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

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Abstract

Artificial control of phage specificity may contribute to practical applications such as the therapeutic use of phages and the detection of bacteria by their specific phages. To change the specificity of phage infection, gene products (gp) 37 and 38, expressed at the tip of the long tail fiber of T2 phage, were exchanged with those of PP01 phage, an *E. coli* O157:H7 specific phage. Homologous recombination between the T2 phage genome and a plasmid encoding the region around *gene 37-38* of PP01 occurred in transformant *E. coli* K12 cells. The recombinant T2 phage, named T2ppD1, carried PP01 gp37 and 38 and infected the heterogeneous host cell *E. coli* O157:H7 and related species. On the other hand, T2ppD1 could not infect *E. coli* K12, the original host of T2, or its derivatives. The host range of T2ppD1 was the same as that of PP01. Infection of T2ppD1 produced turbid plaques on a lawn of *E. coli* O157:H7 cells. The binding affinity of T2ppD1 to *E. coli* O157:H7 was weaker than that of PP01. The adsorption rate constant (k_a) of T2ppD1 (0.17×10^{-9} (ml/CFU min) was almost 1/6 that of PP01 (1.10×10^{-9} (ml/CFU min)). In addition to the tip of the long tail fiber, exchange of gene products expressed in the short tail fiber may be necessary for tight binding of recombinant phage.

Keywords: bacteriophage, *Escherichia coli* O157:H7, receptor, LPS, tail fiber

Introduction

Bacteriophages are viruses that only infect bacteria. Their high level of specificity makes them attractive for practical applications such as phage therapy and bacterial identification (Amy *et al*., 2004, Yoichi *et al*., 2004, Oda *et al*., 2004). Lytic spectra are used to discriminate between different isolates of bacterial species or genera, according to their ability to infect an isolate and form plaques. Differences in infectivity reflect differences in cell surface receptors. Phage attachment involves two separate stages and two different receptors. T-even phages, such as T2 and T4, must bind at least three of their six long tail fibers to primary receptor molecules, and then bind irreversibly to a second receptor (Tétart *et al*., 1998). T-even phages use the heptose residue of the LPS inner core as a secondary receptor. In phages of the T2 family, the *gene 38* product (gp38), which is present at the tip of the long tail fiber, is the determinant of host range (Riede *et al*., 1987). The two conserved regions encompass about 120 amino terminal and 25 carboxyl terminal residues, respectively. The area between these residues is variable and is interrupted by conserved glycine-rich regions (Tétart *et al*., 1998; Morona *et al*., 1984). Alterations causing receptor switches have mostly occurred in the variable regions, which are proposed to be the receptor-recognition domains of gp38 (Riede *et al*., 1987; Montag *et al*., 1987).

A virulent phage PP01, previously isolated from swine stool samples, was found to infect *E. coli* O157:H7 strains with high specificity. PP01 uses the outer membrane protein

OmpC as a receptor (Morita *et al*., 2002). The specific recognition of the *E. coli* O157:H7 OmpC protein by gp38 determines PP01's host range. Enterohemorrhagic *Escherichia coli* serogroup O157:H7 causes bloody diarrhea and hemolytic uremic syndrome in humans. It has been suggested that most *E. coli* O157:H7 infections in humans are foodborne illnesses and that domestic animals are reservoirs of *E. coli* O157:H7 (Hancock *et al*., 1998; Vuddhakul *et al*., 2000). Specificity of infection and virulence of phage are independent characteristics. Artificial introduction of specificity of infection to the virulent phage may facilitate molecular breeding of phage for the therapeutic use.

In this study, introduction of gp37 and 38 into T2 conferred infectivity of the heterogeneous host cell *E. coli* O157:H7.

Materials and methods

2.1. Bacterial strains and bacteriophages

E. coli K12 W3110 was used for propagation and homologous recombination of T2 phage. *E. coli* O157:H7 (ATCC43888) was used for propagation of PP01 phage, isolated from swine stool and infectious towards *E. coli* O157:H7 strains with high specificity and lytic activity (Morita *et al*., 2002). This strain does not produce either Stx 1 or 2 because it lacks the genes encoding 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 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]) was used. Phosphate-buffered saline (PBS) was used for the phage binding assay.

2.2 Construction of the plasmid for alteration of phage tail fiber genes 37 and 38

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.

2.3 Homologous recombination of T2 phage tail fiber genes

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 (Morita *et al*., 2002), 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.

2.4 Sequencing of phage DNA

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).

2.5 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.

2.6 Phage adsorption 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%.

3. Results

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

Figure 1A 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.

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. 1B). 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. 1B). 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). Two restriction fragments encoding the junctions of *g36/37* and *g38/gt*, respectively, were used for sequencing (Fig. 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.*

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 (Morita *et al*., 2002). The host range of three phages, T2ppD1, PP01, and T2, was examined using eleven *E. coli* strains (Table 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. 3), the efficiency of infection was thought to be low. Figure 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. 5). 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}).

4. Discussion

Several practical applications of phage function have been proposed based on its unique characteristics. The idea of phage therapy is to control pathogens such as *Salmonella* and *E. coli* O157:H7 by using virulent phages. Detection of bacteria by green fluorescent labeled phages has also been proposed (Oda *et al*., 2004). Both ideas are based on the specificity of phage infection. The addition of virulent phage to microbial consortia would not disturb bacteria, except for the phage's host cell. Detection of bacteria by phage could be achieved by screening with phage specifically infectious toward target bacteria. Specificity of phage infection relies on an interaction between phage and its host cell. For instance, T even phages use long and short tail fibers for adsorption to the host cell surface. Artificial alteration of phage tail fibers may ease the molecular control of phage specificity.

T2 and PP01 phage adsorption to the host cell surface initiate a specific but reversible interaction between gp38 and phage receptor expressed on the host cell surface, followed by an irreversible interaction between gp12 and lipopolysaccharide (LPS), a component of the host cell outer membrane. 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 (Heinrichs *et al*., 1998). The core OS of *E. coli* K12 is K12-type, while that of *E. coli* O157:H7 is R3-type (Amor *et al*., 2000). 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 (Riede 1987). 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 T even phage. These heptose residues are well conserved among T even phages. Compatibility of gp 12 and LPS is required for the irreversible binding of phage to the host cell.

The O antigen consists of an oligosaccharide repeat unit of about two to six sugars, polymerized to produce the O antigen. Since *E. coli* K12 is missing O antigen, the core OS is located at the tip of LPS. On the other hand, *E. coli* O157:H7 possesses a relatively long O antigen (Morita *et al*., 2002). The LPS O antigen chain length for a strain is usually determined by western blotting of LPS with an O antigen specific antibody, revealing a ladder of bands, each of which represents LPS with a specific number of O units. The adsorption rate constant (ARC) of the Felix O-1 (FO) phage to sensitive *Salmonella* strains was used to investigate the effect of variations in surface antigens (Lindberg *et al*., 1969). The ARC was high when the O antigen contained only one repeating unit, and very low for strains having O antigens with an average of 11 repeating units. This observation indicated that long O antigens

disturb the adsorption of FO phage to its host cell. Low adsorption of T2ppD1 to *E. coli* O157:H7 (Fig. 5) may be due to the disturbance mediated by long O antigens on phage interactions with their 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 to LPS. To examine this possibility, simultaneous alteration of gp12 of T2ppD1 with PP01 gp12 is necessary.

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Figure Legends

Fig. 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 *Eco*RV.

Fig. 2 The position of recombination between PP01 and T2 phage on the distal tail fiber locus. Diagrams 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 *gene37*. Two restriction sites, K: *Kpn*I, S: *Sal*I, are indicated.

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

Fig. 4 *E. coli* O157:H7 ATCC43888 cell lysis by 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.

Fig. 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.

Table 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 possessed pOMPC1
E. coli RK4784 pOMPC1 $T2^r$	RK4784 pOMPC1 T2-resistant mutant
E. coli XL-1 blue	
E. coli O157:H7 (CR-3)	for test of phage-host range
E. coli O157:H19 (A2)	for test of phage-host range
E. coli O157:H37 (CE273)	for test of phage-host range
E. coli K12 (Hfr H)	for test of phage-host range
E. coli B^E	for test of phage-host range
E. coli C600	for test of phage-host range
Bacteriophages	
T2	wild-type
PP01	O157:H7 bacteriophage, (isolated from pig feces in our laboratory)
T2ppD1	T2 phage recombinant with PP01 Distal tail fiber locus

Table 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