### Article / Book Information

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Title: Characterization of a virulent bacteriophage specific for *Escherichia coli* O157:H7 and analysis of its cellular receptor and two tail fiber genes

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Abstract

A virulent phage, named PP01, specific for *Escherichia coli* O157:H7 was isolated from swine stool sample. The phage concentration in a swine stool, estimated by plaque assay on *E. coli* O157:H7 EDL933, was $4.2 \times 10^7$ plaque-forming units g-sample$^{-1}$. PP01 infects strains of *E. coli* O157:H7 but does not infect *E. coli* strains of other O-serogroups and K-12 strains. Infection of an *E. coli* O157:H7 culture with PP01 at a multiplicity of infection of 2 produced a drastic decrease of the optical density at 600 nm due to cell lysis. The further incubation of the culture for 7 hours produced phage resistant *E. coli* O157:H7 mutant. One PP01-resistant *E. coli* O157:H7 mutant had lost the major outer membrane protein OmpC. Complementation by *ompC* from a O157:H7 strain but not from a K-12 strain resulted in the restoration of PP01 susceptibility suggesting that the OmpC protein serves as the PP01 receptor. DNA sequences and homology analysis of two tail fiber genes, 37 and 38, responsible for the host cell recognition revealed that PP01 is a member of the T-even bacteriophages, especially the T2 family.
1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) serogroup O157:H7 has been found to cause bloody diarrhea and hemolytic uremic syndrome in human. EHEC produces two toxins known as Shiga toxin 1 and 2 (Stx1 and Stx2). Production of Stx1 and Stx2 in *E. coli* is conferred by toxin-converting lysogenic bacteriophages [1-4]. The presence of phages carrying the Stx2 gene is also common in sewage from developed countries [5, 6]. The high levels of Stx2 gene in sewage indicate an abundance of such phages circulating among the human population. Natural reservoirs of EHEC are cattle and other domestic animals [7].

In addition to serving as a mediator of horizontal gene-transfer, bacteriophages work as a pressure for controlling bacterial populations by infecting and killing their specific host cell. Phage specificity of infection is determined by interaction between receptors present on the host cell outer membrane and phage host recognition protein [8, 9]. The host range of a phage relies on the ability of the phage to recognize different receptors. T-even coliphages recognize their cellular receptor such as lipopolysaccharide (LPS) and outer membrane proteins with the free ends of the six long tail fibers [10-12]. In T4, the distal part of these fibers consists of a trimer of protein 37 (gene 37 product, Gp37) with the polypeptides arranged in parallel and their C-termini located at the tip of the fiber [13-15]. The situation is different for the phage T2. At the free end of the trimer of Gp37, one copy of protein 38 (gene 38 product, Gp38) is present and serves in receptor recognition [16].

The virulent phage, named PP01, was isolated from swine stool sample using *E.*
coli O157:H7 EDL933 as a host. In this study, we analyzed the host range of PP01 and its characteristics and found that it infects *E. coli* O157:H7 strains but does not infect other serotypic strains, including *E. coli* K-12. Analysis of deduced aa alignment of the tail fiber proteins, Gp37 and Gp38, revealed that the PP01 is related to T2. T2 infects K-12 strains of *E. coli*, but PP01 does not infect K-12. Such kind of particular host range of PP01 is due to the specialized Gp38. Furthermore, PP01-resistant *E. coli* O157:H7 was isolated from the liquid culture of the susceptible strain and was found to have lost the major outer membrane protein OmpC. Complementation of OmpC by the plasmid made it restore the susceptibility. It is suggested that OmpC protein serves as a receptor of PP01 phage.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used for the determination of phage susceptibility were listed in Table 1. EHEC EDL933 was used for the screening of phages infective to O157:H7. *E. coli* O157:H7 ATCC43888, which does not produce either Stx1 or Stx2 because of lack of the genes for these toxins, was used for the propagation of isolated phages and the estimations of phage titer.

2.2. Isolation and purification of bacteriophage from swine stool
To isolate *E. coli* O157:H7-specific phages, 60-bovine, 52-swine, and 5-chicken fecal samples were collected from five separate animal facilities in Japan in 2000. Sludge from Johkasou (compact wastewater treatment facility mainly for domestic or community uses), human stool sample from a public restroom, and activated sludge of a wastewater treatment plant in Japan were also collected every month from January 2000 to January 2001. Samples were pooled in sterile 15 ml tubes at 4 °C and transported to the laboratory within 24 hours. One gram of each sample was mixed with 4 ml of buffer, 1 % NaCl and 0.1 % boric acid pH=10, for detaching phage from the solid substrate. Then the sample was mashed with a sterilized plastic bar, centrifuged, and filtrated through a 0.45-µm filter. The filtrate was subjected to plaque assay on EHEC EDL933 strain. A single plaque was isolated and the phage resuspended in LB broth. The filtrate of centrifuged LB medium was subjected again to single plaque isolation on *E. coli* O157:H7 ATCC43888. Purification of the plaque was repeated one more time. Filtered stocks of coliphages were kept refrigerated.

2.3. Bacteriolysis and isolation of PP01-resistant *E. coli* O157:H7

The *E. coli* O157:H7 ATCC43888 was pre-cultured in LB broth of 2 ml at 37 °C with shaking (120 rpm) overnight. Of this culture 300 µl was inoculated into fresh 30 ml-LB broth. The optical density at 600 nm (OD$_{600}$) was measured to estimate the cell lysis. Bacteriophage PP01 infection with a multiplicity of infection of 2 was performed at an OD$_{600}$ of 0.1. From the culture after overnight incubation, one *E. coli* O157:H7 strain resistant to PP01 named R01s was isolated by the serial dilution.
2.4. Outer membrane proteins and LPS analysis

Bacterial cells grown in 10 ml of LB broth (OD$_{600}$ = 1.0) were collected by centrifugation and suspended in 0.2 ml of 50 mM Tris-HCl (pH = 8.8) containing 20 % sucrose. A 0.02 ml portion of lysozyme (10 mg ml$^{-1}$ in 0.1 M ethylenediaminetetraacetic acid, pH = 8.0) was added, and after incubation for 30 min on ice, 3 ml of 3 mM ethylenediaminetetraacetic acid (pH = 8.0) was added. The mixture was sonicated and centrifuged at 6500 × g for 5 min. The supernatant was treated with sodium sarcosinate (2 % in final concentration) to solubilize the cytoplasmic membranes. The outer membranes were precipitated by the centrifugation (45000 × g, 30 min) and suspended in 0.1 ml of 50 mM Tris-HCl (pH = 8.8) containing 2 mM ethylenediaminetetraacetic acid [20, 21]. The outer membrane fraction was separated on sodium dodecyl sulfate-polyacrylamide (12 %) gel containing 4 M urea and stained with Coomassie brilliant blue R-250.

The LPS was prepared from whole cells by proteinase K digestion at 60 °C for 1 h [20]. The resultant sample was run on sodium dodecyl sulfate-polyacrylamide (12 %) gel and electroblotted onto an Immobilon-P Transfer Membrane (Millipore). Peroxidase-labeled affinity purified antibody to *E. coli* O157:H7 (Kirkegaard & Perry laboratory Inc.) and ELC Western blotting detection reagent (Amersham Pharmacia Biotech) were used for light emitting detection.

2.5. Construction of OmpC expression plasmid

The *ompC* was amplified by polymerase chain reaction (PCR) using KOD
DNA polymerase (Toyobo, Osaka). The chromosomal DNA of *E. coli* O157:H7 ATCC43888 was used as template with primers 5'-CATGCCATGGGCATGAAAGTTAAAGTACTGTCCC-3' and 5'-CCGGTCGACTGATTATCCTCATGCGAAC-3'. Underlined nucleotides indicate sequences of *Nco*I and *Sal*I, respectively. The PCR products were digested with *Nco*I and *Sal*I and inserted into the *Nco*I and *Sal*I-digested pTV118N (Takara, Kyoto). The resultant plasmid, pOMPC1 was introduced into *E. coli* R01s and RK4784 (*ompC* defective K-12). Expression of the *ompC* is under the control of *lac* promoter, which was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) in the final concentration of 1mM. In common with *E. coli* O157:H7 ATCC43888, the *ompC* was also amplified using genomic DNA of *E. coli* K-12 W3110 as template. R01s and RK4784 was transformed by the resultant plasmid, pOMPC2, that encoded the *ompC* derived from *E. coli* K-12.

2.6. Bacteriophage adsorption assay

*E. coli* cells were grown in LB broth overnight. The cells were diluted to $1 \times 10^8$ colony-forming units ml$^{-1}$ with LB broth and inoculated in 10 ml at 37 °C with shaking (120 rpm). Phage infection with the final concentration of $1 \times 10^7$ plaque-forming units (PFU) ml$^{-1}$ was performed after 60 min of incubation. After the infection, samples of 1 ml were removed periodically and centrifuged (15000 × 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 %.
2.7. Cloning and sequencing of tail fiber genes

To clone the phage tail fiber genes, 37 and 38, PCR was conducted. DNA was extracted from bacteriophages with phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was then recovered by precipitation with ethanol. The precipitated DNA was dried and redissolved in distilled water; 1 µl of the DNA solution was then used for the PCR amplification. The primers were as follows: upper primer, 5’-GGGGTACCAAACTTTTTCTCGCAGAATCCT-3’; and lower primer, 5’-GCTCTAGAACACAAATAGAATATC-3’. Underlined nucleotides indicate sequences of KpnI and XbaI, respectively. Since template DNA had KpnI, EcoRI, EcoRI and XbaI sites in this order, five independent DNA fragments (KpnI/KpnI, KpnI/EcoRI, EcoRI/EcoRI, EcoRI/XbaI and XbaI/XbaI) were replaced with the corresponding fragments of the multiple cloning site of pUC118 [22]. Then nucleotide sequence of the resulting fragment was determined using fluorescein isothiocyanate-labeled universal forward (5’-CGCCAGGGTTTTCCCATCGAC-3’) and reverse (5’-GAGCGGATAACAATTTCCACACAGG-3’) primers (Shimadzu, Kyoto). Automated cycle sequencing was performed on a DSQ-2000L sequencer (Shimadzu, Kyoto) with a pretreated sample using a Thermo Sequenase Fluorescent-Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, UK). The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AF349974 for 37 and AF349975 for 38.
3. Results

3.1. Isolation of phage specific for E. coli O157:H7 strains and its characterization

Frequencies of plaque forming samples (positive samples/total samples) on the EHEC EDL933 strain were 41/60:bovine, 26/52:swine, 0/5:chiken, 5/10:Johkasou sludge, 4/10:mixed human stool sample, and 5/19:activated sludge. The titer of the phage ranged from $10^0$-$10^3$ PFU g-sample$^{-1}$. As an exceptional case, one swine sample (named Sample-1) produced $4.2 \times 10^7$ PFU g-sample$^{-1}$. No incidence of enterocolitis was reported during the study period around the area of the animal facilities and wastewater treatment facilities. Dozens of plaques obtained from the various samples were purified on *E. coli* O157:H7 ATCC43888. Of the phages the DNA digestion pattern by restriction enzymes and the lytic action on *E. coli* O157:H7 ATCC43888 were determined. Most of the phages did not produce visible cell lysis on *E. coli* O157:H7 ATCC43888 in LB liquid medium. However, one phage, named “PP01” screened from Sample-1, showed a strong decrease of the culture turbidity (Fig. 1). This coliphage formed relatively large (0.5-1.0 mm) and clear plaques on a cell lawn of *E. coli* O157:H7 strains but did not form a plaque on the lawn of *E. coli* K-12 strains and other related bacteria (Table 1). PP01 is the first isolate of the phage to produce OD$_{600}$ decrease of the *E. coli* O157:H7 culture. After 7 hours incubation of *E. coli* O157:H7 ATCC43888 with PP01 phage, a reascent of the OD$_{600}$ was observed. *E. coli* can develop resistance to the phage by mutation that alters cell surface receptors the phage bind to [10].

To analyze the PP01 receptors, strain R01s was isolated from the *E. coli*
O157:H7 cells resistant to PP01. A phage adsorption assay revealed that PP01 did not bind to R01s cells (Fig. 2). Thus, it seemed that the cell surface receptors were lacking in R01s. Comparing the cell envelopes of R01s and the susceptible strain E. coli O157:H7 ATCC43888, the LPS analysis using the antibody to E. coli O157:H7 that did not react with LPS of E. coli K-12 strain did not show a remarkable difference (Fig. 3-A). However, analysis of the outer membrane proteins revealed that R01s had lost the outer membrane protein OmpC but increased the production of OmpF (Fig. 3-B, lanes 1 and 2). Subsequently, to elucidate the cause of the loss of ompC expression in R01s, PCR amplification of ompC using its genomic DNA was performed. However, no fragment was detected by gel electrophoresis on 1.5 % agarose and stained with ethidium bromide (data not shown). And it is known that the micF, which is inhibitor of OmpF, is located upstream of ompC in E. coli strains. The increase of OmpF production may result from the deletion of micF. Thus we hypothesized that the mutant R01s partially or completely deletes ompC including its upstream region but the details about the mutation remained to be determined. According to the results mentioned above, OmpC protein is assumed to be the cell surface receptor of PP01 phage.

This hypothesis was verified in following way. The plasmid pOMPC1, which codes ompC of E. coli O157:H7 ATCC43888, was constructed and introduced into both R01s and RK4784 (ompC mutant of K-12). These transformants were grown in the presence of IPTG to induce the OmpC expression. According to the electrophoretic analysis, the distinct band corresponding to OmpC was observed in the R01s/pOMPC1 and RK4784/pOMPC1 (Fig. 3-B, lanes 3 and 6). The PP01 phage susceptibility of R01s was restored by the complementation of OmpC and PP01 was absorbed on R01s/pOMPC1 (Fig. 2). Furthermore, the efficiency of plating (e.o.p.), defined as a
ratio of PFU value of each strain to that of ATCC43888 of PP01, on R01s/pOMPC1 was about nine times higher than that on wild type strain ATCC43888 (Table 2). Apparently, the overproduction of plasmid-derived OmpC resulted in the increase of cell surface receptor, and thereby facilitated the PP01 infection of R01s/pOMPC1 compared to that of ATCC43888. *E. coli* K-12 RK4784, which does not produce the OmpC protein, was insensitive to PP01. However, RK4784 became susceptible to PP01 by the production of OmpC of O157:H7 ATCC43888 and the e.o.p. was almost the same as that with ATCC43888 (Table 2). In addition, PP01 adsorption to RK4784/pOMPC1 was observed, however the adsorption was lower than that of ATCC43888 (Fig. 2).

In the same manner as described above, OmpC of *E. coli* K-12 was produced in R01s and RK4784 by the plasmid, pOMPC2 (Fig. 3-B, lanes 4 and 7). In both, expression of *ompC* from *E. coli* K-12 did not produce susceptibility to PP01 (Table 2). The aa sequence of OmpC from different *E. coli* O157:H7 strains (DDBJ/EMBL/GenBank accession number AE005174 and NC_002695) shows no variation and the OmpC sequence of *E. coli* O157:H7 ATCC43888 is also identical. However, the OmpC alignment of *E. coli* O157:H7 strains differs from that of *E. coli* K-12 by substitutions at 13-aa and two gaps with one 4-aa deletion and one 4-aa insertion (Fig. 4). Thus, PP01 utilized only OmpC derived from O157:H7 and not that of K-12 as its receptor.

3.2. Cloning and sequencing of PP01 phage tail fiber genes

The final step of phage infection is host cell lysis with the release of newly produced phage particles. Lysis is critical for phage propagation, so that proteins
involved in this process seem to be conserved among related phages. Lysis by T4 phage requires the action of two gene products, gene e product and gene t product [23]. PCR amplification of gene e and gene t by using two primer sets based on the DNA sequence of gene e and gene t of T4 phage and PP01 DNA as a template, produced 500 bp and 650 bp DNA fragments, respectively (data not shown), suggesting that the PP01 phage carries a host cell lysis system similar to that of T4 phage. PP01 is possibly a member of the T-even phages. For T-even phages, the five contiguous genes 34, 35, 36, 37, and 38 and the unlinked gene 57 controls tail fiber structure and assembly [14]. The sequences of 37 and 38 vary and are responsible for host specificity among T-even phages, whereas those of 36 and t flanking this locus are quite conserved. Based on this information, a primer set that would enable us to amplify the DNA fragment covering the 3’ ends of 36, 37, 38, and the 5’ end of t, which encodes holin protein, was constructed. DNA fragment of 4.3 kb was amplified and detected by gel electrophoresis on 0.7 % agarose and stained with ethidium bromide (data not shown). Cloning of the fragment into the pUC118 multiple cloning site was conducted after digestion by restriction endonucleases KpnI and XbaI, which produced 3 fragments with nucleotide sizes of 1370 bp, 2340 bp, and 590 bp. The 2340 bp fragment was truncated further by EcoRI digestion, and the products were recloned into pUC118. Cloned DNA fragments into pUC118 were subjected to DNA sequencing. Nucleotide sequencing of the phage tail fiber locus of 4.3 kb and subsequent computer analysis allowed the identification of two adjacent open reading frames, one (putative 37) of 3327 bp and the other (putative 38) of 777 bp with a 33-nucleotide interval. The putative 37 gene of PP01 encoded 1109 aa. The size of the Gp37 was between that of T4 (1026 aa) and T2 (1341 aa) and was almost the same as that of Ac3 (1103 aa) and AR1 (1104 aa) [12, 24]. High identity of
aa sequences in the N-terminal first 50 aa of Gp37 and those of the 5 phages was observed. On the other hand, only a trace similarity was observed in the other regions between PP01 Gp37 and that of T2 (14.7 %) and that of T4 (15.2 %). Relatively high identity to PP01 Gp37 was observed with Ac3 Gp37 (81.2 %) and AR1 Gp37 (85.8%).

Gp 38 of phage T2, which uses the *E. coli* outer membrane protein OmpF as receptor, and also Gp38 of several T-even-type phages, which use the outer membrane protein OmpA as receptor, determine their host range [10]. To elucidate the host specificity of the PP01 phage, the aa sequence of Gp38 of PP01 was analyzed. It consists of 259 aa. This size is identical to that of the corresponding protein of phages Ac3 and AR1 and is close to that of T2 (262 aa) but is different to that of T4 (183 aa). The aa sequence identity between Gp38 of PP01 and that of other T-even phages is 94.2 % (AR1), 70.6 % (Ac3) and 48.5 % (T2). Similar as PP01, AR1 is also specific for *E. coli* O157:H7 and a member of the T2 family. In contrast, Ac3 is not specific for *E. coli* O157:H7. The N-terminal sequences of Gp38 of *E. coli* O157:H7 specific phages (PP01 and AR1) and Ac3 were relatively conserved. On the other hand, missing residues and alterations were observed in the middle of the polypeptides in the regions that are of a repetitive nature and contain several stretches rich in alanine, asparagine and in particular glycine [16].

4. Discussion

Relatively abundant phages specific for *E. coli* O157:H7 were detected in feces from animals or from humans in this study and other reports [25, 26]. In general phages
can be categorized into two groups, temperate phage and virulent phage, based on their life cycle. Temperate phage can mediate horizontal transfer of genes such as *stx*. On the other hand, virulent phages may play a role in controlling the host cell population in a limited environment, including gastrointestinal ecosystems. As an exceptional case, a swine stool sample used in this study contained $4.2 \times 10^7$ PFU g-sample$^{-1}$ of the virulent *E. coli* O157:H7 specific phage PP01, suggesting that PP01 may suppress its host *E. coli* O157:H7 in the swine gastrointestinal tract.

Phage infection is started by the adsorption on the bacterial cell surface and the host range is controlled by the specificity of the interaction of the phage and its receptor. Generally, phages use outer membrane proteins and/or LPS as their receptor. The PP01 resistant strain derived from the sensitive *E. coli* O157:H7 ATCC43888 (strain R01s) lacked the major outer membrane protein OmpC and did not bind PP01 phage. And not only R01s but also OmpC defective mutant of *E. coli* K-12 (RK4784) became susceptible to PP01 by the expression of ompC derived from O157:H7. OmpC from K-12 did not serve as a PP01 phage receptor. The immunoblotting analysis using an antibody raised against the *E. coli* O157:H7 LPS revealed that the LPS of R01s was almost identical to that of *E.coli* O157:H7 ATCC43888. The attachment of T-even bacteriophages to *E. coli* cells is mediated by two phage structures: the long tail fibers and the short tail fibers. The specificity of phage infection arises from the interaction between the long tail fiber and the cell receptor. In contrast, interaction of the short tail fiber and any cell component is not responsible for specificity [27, 28]. Since the production of OmpC of *E. coli* O157:H7 (ATCC43888) in *E. coli* K-12 (RK4784) cells supposed PP01 phage infection, it was concluded that OmpC was the receptor for the distal long tail fibers of PP01. However, PP01 adsorbed only slowly to RK4784 cells
producing the OmpC of O157:H7, so that the effective adsorption might need other elements of the cell surface besides OmpC.

T-even phages recognize their receptors with a protein at the distal part of the tail fiber. From the deduced aa sequences of the tail fiber proteins, it became clear that PP01 is member of the T2 family. In T2, Gp38 bound to the tip of the tail fibers act in receptor recognition [10, 14]. The general architecture of Gp38 of T2 family is the same. About 120 N-terminal and 20 C-terminal residues are conserved, the region in between these being variable. This area is flanked and interrupted by glycine-rich stretches [16]. The glycine-rich stretches could cause an increased flexibility of the protein at these sites, which may facilitate orientation and binding to the receptor. Variance of Gp38 between Ac3 and E. coli O157:H7 specific phages including PP01 has mostly been found in this area, suggesting that these areas are responsible for specific recognition of E. coli O157:H7 OmpC protein.

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References


Figure Legends

Fig. 1. *E. coli* O157:H7 lysis by PP01 phage. Culture was incubated at 37 °C. *E. coli* O157:H7 was infected with a PP01 phage. Cultures were incubated with (closed) or without (open) phage infection. At the time indicated by arrow, PP01 phage was added to the culture.

Fig. 2. PP01 adsorption assay. Relative phage titer in the supernatant of a mixture containing PP01 and each strain was determined. Phage concentration at the time 0 was ca. 10^7 PFU ml^{-1}. Symbols: ATCC43888 (open circle). R01s (open square). R01s/pOMPC1 (closed square). RK4784 (open triangle). RK4784/pOMPC1 (closed triangle).

Fig. 3. The bacterial envelope analysis. (A) LPS analysis using the antibody to *E. coli* O157:H7, (B) Outer membrane protein analysis. Lanes in figure A: 1, ATCC43888. 2, R01s. 3, RK4784. Lanes in figure B: 1, ATCC43888. 2, R01s. 3, R01s/pOMPC1. 4, R01s/pOMPC2. 5, RK4784. 6, RK4784/pOMPC1. 7, RK4784/pOMPC2. Molecular mass standard is represented on the left.

Fig. 4. Alignment of OmpC from *E. coli* O157:H7 (O157) and K-12 (K-12). Amino acid substitutions are shown in asterisk and gapping residues are dashed.
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a Obtained from H. Watanabe (National Institute of infectious Disease, Japan).
b Obtained from F. Blattner (University of Wisconsin).
c Obtained from M. Nakazawa (National Institute of Animal Health, Japan).
Table 2
Efficiency of plating (e.o.p.) of PP01 phage

<table>
<thead>
<tr>
<th>Strain</th>
<th>e.o.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC43888</td>
<td>1.00</td>
</tr>
<tr>
<td>R01s</td>
<td>N.D.  b</td>
</tr>
<tr>
<td>R01s/pOMPC1</td>
<td>8.80 ± 0.42</td>
</tr>
<tr>
<td>R01s/pOMPC2</td>
<td>N.D.  b</td>
</tr>
<tr>
<td>RK4784</td>
<td>N.D.  b</td>
</tr>
<tr>
<td>RK4784/pOMPC1</td>
<td>1.40 ± 0.041</td>
</tr>
<tr>
<td>RK4784/pOMPC2</td>
<td>N.D.  b</td>
</tr>
</tbody>
</table>

a The plaque titers were expressed relative to that obtained on ATCC43888 which was $1 \times 10^{10}$ PFU ml$^{-1}$.

b No plaque was detected. The value was less than $10^{-9}$. 