of plaques under 100/plate so that the picked up plaque would not be contaminated by the adjacent ones.

3.3.2 Isolation of recombinant T2 phage

Isolated plaque suspensions were subjected to a second selection step based on

the PCR. The origin of gene *12* was examined to screen the recombinant T2 phage. Primer sets for both T2 and PP01 were used in the same PCR mixture. The primer set for PP01 produced a 1.8kbp fragment exclusively from PP01-gene *12* and the primer set for T2 amplified a 0.48kbp fragment exclusively from T2-gene *12* (Fig. 3-2). Twenty-five of fifty-four isolates gave T2-gene *12* bands and the other samples gave PP01-gene *12* bands. Those isolates that gave PP01-gene *12* bands were supposed to have a genome mainly composed from a PP01 e-/GFP originating fragment. Further, the recombinant T2 phage that acquired the PP01-originating fragment containing gene *38* were predicted to give the band of T2-gene *12*. Two isolated phages which gave the bands corresponding to T2-gene *12* (Fig. 3-2) (designated as TPr03 and TPr04) were subjected to further analysis.

3.3.3 Identification of the recombination sites

Schematic diagrams are shown in figure 3-3 and selected PCR-RFLP results are shown in figure 3-4. TPr03 and TPr04 were infective to *E. coli* O157:H7, suggesting that both the isolates possessed a similar adhesin to that of PP01. To verify this prediction, gene fragments containing the whole sequence of gene *37* and *38* were amplified using the primers 37f and 38r. RFLP patterns obtained by *Fok*I digestion of the fragments were compared with those of T2 and PP01. For both TPr03 and TPr04, RFLP of this region was absolutely the same as that of PP01 and different from T2, supporting the prediction mentioned above. It was also supposed that no recombination site was included in gene *37* and gene *38* for both isolates.

More extensive regions around gene *38* were also examined by PCR-RFLP analysis. Two primer sets of 37f, rIIBr and 38r, 59f were used to amplify 12kbp fragments from both sides of gene *38*. RFLP of TPr03 and TPr04 obtained by the

digestion of these fragments with *Rsa*I and *Msp*I were compared with those of T2 and PP01. RFLP obtained from downstream region of gene *38* (clockwise direction in Fig. 3-3 B) for TPr04 showed limited homology with both T2 and PP01 (Fig. 3-4 A), suggesting that the recombination site was included in this region. RFLP of the other side of gene *38* for TPr04 and both sides of gene *38* for TPr03 showed no difference from those of PP01 (Fig. 3-4 B).

The gene *38*-downstream recombination site of TPr04 was determined to locate between gene *37* and gene *rIIB*. Gene *motA* and *52* were located in the middle between gene *37* and *rIIB* and the origin of the fragment from gene *motA* to *52* (primer: motAf, 52r) (2.1kbp) was examined. The RFLP of the fragments was identical to that of T2. However RFLP of the fragment from gene 37 to 52 (primer: 37f, 52r) (8.7kbp), which include the fragment from gene *motA* to *52* was almost the same as that of PP01 (Fig. 3-4 C), suggesting that the recombination site located near gene *motA*. Finally the fragment from gene *arn.1* to *motA* (primer: arn1f, motAr) (1.5kbp) was sequenced and the recombination site was identified as lying in the region between 58bp downstream and 27bp upstream from the gene *motA* stop codon.

The other recombination sites did not locate to the 24kbp region around gene *38*. In order to determine the approximate recombination sites in the gnome, some regions were picked up appropriately based on the T4 genome map and examined for the origin.

For the gene *38*-downstream recombination site of TPr03, gene *soc* region (primer: socf, socr) (0.86kbp) was determined as being of T2 origin and the region from gene *39* to *dda* (primer: 39f, ddar) (6.2kbp) was found to contain the recombination site. One of the *Eco*RI fragment of this region for TPr03 was not observed for T2 and PP01 (Fig.3-4 D), suggesting that this fragment contained the recombination site. Therefore this *Eco*RI fragment was cloned into pUC118 vector and sequenced. Next, primers for

the region from gene *motB.2* to *dexA* were designed based on the sequence result (primer: motB2f, dexAr) (1.2kbp). PCR fragments using these primers were sequenced and the recombination site was identified as lying in the region from 20bp to 31bp upstream from gene *motB.2* initial codon.

The gene *38*-upstream recombination sites of TPr03 and TPr04 were also identified with a similar methodology. For TPr04, the gene *alt* region (primer: altf, altr) (0.78kbp) and gene 23 region (primer: 23f, 23r) (1.3kbp) were determined as being of PP01 origin (Fig.3-4 E) and the region from gene *15* to *19* (primer: 15f, 19r) (5.2kbp) contained the recombination site. Sequence analysis of the region of gene *18* (primer: 18f, 18r) (0.84kbp) revealed that the recombination site lay in the region from 881bp to 1084bp downstream from gene *18* stop codon.

Gene *alt* region was determined as T2 origin for the gene 38-upstream recombination site of TPr03 (Fig.3-4 E) and recombination site was contained in the region from gene *59* to *NrdA.1*(primer: 59f, NrdA1r) (6.5kbp). Sequence analysis of the region from gene *32* to *frd.1* (primer: 32f, frd1r) (1.4kbp) determined that the recombination site lay in the region from 47bp to 125bp upstream from the initial codon of gene *frd.3*. The recombination sites of TPr03 and TPr04 are shown in Figure 3-5.

The results of the recombination site analysis suggested that T2-originating regions constituted 82% of the TPr03 genomes and 62% of the TPr04 genome. TPr03 had only tail fiber genes, whereas TPr04 acquired also head and tail tube genes.

3.3.4 Host range and binding ability of the recombinant phage

Host range of TPr03 and TPr04 was examined against 10 *E. coli* strains (Table 3-2). Both isolated phages showed exactly the same host range as PP01. TPr03 and TPr04 cannot infect *E. coli* RK4784. However, this strain was susceptible to these

phages as well as PP01 infection due to *E. coli* O157:H7-OmpC expression from plasmid. This observation was consistent with the results of sequence analysis that showed adhesin genes of both phages were identical to PP01.

The binding rate against *E. coli* RK4784 harboring pOMPC1 was investigated with the method detailed in chapter 2 to explore the infectivity of the new phages generated by the superinfection experiment. Figure 3-6 shows the results of the phage binding assay. Both TPr03 and TPr04 showed similar adsorption behavior to PP01. First order binding constants were calculated using the same equations presented in chapter 2 as 6.6×10^{-9} mL min^{-1} for TPr03, 8.5×10^{-9} mL min^{-1} for TPr04, 3.7×10^{-9} mL min^{-1} for T2 and 1.52×10^{-8} mL min^{-1} for PP01, respectively. Though both isolated phages were thought to possess the same adhesin as PP01, their first order binding constants were lower.

3.4 Discussion

Bacteriophage genomes show mosaic structures and analogy in the functions of homologous sequences among phages imply the magnitude of the horizontal gene transfer for the bacteriophage evolution. Even though the lysogenic phages have more chances to recombine with the genome of another phage, lytic phages also show the mosaic structure in the genome, especially in the tail fiber genes [21, 49]. In this study, genomic recombination between two virulent phages through superinfection was demonstrated. Employment of lysozyme inactivated mutant of PP01 (PP01 e^-/GFP) and the diagnostic PCR made it easier to isolate the recombinant T2 phage that acquired the infectivity to *E. coli* O157:H7. PCR-RFLP analysis followed by the DNA sequence identified the genes where the recombination sites located as gene *motB.2* and gene

Frd.3 for TPr03 and gene *motA* and gene *18* for TPr04. The complete genome sequences of bacteriophage RB69, RB49, 44RR2.8t, KVP40 and Aeh1 are available and all of them are morphologically related to T4 [50]. The four relevant gene regions of T4 where recombination occurred were compared with those of these 5 phages. The T4-gene *18* showed high homology with these phages (76% with RB69, 64% with RB49, 62% with 44RR2.8t, 55% with KVP40 and 55% with Aeh1). However the other 3 gene regions were not identified except the region around gene *motA* in RB69, suggesting that these gene regions are not conserved. Apparently recombination events through superinfection are not related to the conservation. It was suggested that TPr03 had only tail fiber genes of PP01, whereas TPr04 acquired also head and tail tube genes. It seems, at least between T2 and PP01, assemblages of the products of each structural gene clusters are highly compatible.

Identification of the recombination sites suggested that T2 phage acquired infectivity towards *E. coli* O157:H7 through the exchange of genes, including the adhesin gene (gene *38*) with superinfected phage (PP01 e^-/GFP). Mosaic structure in the tail fiber region of various T4-type phages was revealed by comparative sequence analysis and it is considered that the conserved motifs facilitate the swapping of the adhesin genes among diverse phages [7, 11, 28]. However recombination of considerably extended regions including the adhesin gene was observed in our experiment, suggesting that recombination events occur more drastically during superinfection.

TPr03 and TPr04 showed absolutely the same host ranges against 10 *E. coli* strains as that of PP01. Fayard *et al.* demonstrated that *Streptococcus thermophilus* temperate phages subjected to superinfection of hosts lysogenized with different temperate phages caused host range expansion along with a change in DNA restriction

profiles [18]. This observation indicated an apparent recombination between the two temperate phages. Coliphage K1-5 possesses two tail fiber genes, which enable it to infect both K1 and K5 strains of *Escherichia coli* [46]. Each tail fiber gene of K1-5 showed significant homology to that of bacteriophage ΦK1E or ΦK5, suggesting that the bacteriophage ΦK1E or ΦK5 acquired other tail fiber genes, resulting in K1-5 phage generation. Host range expansion of T4 phage was also observed by duplication of a small domain in the tail fiber gene [51]. These investigations suggest that recombination events sometimes cause host range expansion. However the host ranges of the two isolates TPr03 and TPr04 only shifted to match that of PP01. Host range expansion by recombination can be caused but host range shift caused by adhesin gene swapping may be more likely to occur though experimental evidence of host range shift through superinfection has not been reported previouly. It is the first experimental evidence of the host range shift through superinfection by two different bacteriophages.

TPr03 and TPr04 showed an intermediate first order binding constant against *E. coli* RK4784 pOMPC1. Gp38 of PP01 recognizes OmpC of *E. coli* O157:H7. On the other hand, Gp38 of T2 recognizes outer membrane protein OmpF of *E. coli* K12. Therefore, it was suggested that the difference in the receptor caused a divergence in the binding ability between T2 and the other phages. Further, both isolated phages were thought to possess the same adhesin as PP01, however their first order binding constants were lower. Gp12 and Gpwac, a fibril exerted from the phage neck, also engage in binding to the host cell [9, 30, 43]. The end of Gpwac is thought to interact with Gp36 in the long tail fiber and hold them in retracted configurations that prevent phage infection [9]. Both TPr03 and TPr04 were suggested to possess gene *12* and *wac* derived from T2. This means those relevant proteins would have to interact with unnatural targets which might affect the binding ability. The affection of the different origin in

Gp12 have also been discussed in chapter 2. In the adsorption assay of the present study, the host bacteria *E. coli* RK4784 was a derivative of *E. coli* K12. Therefore Gp12 of both the recombinant phages derived from T2 should have interacted with its natural receptor. Two possible reasons for the lower binding ability of both TPr03 and TPr04 than that of PP01 can be supposed. One is that simply, the binding ability of PP01-Gp12 may be higher than that of T2-Gp12. The other reason is that, in spite of the apparent high compatibility among structural proteins, the efficiency of the signal transmission from the tail fiber to the baseplate may become lower because of the unnatural interaction, causing inefficient conformational change for the irreversible binding. Simultaneous recombination of the distal tail fiber and baseplate may verify these hypotheses.

In this research, host range shift caused by genomic recombination through superinfection was observed. Recombination events were apparently not site-specific and exchange of extremely large fragments (up to 38 percent of the whole genome) seemed to be possible. These observations suggest that the phage-breeding system presented here can be used as a new genome manipulation technique to generate phage which inherit characteristics from both parent phages. Also the results observed in this research indicated the wider possibility of the occasional occurrence of spontaneous virulent phage superinfection followed by genomic recombination between the phages, thus generating new phage characteristics.

Table 3-1. Primers

Primer	Sequence	Target gene
For the screening of recombinant phage		
T2g12f	5'-AGCTAAATTTACTGTTGCTCTTAATAATGT-3'	*12*
T2g12r	5'-GCATCGCCTCCACTCTGTGAACCGGCGGTT-3'	*12*
PP01g12f	5'-CGGGATCCATGAGTAATAATACATATCAA-3'	*12*
PP01g12r	5'-GCTCTAGAGCTGTTTTAAGAATTCCTATATTTTCG-3'	*12*
For the identification of recombination sites		
37f	5'-GGGGTACCATTCTGTTCAAGATTTCGGCTATTATTAA-3'	*37*
38r	5'-GCTCTAGATGATATTCTAGGTGCTGCCAT-3'	*38*
rIIBr	5'-GAAGATGGTTACTTCTATGCTTGG-3'	*rIIB*
59f	5'-GAATCAAGCAAGATAAACGTCTCAAACG-3'	*59*
socf	5'-CGGAATTGGAAGAAATCTTTAAACTTTATTATCTG-3'	*soc*
socr	5'-TCTAAGCTTGGTTTAATCCAACGATTTAACAT-3'	*soc*
39f	5'-CGGGATCCCACCATTCTTAGTATGAATGTT-3'	*39*
ddar	5'-CGGGATCCATGACATTTGATGATTTGACCG-3'	*dda*
motB2f	5'-CGGGATCCCAGATACACGAGCTAATTCACC-3'	*motB.2*
dexAr	5'-CGGGATCCGCACATGATTCTATTCATGACT-3'	*dexA*
altf	5'-CGGGATCCTAAAATTTCCCGTAGAGCAATT-3'	*alt*
altr	5'-CGGGATCCTATTTTTAACTAGCGCTTCGTA-3'	*alt*
NrdA1f	5'-CGGGATCCTTAATTGCATTAAGTCCTCAAC-3'	*NrdA.1*
59r	5'-CGGGATCCGATAAAACAGCATATGAATGGA-3'	*59*
Frd1f	5'-CGGGATCCGCAGTATTCTTCTGACATACTC-3'	*Frd.1*
32r	5'-CGGGATCCGCTGCTAAGAAAGCTGATAAAG-3'	*32*
motAf	5'-CGGGATCCACCGTTTTTAGCGATTTTACAT-3'	*motA*
52r	5' CGGGATCCGTATCATTGATAATGAAGCATT-3'	*52*
arn1f	5'-CGGGATCCTTATGCTTTGCGACTAAATTGT-3' -3'	*arn.1*
motAr	5'-GGAAATATGCGAATTTTTGG-3'	*motA*
23f	5'-CGGGATCCAAGCGATTATCGCTAAAATCTT-3'	*23*
23r	5'-CGGGATCCTAACGAGTTTTGAATCCCATTA-3'	*23*
15f	5'-CGGGATCCGATGGGCGATTTGTTTTCAAA-3'	*15*
19r	5'-CGGGATCCGTGTTACATCATCTACAAACAT-3'	*19*
18f	5'-CGGGATCCACTGAAGGTAAAATTACTGAAG-3'	*18*
18r	5'-CGGGATCCAATACTAAGCAATCTTGGCG-3'	*18*

Table 3-2. Host range of T2, PP01, and two recombinant phages TPr03, TPr04

Strain	TPr03	TPr04	T2	PP01
E. coli O157:H7 (ATCC43888)	+	+	-	+
E. coli K12 (w3110)	-	-	+	-
E. coli C	+	+	+	+
E. coli O157:H7 (CR-3)	+	+	-	+
E. coli O157:H19 (A2)	+	+	-	+
E. coli O157:H37 (CE273)	-	-	-	-
E. coli K12 (Hfr H)	-	-	+	-
E. coli BE	-	-	+	-
E. coli C600	-	-	+	-
E. coli RK4784	-	-	+	-
E. coli RK4784 (pOMPC1)	+	+	+	+

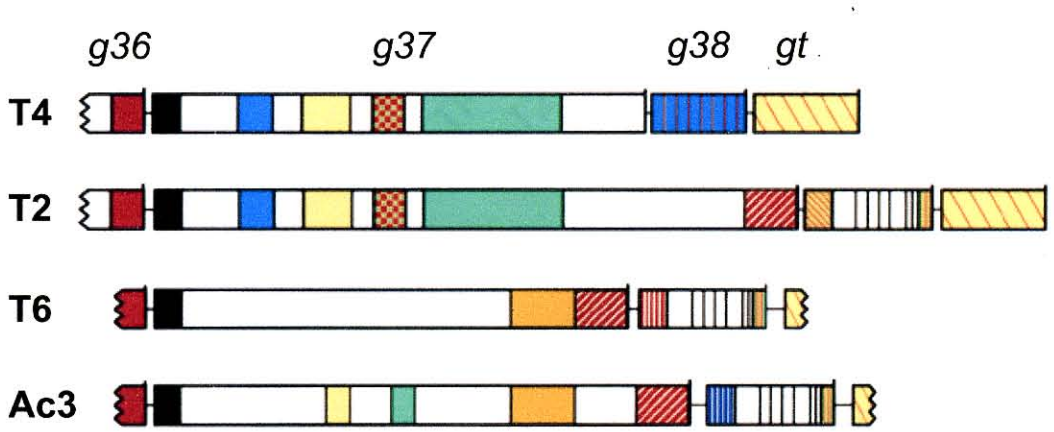

Figure 3-1. Diagrams of the different versions of the loci involved in distal tail fiber formation in the T-even phage T2, T4, T6 and Ac3. Genes 36, 37 and 38 of this locus are indicated by boxes that are proportional to the length of the coding sequence of the phage gene (labelled g36, g37 and g38). An identical pattern of hatching in the different boxes, whose sizes are not necessarily the same, indicates patches of homology in different versions of the gene (reproduced from reference No. 49).

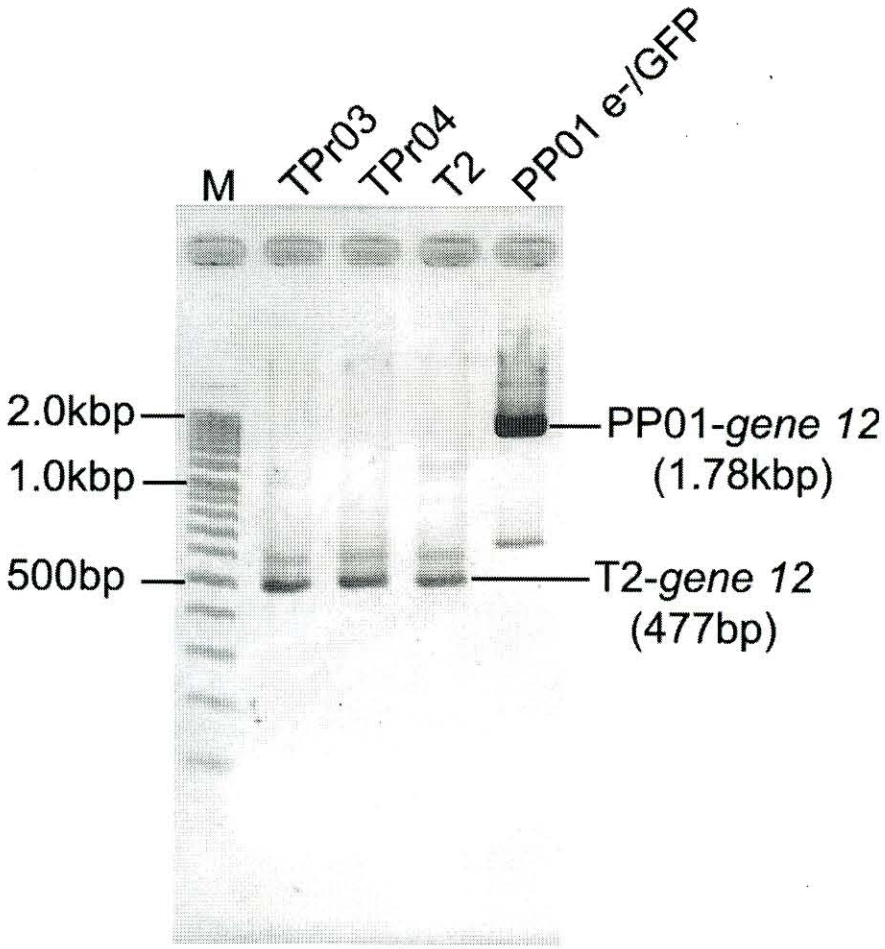

Figure 3-2. Specific amplification of the fragments in gene *12*. Lysate solutions were directly used as template for the PCR and both the primer sets for T2 and PP01 were included in the same PCR mixture. Target regions for T2 (477bp) and for PP01 (1.78kbp) could be amplified exclusively.

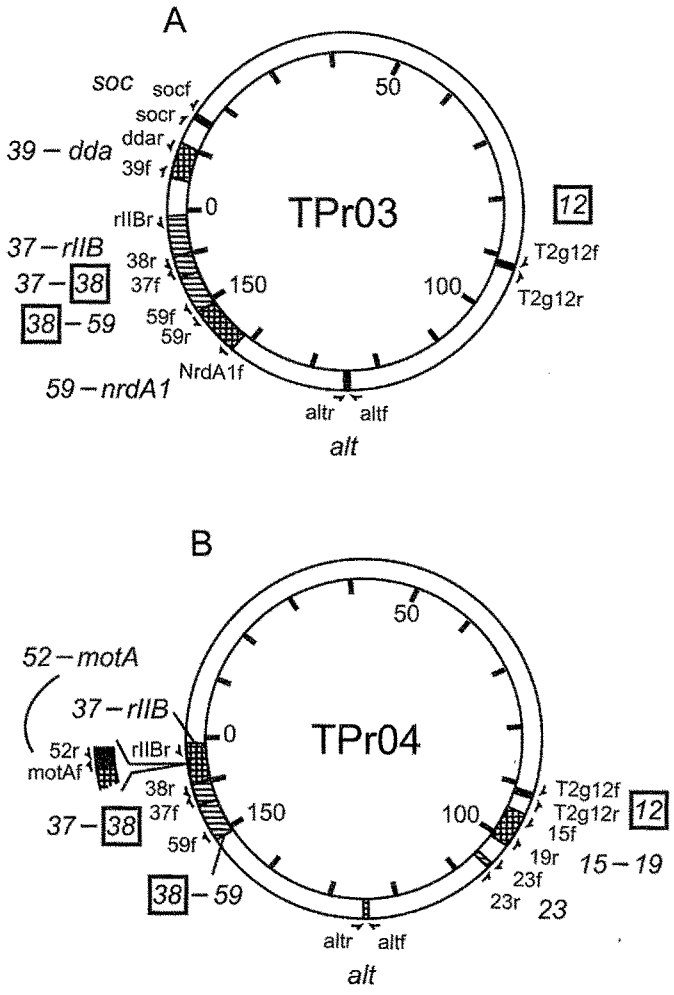

Figure 3-3. Schematic diagram of the recombination sites identification (A; TPr03 and B; TPr04). Scales in the genome map are assigned every 10kbp from the border between gene *rIIA* and *rIIB*. The gene regions examined by PCR-RFLP analysis are indicated with boxes filled with patterns according to their origin (T2 regions; black, PP01 regions; striping and recombination sites-containing regions; netted) and primers used for the amplification of these regions are also depicted beside the boxes.

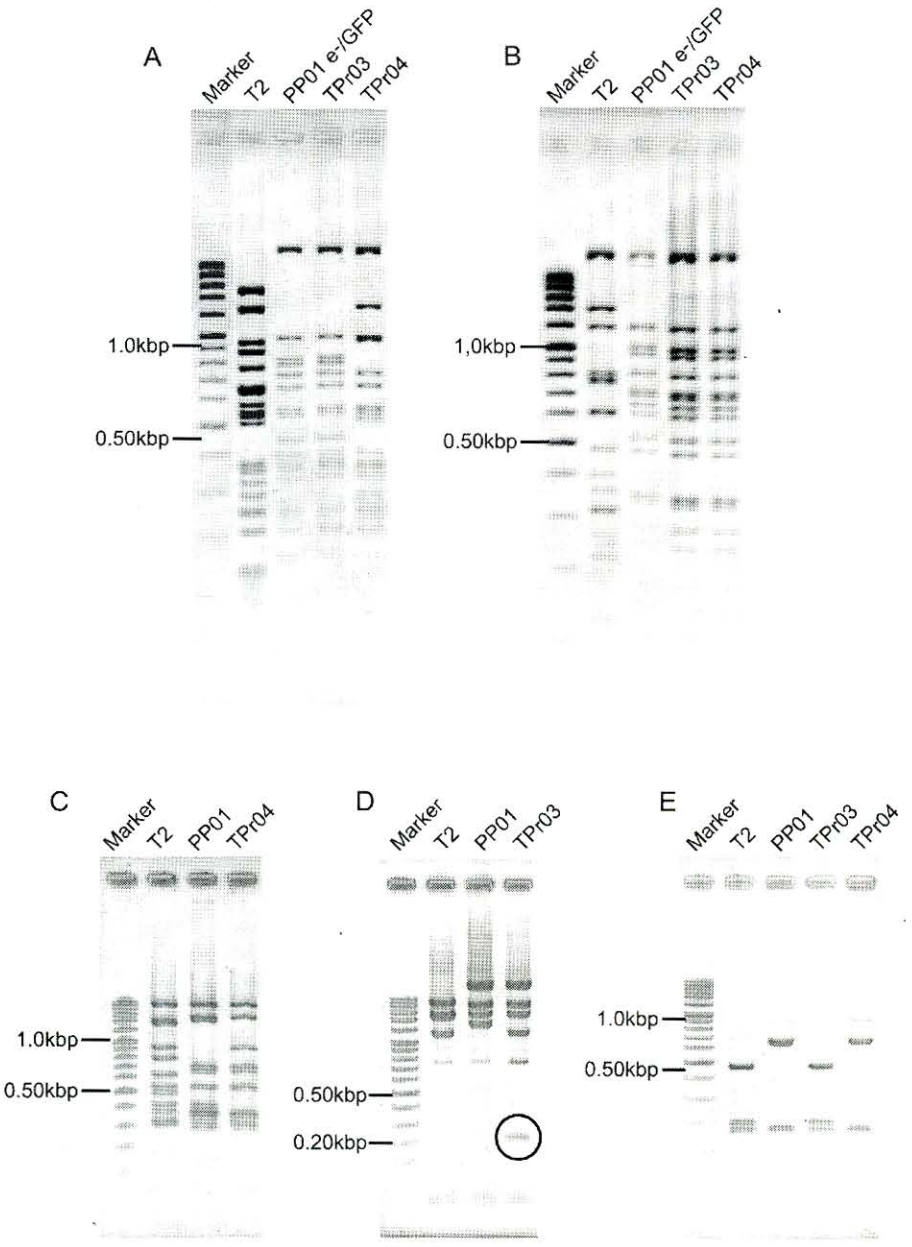

Figure 3-4. RFLP results: A; RsaI digestion of gene 37 to rIIB region, B; MspI digestion of gene 59 to 38 region, C; *Msp*I digestion of gene *52* to *37* region, D; *Eco*RI digestion of gene *39* to *dda* region and E; *Ase*I digestion of gene *alt* region. In the RFLP results of the *Eco*RI digestion of the gene *39* to *dda* region, the gene fragment that was supposed to contain the recombination site is indicated by a circle (D).

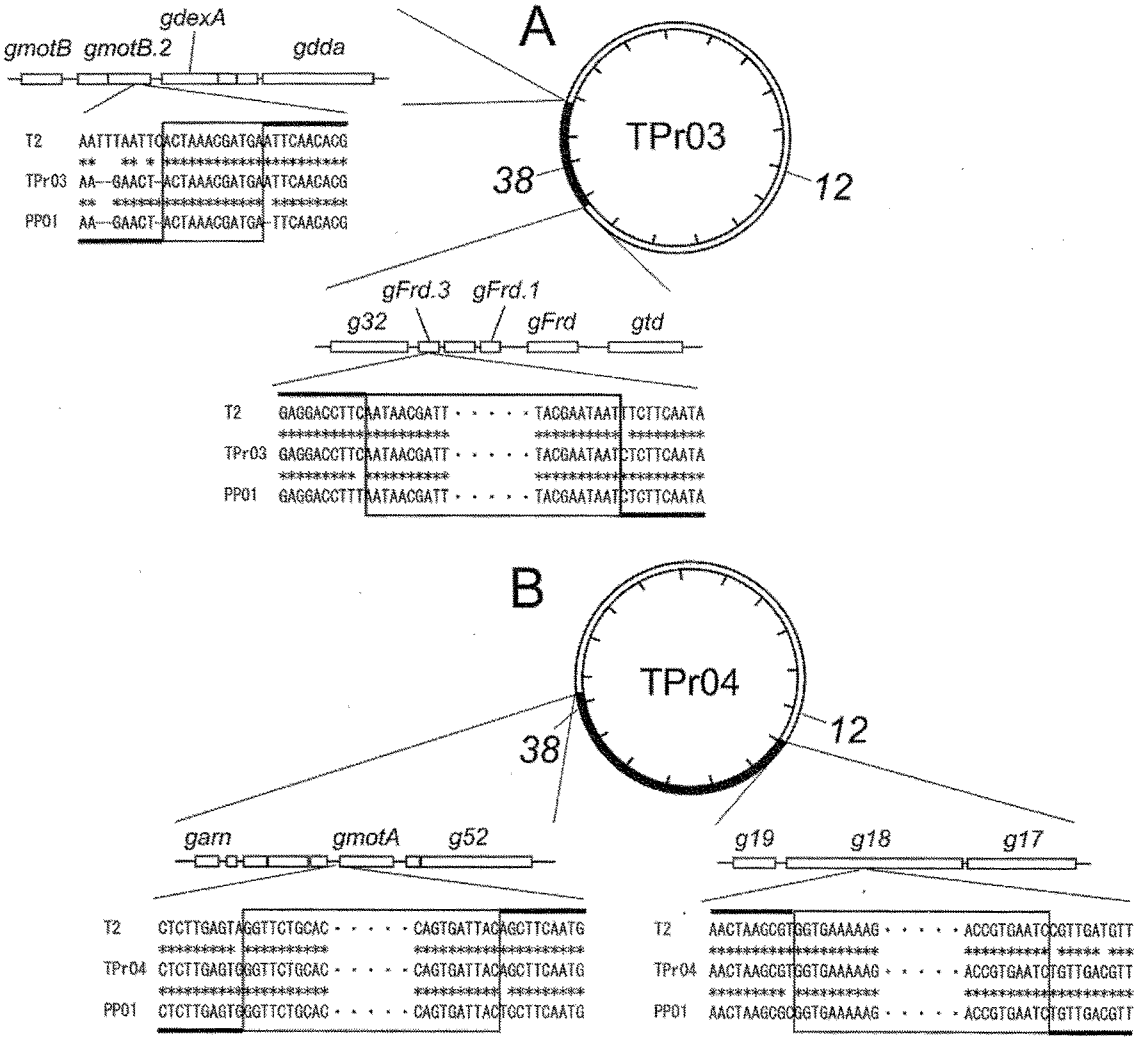

Figure 3-4. The positions of recombination events occurring between T2 and PP01-e⁻/GFP that generated the chimeric hybrid genome. Approximate locations of the recombination sites of TPr03 (A) and TPr04 (B) in the genomes are shown based on T4 genome map. The regions suggested to be identical to PP01 are indicated by the filled boxes.

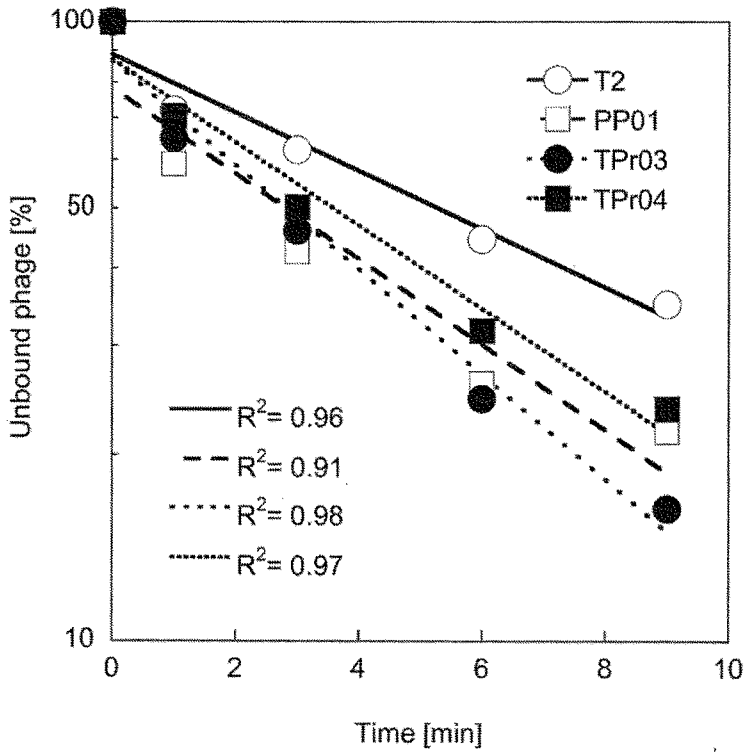

Figure 3-5. Binding of recombinant phages to exponentially growing *E. coli* RK4784 harboring pOMPC1 at a multiplicity of infection of 0.01, in LB medium at 37 °C. Binding rates of TPr03 (closed circle) and TPr04 (closed square) were compared with T2 (open circle) and PP01 (open square). Fraction of unbound phage was plotted against the incubation time.

Chapter 4
Conclusions

4.1 General conclusions

The basis of the increasing interest in the applications of bacteriophage is its high specificity to the host bacteria. However the flexibility of the specificity has also been observed by many researchers. To assess the possibility of manipulating the specificity, the flexibility of the T-even phage host recognition was evaluated in this study.

The distal tail fiber gene region of PP01, an *E. coli* O157:H7 specific phage, was cloned into a vector and almost the entire gene *37* to gene *38* was recombined with the T2 phage genome. The resulting recombinant T2 phage (T2ppD1) showed exactly the same host range as that of PP01. Comparison of the cell lysis curve against *E. coli* O157:H7 between T2ppD1 and PP01 indicated the lower lytic ability of T2ppD1 and it was supposed to be caused by significantly lower binding ability of T2ppD1 as compared to PP01.

To investigate the phenomenon which cause the host recognition change of T-even phage in nature, the occurrence of the host recognition alteration during superinfection of T2 and PP01 phage was studied. The use of lysozyme-inactivated PP01 phage and diagnostic PCR lead to the isolation of the recombinant T2 phages (TPr03 and TPr04). PCR-RFLP analysis and sequence analysis revealed the recombination sites of both TPr03 and TPr04. Recombination of a DNA fragment that extended to up to 38% of the whole genome was observed. The host ranges of two recombinant phages just shifted to that of PP01. Both recombinant phages showed higher binding ability to *E. coli* RK4784 pOMC1 than T2 wild type and it was suggested that the difference in the receptor for each phage against *E. coli* RK4784 pOMPC1 (i.e. OmpF for T2 and OmpC for TPr03 and TPr04) affected the affinity

between the phage and the host bacteria.

4.2 Prospective points for further study

Both the recombinant T2 phages isolated in the study of Chapter2 (T2ppD1) and Chapter3 (TPr03 and TPr04) showed lower binding abilities than PP01 wild type. All the recombinant phages possess baseplate genes of T2 origin. Especially, gene *12* is responsible for the second step of the binding to the host bacteria and may affect the binding ability directly. Therefore simultaneous exchange of the long tail fiber genes and baseplate genes (or gene *12*) may avoid the decrease in the binding ability.

In this study, the occurrence of the host range shift during superinfection was demonstrated. In order to evaluate the frequency of host range shift and the variety of the recombination during superinfection, the rate of the emergence of recombinant T2 phage should be analyzed and recombination site analysis with more recombinant T2 phage should be performed, which will provide important information about the significance of the phenomenon observed in this study for bacteriophage evolution.

From the view of bacteriophage evolution in nature, the effect of point mutation also should be considered to understand the phenomena which cause the host recognition shift. Drexler *et al.* and Morona *et al.* demonstrated host range mutation due to a single amino acid alteration in the gene 38 of bacteriophage Ox2, a T2-type phage [16, 17, 36]. An advantage of this approach would be that it would allow specific mutagenesis of a single amino acid, which could modify host specificity of phage without reducing the binding ability.

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List of Publications

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Alteration of tail fiber protein gp38 enables T2 phage to infect *Escherichia coli* O157:H7.

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