Amino acid alterations in Gp38 of host range mutants of PP01 and evidence for their infection of an *ompC* null mutant of *Escherichia coli* O157:H7

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Abstract

The previously isolated T-even type coliphage PP01, specifically infective to *Escherichia coli* O157:H7, uses the outer membrane protein OmpC as a receptor. The characterization of a spontaneous PP01-resistant strain indicated that it had lost *ompC* due to the deletion of a 14-kbp region upstream of and partially including *ompC*. Two host range mutants, able to infect an *ompC* null mutant, were isolated. Sequencing of gene 38, which codes for the receptor recognition protein Gp38, indicated three mutations in one mutant and two in the other. Both mutant proteins had a Gly208Arg, a Gly161Arg or Gly101His replacement, respectively, and the one mutant phage in addition a Trp189Arg replacement. These alterations suggest that the host range was mediated by a more positively charged Gp38.

Keywords: *Escherichia coli* O157:H7; Virulent bacteriophage; Resistant cell; Host range mutant; Receptor

1. Introduction

Enterohemorrhagic *Escherichia coli* serogroup O157:H7 is known to cause 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 [1,2]. Phages specific for *E. coli* O157:H7 have been detected in feces from animals at relatively abundant levels [3-5]. In general, phages can be categorized into two groups: temperate and virulent phages, based on their life cycle. Temperate phages can mediate horizontal transfer of genes such as *stx* [6]. On the other hand, virulent phages may play a role in controlling the host cell population in gastrointestinal or other environments. Phage-mediated control of pathogenic organisms may potentially be exploited therapeutically. Several investigations have had success in animal models [7-11].

A virulent phage, previously isolated from swine stool samples, was found to infect *E. coli* O157:H7 strains with high specificity. Phage infection is initiated by the specific adsorption on the bacterial cell surface. The distal long tail fibers of T-even phages are responsible for recognizing specific cell surface receptors. In phages of the T2 family, the gene 38 product (Gp38), which is present at the tip of long tail fiber, is the determinant of the host range [12]. Analysis of deduced amino acid alignments of the tail fiber proteins revealed the PP01 was related to T2. Moreover, the specific recognition of the *E. coli* O157:H7 OmpC protein by Gp38 determines its host range [13]. However, after long co-incubation of cell and phage, PP01-resistant cells appeared spontaneously. Analysis revealed that an *ompC* null mutation endowed the *E. coli* O157:H7 cells with resistance against PP01 infection. On the other hand, host range mutants of PP01, which were infective to the *ompC* null mutant, were also isolated. According to analyses of host range mutants in the T2 family, mutations in gene 38 are usually either insertions caused by small duplications or amino acid alterations caused by DNA base-pair alterations. These mutations alter the receptor for the phage [14-18].
Understanding the mechanisms of mutations controlling the infectivity of phages may facilitate the design of successful phage-based therapies. In this study, we elucidated the cause of the loss of ompC expression in mutant E. coli O157:H7 cells and found that mutational alterations on Gp38 endow the PP01 phage mutants with a broad host range.

2. Materials and methods

2.1. Bacterial strains and bacteriophages

E. coli O157:H7 ATCC43888, which does not produce either Stx1 or Stx2 because of a lack of the genes for these toxins, was used for the propagation of phages and the estimation of phage titers. The PP01-resistant strain, R01s, was obtained from ATCC43888, as described previously [13]. This strain has lost the production of outer membrane protein, OmpC. In addition, E. coli K-12 W3110 [19] was used for the determination of phage susceptibility. All strains were grown in Luria–Bertani (LB) broth at 37°C.

The bacteriophage PP01 and its host range mutants, M01f and M01g, were obtained independently by plating PP01 onto R01s. The host range mutants, M01f and M01g, were from the laboratory stock. The PP01-resistant strain, O157:H7 ATCC43888, was generated with primers (5'-CCCACTGGCCGTAACCGGTCAGCTGGTC-3' and 5'-CCCAAGCTTTGATTATCCTCATGCGA-3') for this reaction were 5'-CGGGATCCCCTGGCCCCTTGGAG-3' and 5'-CCCAGGATCCGCCCCCACAATGTGGTC-3' and 5'-CCAAAGCTTGGGACAGTACTTTAA-3', respectively, except for the primers, probe DIG-up, specific for the 1-kbp region upstream of ompC, was prepared. The primers for this reaction were 5'-CCCAACGTTGGGACAGTACTTTAA-3' and 5'-CCAAAGCTTGGGACAGTACTTTAATTCATC-3'.

Genomic DNA was digested with restriction enzymes and analyzed by electrophoresis on a 1.5% agarose gel. The agarose gel containing DNA was soaked in the denaturation solution (0.5 M NaOH; 1.5 M NaCl) for 15 min, followed by washing in the neutralization solution (1.0 M Tris; 1.5 M NaCl, pH 8.0) for 15 min. DNA was then transferred to a nylon membrane (Millipore) by capillary action with 20×SSC (3 M NaCl; 0.3 M sodium citrate, pH 7.0) and was fixed to the membrane by UV cross-linking at 1200 J cm⁻². For prehybridization, membranes were treated with DIG-Easy-Hyb (Roche Diagnostic) and incubated at 55°C for 30 min, followed by hybridization at 68°C overnight with DIG-labeled probe DNA. Membranes were washed in 2×SSC with 0.1% (w/v) sodium dodecyl sulfate at room temperature (two times, 5 min), followed by two washes for 15 min each at 68°C with 0.1×SSC with 0.1% (w/v) sodium dodecyl sulfate. Then, after, detection of the hybridized probes was conducted with the DIG-Luminescent-Detection-Kit (Roche Diagnostic) according to the manufacturer’s instructions.

2.3. Cloning and sequencing

The KpnI/PstI fragment of the R01s genome, hybridized with DIG-ompC, was extracted from agarose gel by GenElute-Agarose-Spin-Columns (Sigma). This fragment was then ligated into pUC118 treated with KpnI and PstI.

PCR was used to clone the tail fiber gene 38 of host range mutants. DNA was extracted from bacteriophages with phenol-chloroform-isooamyl alcohol (25:24:1), and the DNA was then recovered by precipitation with ethanol. The precipitated DNA was dried and redissolved in distilled water; the DNA solution was then used for the PCR amplification. The upper primer was 5'-CCGGATCCGCGCCCCACAATGTGAGCTCAGGCACC-3', and the lower primer 5'-CCCAAGCTTGGCATAACCTTGACCTCAGTCTGC-3'. Underlined nucleotides indicate sequences of BamHI and HindIII, respectively. PCR fragments were cloned into the BamHI/HindIII site of pUC118. The resultant plasmids were designated as pMF38 and pMG38, each harboring the gene 38 of M01f or M01g, respectively.

Nucleotide sequences of the cloned fragments were determined using fluorescein isothiocyanate-labeled universal forward (5'-GACGAGCTTGGGACAGTACTTTAA-3') and reverse (5'-GACGAGCTTGGGACAGTACTTTAA-3') primers (Shimadzu). Automated cycle sequencing was performed on a DSQ-2000L sequencer (Shimadzu) with a pretreated sample using a Thermo Sequenase Fluorescent-Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech).

2.4. Identification of the gene deletion

According to the sequence of the KpnI/PstI fragment of the R01s genome that hybridized with DIG-ompC, the upper primer (5'-CCGGATCCGCGCCCCACAATGTGAGCTCAGGCACC-3') and the lower primer (5'-ATATTCCGACTTGCCGTAACCTTGACTTTACG-3') were constructed for the amplification of a DNA fragment including the 14-kbp deletion region by PCR using TaKaRa LA Taq® (Takara Bio) with the genomic DNA of E. coli O157:H7 ATCC43888 as a template.

In addition to the PCR amplification, DNA hybridization was carried out. The deletion region was divided into three parts – the upper (del1, ca. 3300 bp), middle (del2, ca. 4100 bp) and lower part (del3, 5000 bp) – to generate the DIG-labeled probe using the PCR DIG-Probe-Synthesis-Kit (Roche Diagnostic) and the genomic DNA of
E. coli O157:H7 ATCC43888 as a template. The primers for the probes were as follows: 5'-GGGCCCGGGCGAAGCCTTCTGGTGCAGGCGATA-3' and 5'-CCCAAGCTTGGCACCATCAGCGGTACGC-3' were for DIG-del1; 5'-GGGGATCCAGGCGTAACTGACGACAA-3' and 5'-CCCAAGCTTGCCCATACGCGTTAGC-3' were for DIG-del2; 5'-GGGGATCCACGTATTTTCATTCCGCGG-3' and 5'-CCCAAGCTTACAACTCAACGCT-3' were for DIG-del3. The experimental procedures for hybridization and detection were described above.

2.5. Construction of chimeric phages

E. coli O157:H7 ATCC43888 strains carrying either pMF38 or pMG38 were infected with PP01 and incubated overnight. The overnight culture was centrifuged (12,000×g for 5 min at 4°C) and phages infective to the R01s strain were isolated by the plaque assay. A single plaque was isolated, and the phage was resuspended in LB broth. The resuspension was centrifuged, filtered, and subjected again to single plaque isolation on the LB broth. The resuspension was centrifuged, filtered, and subjected again to single plaque isolation on the R01s strain. Phages that formed plaques on R01s seemingly acquired the gene 38 of the host range mutant by homologous recombination. The gene 38 of the chimeric phage was confirmed to be identical to that of host range mutant. Chimeric phages were designated as PP01mf38 and PP01mg38, each harboring gene 38 of M01f or M01g, respectively.

2.6. Bacteriophage adsorption assay

R01s cells were grown in LB broth overnight. The cells were diluted to 1×10^8 colony-forming units ml^-1 with fresh LB broth at 37°C with shaking (120 rpm). Phage infection with the final concentration of 1×10^9 plaque-forming units ml^-1 was performed after 60 min of incubation. After the infection, samples of 1 ml were removed periodically and centrifuged (15,000×g for 1 min at 4°C). The phage titer of the supernatant was determined by the plaque assay with E. coli O157:H7 ATCC43888. The phage titer at time 0 was defined as 100%.

3. Results

3.1. Evidence for the loss of OmpC production in R01s strain

In our previous work, analysis of the outer membrane proteins revealed that R01s had lost the outer membrane protein OmpC. In addition, PCR amplification of ompC using R01s genomic DNA failed. Thus we hypothesized that in R01s, a region partially or completely containing ompC is deleted.

Initially, deletion of genes upstream of ompC was elucidated using the probe DIG-up corresponding to the 1-kbp region upstream of ompC. Chromosomal DNA from E. coli O157:H7 ATCC43888 gave strong hybridization signals in a Southern blot; however, no hybridization signal was obtained for R01s. On the other hand, probe DIG-ompC, specific for the ompC of E. coli O157:H7, hybridized with chromosomal DNA of both strains (data not shown). Additionally, the KpnI/PstI digest of the R01s genome generated a ca. 700-bp fragment hybridizing with DIG-ompC, which was not observed in the ATCC43888 genome digested with the same restriction enzymes. This 700-bp fragment was cloned, and its DNA sequence was determined. This revealed that the fragment, except for the first 261 bp, was identical to an internal region of ompC (204–641 bp of the 1101-bp ompC); however, the first 261 bp had no relation with ompC. According to a FASTA search, this sequence showed 100% identity over 260 bp to a region (913–1172 bp out of 4605 total bp) of a putative membrane protein gene found at ca. 14 kbp upstream of ompC in E. coli O157:H7 strains (Fig. 1). These results imply a 14-kbp deletion, including the first 203 bp of ompC.

To identify the deletion, an upper primer complementarity to the putative membrane protein gene and a lower primer complementary to the internal region of ompC were constructed. Then, the ca. 14-kbp from the E. coli O157:H7 ATCC43888 genome was amplified by PCR and detected by gel electrophoresis (data not shown). Moreover, the three probes specific for three different regions of the 14-kbp deletion region, hybridized successfully with the genomic DNA of E. coli O157:H7 ATCC43888. In the case of R01s, however, only faint signals derived from non-specific hybridization were observed (data not shown). Therefore, the 14-kbp deletion present in the R01s strain was verified and did not translocate to another chromosomal region. It is known that micF, which codes for an inhibitor of ompF expression, is located upstream of ompC in E. coli strains. The 93-nucleotide micF-RNA represses OmpF synthesis by decreasing the level of ompF mRNA [20]. From the results mentioned above, the 14-kbp deletion, including the first 203 bp of ompC, caused the loss of the OmpC production and increase of OmpF production in the R01s strain.
Table 1
Mutations on gene 38 of the host range mutants

<table>
<thead>
<tr>
<th>Phage</th>
<th>Codon at 161 aa (aa alteration)</th>
<th>Codon at 189 aa (aa alteration)</th>
<th>Codon at 208 aa (aa alteration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP01</td>
<td>CAA</td>
<td>TGG</td>
<td>GGG</td>
</tr>
<tr>
<td>M01f</td>
<td>CGA (Gln161Arg)</td>
<td>CGG (Trp189Arg)</td>
<td>AGG (Gly208Arg)</td>
</tr>
<tr>
<td>M01g</td>
<td>CAC (Gln161His)</td>
<td>TGG</td>
<td>CGG (Gly208Arg)</td>
</tr>
</tbody>
</table>

3.2. Alteration of receptor recognition by amino acid alterations on Gp38

The host range of a phage relies on the ability of the phage to recognize different receptors. In PP01, Gp38 bound to the tip of the distal tail fibers may act in recognition of the cellular receptor OmpC [13]. Thus, the host range mutants of PP01 which could infect not only ATCC43888 but also the R01s strain (defective in OmpC production), may have mutations in Gp38. Two host range mutants, M01f and M01g, were isolated from independent cultures and the sequence of their gene 38 was determined (Table 1). The M01f mutant had three mutations in gene 38 resulting in the three amino acid replacements Gln161Arg, Trp189Arg and Gly208Arg. Interestingly, all three amino acids were changed to arginine, which is a basic amino acid and is positively charged at neutral pH. The two mutations on M01g were identified as Gln161His and Gly208Arg. Note that histidine is also a basic amino acid. The two amino acid mutations in M01g-Gp38 occurred at the same positions as two of three mutations in M01f-Gp38; however, the base-pair alterations in their DNA were different. These findings imply that switching amino acid residues Gln161 and/or Gly208 into basic amino acids might play an important role in host range extension.

To confirm that only the mutations in gene 38 extended the host range, a fragment of gene 38 from each mutant was cloned into a plasmid. Then, E. coli ATCC43888 cells carrying these plasmids were infected with PP01. Chimeric phages were found at frequencies typical of homologous recombination, which is well above those of spontaneous mutations [22,23]. Therefore, the mutations in gene 38 seemed to be the determinant for the broad host range. Instead of M01f and M01g, the chimeric phages, PP01m38 and PP01mg38, were further characterized to clarify the effect of mutations in gene 38.

The host ranges of PP01m38 and PP01mg38 were examined by the spot test assay demonstrating that they were able to infect R01s. Both of them were also infectious to E. coli O157:H7 ATCC43888; however, like wild-type PP01, they did not form plaques on E. coli K-12 W3110 strain (Table 2). The receptor for wild-type PP01 was OmpC derived from O157:H7. However, chimeric phages gained the ability to use other unidentified cellular receptor(s), but did not lose the specificity to E. coli O157:H7.

To examine the affinity of each phage for the R01s strain, the phage adsorption assay was conducted (Fig. 2). While PP01 did not bind to R01s cells, adsorption of chimeric phages was evident from the decrease of phage titer. Comparing their affinity, adsorption of PP01mg38 was lower than that of PP01m38. Gp38 of PP01m38 was more positively charged, than that of PP01mg38 due to the three amino acid alterations into arginine. Generally, the cell surface of Gram-negative bacteria is covered with negatively charged outer membrane elements, e.g. lipopolysaccharide [24]. Therefore, the charge-dependent interaction between Gp38 and the bacterial cell surface might enhance phage access to the bacteria.

4. Discussion

In the T2 family, the general architecture of Gp38 of the T2 family is the same. The two conserved regions encompass about 120 amino-terminal and 25 carboxyl-terminal residues, respectively. The area between these is variable and is interrupted by conserved glycine-rich regions [23,25]. Amino acid insertions by small duplications or amino acid alterations by DNA base-pair alterations causing receptor switches have mostly occurred in the variable regions, which are proposed to be the receptor-recognizing domains of Gp38 [14–18]. The Gp38 of the PP01 host range mutant also has amino acids alterations within the variable regions. This result supports that these regions are responsible for specific attachment with the E. coli O157:H7 OmpC protein. It is unclear what the host range mutants of PP01 recognize as a receptor. Host range mutants of the T-even type coliphage Ox2 had switched receptors in a sequential manner. Ox2 uses OmpA as a receptor, and subsequent switches were from OmpA to OmpC, from OmpC to OmpP and from OmpP to lipopolysaccharide of the E. coli K-12 [14–16]. Similarly, the host range mutants of PP01 may recognize other elements, such as outer membrane proteins or lipopolysaccharides peculiar to the E. coli O157:H7 cell surface instead of OmpC.

Table 2
Infectivity of PP01 and chimeric phages with mutant tail fiber gene 38

<table>
<thead>
<tr>
<th></th>
<th>PP01</th>
<th>PP01m38</th>
<th>PP01mg38</th>
</tr>
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<tbody>
<tr>
<td>ATCC43888</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R01s</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W3110</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Plaque formation was confirmed by spot test assay. +, –: presence or absence of plaque formation.
seemed to be continuously evolving in a mutual, ever-escalating ‘arms race’.

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References


