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Rapid Detection of *Escherichia coli* O157:H7 by Using Green Fluorescent Protein-Labeled PP01 Bacteriophage

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A previously isolated T-even-type PP01 bacteriophage was used to detect its host cell, *Escherichia coli* O157:H7. The phage small outer capsid (SOC) protein was used as a platform to present a marker protein, green fluorescent protein (GFP), on the phage capsid. The DNA fragment around *soc* was amplified by PCR and sequenced. The gene alignment of *soc* and its upstream region was *g56-soc.2-soc.1-soc*, which is the same as that for T2 phage. GFP was introduced into the C- and N-terminal regions of SOC to produce recombinant phages PP01-GFP/SOC and PP01-SOC/GFP, respectively. Fusion of GFP to SOC did not change the host range of PP01. On the contrary, the binding affinity of the recombinant phages to the host cell increased. However, the stability of the recombinant phages in alkaline solution decreased. Adsorption of the GFP-labeled PP01 phages to the *E. coli* cell surface enabled visualization of cells under a fluorescence microscope. GFP-labeled PP01 phage was not only adsorbed on culturable *E. coli* cells but also on viable but nonculturable or pasteurized cells. The coexistence of insensitive *E. coli* K-12 (W3110) cells did not influence the specificity and affinity of GFP-labeled PP01 adsorption on *E. coli* O157:H7. After a 10-min incubation with GFP-labeled PP01 phage at a multiplicity of infection of 1,000 at 4°C, *E. coli* O157:H7 cells could be visualized by fluorescence microscopy. The GFP-labeled PP01 phage could be a rapid and sensitive tool for *E. coli* O157:H7 detection.

Enterohemorrhagic *Escherichia coli* (EHEC) of serogroup O157:H7 has been found to cause bloody diarrhea and hemolytic uremic syndrome in humans. EHEC produces two toxins, namely Shiga toxins 1 and 2 (Stx1 and Stx2). The natural reservoirs of EHEC are cattle and other domestic animals (21, 22). Although the predominant mode of transmission to humans is via consumption of contaminated meat, infection via contaminated water has also been documented (1).

Rapid and sensitive detection of *E. coli* O157:H7 is essential for minimizing the outbreak of infection, for surveillance, and for sanitary supervision. Three methods, culturing, PCR analysis, and immunoassay, are available for the detection of *E. coli* O157:H7. These culture methods are laborious and expensive and require a minimum of 3 days to perform (4). PCR analysis of *E. coli* O157:H7 has often been aimed at detecting the genes for Stx1 and Stx2 (6). Although these assays may be useful for the examination of human or animal fecal samples, their usefulness for the examination of environmental samples is limited due to the widespread presence of these genes in nonpathogenic bacteria. Enzyme immunoassays have also been used for detecting *E. coli* O157 in enrichment cultures of food and environmental samples (2). Although sensitive, these assays are laborious and expensive and tend to yield positive results that cannot be confirmed by culturing (2).

E. coli O157:H7 enters a viable but nonculturable (VBNC) state after a lengthy exposure to oligotrophic fresh- and seawater at an ambient temperature. Although the role of VBNC cells in food or water safety is not fully known, VBNC *E. coli* O157:H7 was shown to occur widely in a natural freshwater environment in Tokyo, Japan (12). Direct viable cell counts of *E. coli* O157:H7, determined by acridine orange staining, remained essentially the same for 12 weeks at 25°C, whereas viable cell counts on tryptic soy agar plates decreased to undetectable levels within 12 weeks (7). Direct viable cell counts, however, can be applied only to axenic cultures and not to *E. coli* in natural environments, where mixed bacterial populations exist. Conventional culture methods also fail to detect VBNC *E. coli* O157:H7 in the natural environment.

A virulent phage (PP01), previously isolated from swine stool samples, was found to infect *E. coli* O157:H7 strains with a high specificity (15). In phages of the T2 family, the gene *38* product (Gp38), which is present at the tip of long tail fibers, is the determinant of host range (19). Analysis of deduced amino acid alignments of the tail fiber proteins revealed that the PP01 phage is related to the T2 phage. Moreover, the specific recognition of the *E. coli* O157:H7 OmpC protein by Gp38 determines PP01's host range (14). In this study, PP01 was used for the detection of *E. coli* O157:H7 in viable and VBNC states. One of the outer capsid proteins, named small outer capsid (SOC) protein, was fused with green fluorescent protein (GFP). Labeled recombinant PP01 phage provided a rapid and sensitive method for *E. coli* O157:H7 detection.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The *E. coli* O157:H7 ATCC 43888 bacterial strain was used as the host for the phage. This strain does not produce either Stx1 or Stx2 because of the absence of genes for these toxins, but it possesses an envelope structure similar to that of EHEC O157:H7. The PP01 phage, isolated from swine stool and infectious to *E. coli* O157:H7 strains with high specificity and lytic activity (15), was employed for the construction of GFP-labeled bacteriophage.

In batch cultures, *E. coli* O157:H7 ATCC 43888 was cultured overnight in 2 ml of Luria-Bertani (LB) broth at 37°C with shaking (120 rpm). The optical density of the medium at 600 nm (OD_{600}) was measured with a Klett spectrophotometer (Hitachi High-Technologies Corp.) to estimate the cell concentration. Bacterio-

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TABLE 1. Phages, *E. coli* strains, and plasmids used in this study

Phage, strain, or plasmid	Properties or use
Phage	
PP01	Virulent, *E. coli* O157:H7 specific
E. coli strains	
O157:H7 (ATCC43888)	Host cell for propagation of PP01
XL-1 Blue	General cloning host
K-12 (W3110)	Nonsusceptible model bacteria
O157:H37 (CE237)	Host range assay
O157:H19 (A2)	Host range assay
O157:H7 (CR3)	Host range assay
BE	Host range assay
HfrH	Host range assay
P. aeruginosa PAO1	Host range assay
Plasmids	
pUC118	General cloning vector
pQB2	Expression vector for GFP
pQB2′	720-bp PCR end-modified *gfp* cloned into *Kpn*I site of pQB2
pQB-GFP/SOC	Expression vector of GFP/SOC fusion protein
pUC-GFP/SOC1	pUC118 inserted with 1.1-kb *GFP*/*SOC* from pQB-GFP/SOC
pUC-GFP/SOC	0.5 kbp upstream of *soc* inserted into pUC-GFP/SOC1
pUC-SOC (*Kpn*I)	1-kb *soc*, including its surrounding regions inserted into pUC118
pUC-SOC/GFP	pUC-SOC (*Kpn*I) with *gfp* inserted

phage PP01 infection at a multiplicity of infection (MOI) of 2 was performed at an OD_{600} of 0.1. For dilution and preservation of the 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.

Sequencing of phage DNA. The plasmids used in this study are listed in Table 1. PP01 phage DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. PP01 phage DNA was diluted with distilled water and used as a template for PCR. The oligonucleotide primers used for PCR and sequencing are listed in Table 2. The DNA fragment encoding PP01 SOC protein was amplified by use of the primer set of $g56^+$ and mrh^-. The primer set was designed based on the DNA sequence of T2 phage genome DNA. The PCR fragment was digested with *Pst*I and *Xba*I and inserted into the *Pst*I and *Xba*I sites of pUC118 to obtain pUC-SOC, which encodes the PP01 *soc* gene and its surrounding region. Sequencing of the cloned DNA was performed by using a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit and a 7-deaza-dGTP kit (Amersham Pharmacia Biotech). The sequencer used was DSQ-2000L (Shimadzu, Kyoto, Japan).

Construction of plasmid. The *gfp* gene encoding GFP was amplified by PCR using the primer set of gfp^+(Kpn) and gfp^-, with plasmid pQB2 (11) as the template. KOD DNA polymerase (Toyobo, Osaka, Japan) was used for PCR. The PCR fragment was digested with *Kpn*I and reinserted into the *Kpn*I site of pQB2 to produce pQB2′, which lacks a stop codon for *gfp*. The DNA fragment encoding PP01 SOC and its downstream region (about 100 bp) was amplified by use of the primer set of $socN^+$ and $mrh2^-$, with the PP01 phage genome as a template. The PCR fragment was digested with *Pst*I and *Hin*dIII and inserted into the *Pst*I and *Hin*dIII sites of pQB2′ to produce pQB-GFP/SOC. The PCR fragment amplified using pQB-GFP/SOC as the template and the primer set of gfp^+(*Xba*I) and $mrh2^-$ was digested with *Xba*I and *Hin*dIII and inserted into the *Xba*I and *Hin*dIII sites of pUC118 to produce pUC-GFP/SOC1. Then the PCR fragment amplified using the PP01 phage genome as the template and the primer set of $g56^+$ and $socN^-$ was digested with *Xba*I and inserted into the *Xba*I site of pUC-GFP/SOC1 to produce pUC-GFP/SOC.

The DNA fragments encoding PP01 *soc*, its upstream region (about 200 bp), and its downstream region (about 100 bp) were amplified by use of the primer sets $g56^+$-$socC^-$ and $socC^+$-mrh^-, respectively, with the PP01 phage genome as the template. The two PCR fragments were digested with *Xba*I-*Kpn*I and *Kpn*I-*Pst*I, and the two fragments were inserted into the *Xba*I and *Pst*I sites of pUC118 to produce pUC-SOC/*Kpn*I. Then the *gfp* gene digested with *Kpn*I from pQB2 was inserted into the *Kpn*I site of pUC-SOC/*Kpn*I to produce pUC-SOC/GFP.

Homologous recombination. The protocol used to integrate *gfp* into the phage genome is outlined in Fig. 1. *E. coli* O157:H7 (ATCC 43888) was transformed with two plasmids, pUC-GFP/SOC and pUC-SOC/GFP, by electroporation. The transformant *E. coli* cells were incubated in LB medium supplemented with 50 mg of ampicillin per liter. When the OD_{600} reached 0.1, the PP01 phage was added at an MOI of 0.01. After 5 h of incubation, chloroform was added to lyse the cells and the culture was centrifuged to remove cell debris. The cell lysate was diluted with SM buffer to obtain a phage concentration of 10^4 PFU/ml. The diluted phage lysate was mixed with *E. coli* O157:H7 in 0.7% agar and overlaid on an LB plate. The recombinant phage was detected by plaque hybridization with a digoxigenin (DIG)-labeled probe. The plaques were transferred to a nylon membrane (Roche Diagnostics) and immersed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min, in neutralizing buffer (1.0 mM Tris-HCl, 1.5 M NaCl, pH 7.5) for 15 min, and in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) for 10 min. Phage DNA was cross-linked on the membrane by UV radiation (1,200 J/cm^2) and incubated in 2 mg of proteinase K solution per liter for 1 h at 37°C. The membrane was rinsed with distilled water and prehybridized in DIG-Easy-Hyb buffer (Roche Diagnostic) for 1 h at 55°C. The membrane was hybridized with the DIG-labeled *gfp* probe overnight at 55°C. The probe DNA was amplified by use of a PCR DIG-Probe-Synthesis kit (Roche Diagnostic), using pQB2 as the template and gfp^+(*Kpn*I) and gfp^- as the primer set. The hybridized membrane was washed twice with 2× SSC containing 0.1% sodium dodecyl sulfate for 5 min at room temperature and once with 0.1× SSC containing 0.1% sodium dodecyl sulfate for 15 min at 68°C. Then the hybridized spot was detected by use of a DIG-Luminescent-Detection kit (Roche Diagnostic).

Purification of the GFP-labeled phage. A luminescent plaque was isolated and purified twice. Integration of *gfp* into the phage genome was reconfirmed by

TABLE 2. Oligonucleotide primers used for PCR

Primer	Sequence (5′ → 3′)[a]
$g56^+$	GC**TCTAGA**GAAGAAATCTTTAAACTTTATTATCTG
$mrh2^-$	TCT**AAGCTT**GGTTTAATCCAACGATTTAACAT
mrh^-	TGA**AAGCTT**CAAGCATCTTCTTCAGAACTT
$socN^+$	AA**CTGCAG**GCATGGCTAGTACTCGCGGTTA
$socN^-$	CA**TCTAGA**TCTCCTTTTATTTAAATTACATGAC
$socC^+$	GG**GGTACC**AGACTCTTCGGGAGTCCTTT
$socC^-$	TT**GGTACC**CAGTTACTTTCCACAAATCTT
gfp^+(Xba)	CT**TCTAGA**TGAGTAAAGGAGAAGAACTTTT
gfp^+(Kpn)	GG**GGTACC**CATGAGTAAAGGAGAAGAACTTTT
gfp^-	GG**GGTACC**TTTGTATAGTTCATCCATGCCA

[a] Restriction sites are shown in bold.

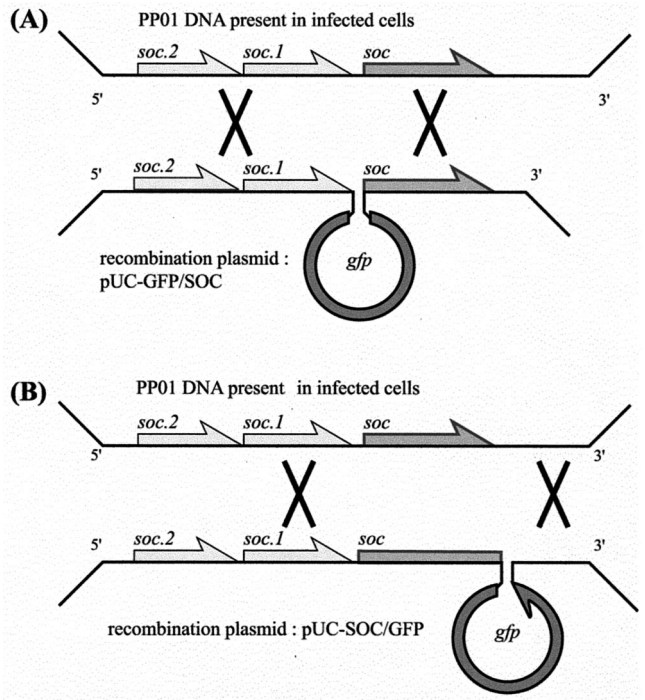

FIG. 1. Outline of the homologous recombination process leading to insertion of *gfp* upstream (A) or downstream (B) of the major capsid protein gene *soc*. The multiplication signs indicate the recombination events (double crossover) between phage DNA (top) present in infected cells and pUC-GFP/SOC (A) or pUC-SOC/GFP (B).

plaque hybridization. The isolated phage was added to the *E. coli* O157:H7 culture in 250 ml of LB broth at an MOI of 0.01 and incubated for 6 h. Chloroform (2.5 ml) was added to the culture, which was further incubated for 1 h at 4°C. Cell debris was separated by centrifugation (9,500 × *g*, 10 min, 4°C). The phage was precipitated by the addition of 25 g of polyethylene glycol 6000 and 10 g of NaCl, and the culture was allowed to stand overnight at 4°C. The phage was separated by centrifugation (16,000 × *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 (10,000 × *g*, 20 min, 4°C) to remove cell debris. The phage was separated by cesium chloride (CsCl) density gradient (1.45, 1.5, and 1.7 g/ml) centrifugation (111,000 × *g*, 2 h, 4°C). CsCl was removed by dialysis in the SM buffer.

Phage adsorption assay. *E. coli* O157:H7 cells in the logarithmic growth phase (10^7 CFU/ml) were preserved on ice until use. The cell culture (400 μl) was prewarmed at 25°C for 10 min and mixed with the same amount of phage solution (10^5 PFU/ml) in SM buffer. The mixture was incubated at 25°C. After infection, 110 μl of the mixture was sampled periodically, and the samples were centrifuged (174,000 × *g*, 1 min, 4°C). The phage titer of the supernatant was determined by plaque assay using *E. coli* O157:H7 (ATCC 43888), and the phage titer at time zero was defined as 100%.

Phage stability under alkaline conditions. Phage solution (10^7 PFU/ml; 10 μl) was mixed with 990 μl of alkaline SM buffer (pH 10.6) and incubated at 37°C. The mixture was sampled periodically and diluted 1:100 with SM buffer (pH 7.5). The stability was estimated by comparing the phage titer in alkaline SM buffer with that in neutral SM buffer (pH 7.5).

Detection of *E. coli* O157:H7 by using GFP-labeled PP01 phage. *E. coli* O157:H7 cell culture (10^7 CFU/ml) in the logarithmic growth phase was allowed to stand on ice until use. The cell culture was prewarmed at 25°C for 10 min and then mixed with the same amount of phage solution (5×10^9 PFU/ml). The mixture was allowed to stand at 25°C for 10 min, followed by centrifugation (17,400 × *g* for 1 min at 4°C), washing with PBS, and resuspension in PBS. Luminescent *E. coli* O157:H7, due to adsorbed GFP-labeled PP01 phage, was observed under an epifluorescence microscope (BX60; Olympus, Tokyo, Japan) equipped with a filter (U-MWIBA/GFP; Olympus). Photographs were taken with a digital still camera, with an exposure setting of 1/5 s for phase-contrast microscopy and 2 s for fluorescence microscopy.

Establishment of VBNC state. An *E. coli* O157:H7 cell culture (10^7 CFU/ml; 30 ml) in the logarithmic growth phase was centrifuged (12,000 × *g* for 3 min at 4°C) and resuspended in the same amount of PBS. After 1 week of incubation at 4°C, cell viability was estimated by counting of colonies on LB plates.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper for *soc* will appear in the DDJB/EMBL/GenBank nucleotide sequence databases under accession number AY247798.

RESULTS

Construction of GFP-labeled PP01. For amplification of the DNA fragment encoding the SOC protein of PP01 phage, two oligonucleotide primers, $g56^+$ and mrh^-, were constructed. The $g56^+$ primer is complementary to the 3′ region of gene *56* from the T4 phage. *g56* is well conserved among the T-even phages and is located upstream of *soc*. The mrh^- primer was designed based on the sense alignment of the 3′ region of gene *mrh* encoded by the T4 phage. The PCR product of 1.9 kb was digested with *Pst*I cloned into the same site of pUC118 and was used for sequencing. Two open reading frames, *soc.1* and *soc.2*, were found between *g56* and *soc*. The nucleotide sequence identities of *soc* genes among the T-even phages, PP01, RB15, T4, and T2, are shown in Fig. 2. The amino acid sequence identities between PP01 SOC and that of other T-even phages were 95.1% (RB15), 97.5% (T4), and 84.8% (T2). The numbers of amino acids making up the SOC proteins were 82 (PP01), 82 (RB15), 81 (T4), and 86 (T2).

Although SOC is not an essential component for phage replication, it plays an important role in the stability of the phage capsid (8). To clarify the effect of N- and C-terminal fusion of GFP to SOC on phage stability, two recombinant PP01 phages, PP01-GFP/SOC and PP01-SOC/GFP, were constructed. PP01-GFP/SOC integrated GFP to the N terminus of SOC. In contrast, PP01-SOC/GFP integrated GFP to the C terminus of SOC.

To introduce *gfp* adjacent to *soc*, homologous recombination between the plasmid and the phage genome was conducted. The frequencies of recombination were approximately 0.3% for PP01-GFP/SOC and 0.5% for PP01-SOC/GFP. Several positive plaques, which emitted green fluorescence, were isolated from the agar plate and purified. Integration of *gfp* into the phage genome was confirmed by plaque hybridization and sequencing of the PCR-amplified DNA region around the *soc-gfp* and *gfp-soc* junctions (data not shown).

Characterization of GFP-labeled PP01. The turbidity and fluorescence changes of the *E. coli* O157:H7 culture after phage infection were measured (Fig. 3). Three phages, PP01-wt, PP01-GFP/SOC, and PP01-SOC/GFP, were added to the medium at time zero at an MOI of 0.1. Incubation was performed at 28°C to increase the fluorescence intensity. After a 1.5-h incubation of *E. coli* O157:H7 with one of the phages, a decrease in OD_{600} was observed. Since the LB medium contains self-luminous compounds, the initial fluorescence intensity of the culture was 125 (no dimension). The PP01-wt infection did not influence the fluorescence intensity of the culture. On the other hand, PP01-GFP/SOC and PP01-SOC/GFP infections increased the fluorescence intensity of the culture. Fluorescence intensity of the culture reached 220 (no dimension) 3 h after infection of PP01-GFP/SOC and remained al-

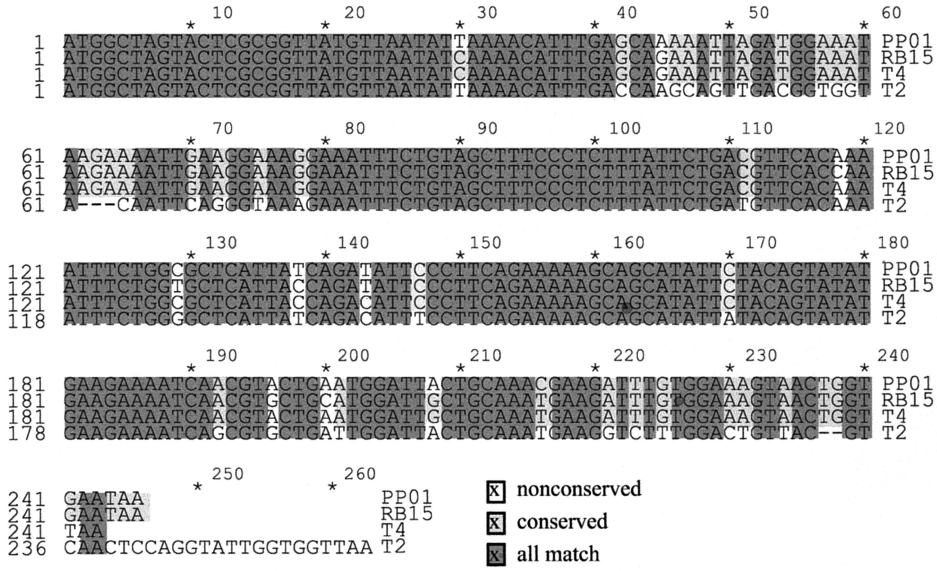

FIG. 2. Nucleotide sequences of upstream and downstream *soc* regions from phages PP01, RB15, T4, and T2. Dashes indicate nucleotides that are deleted. Completely conserved nucleotides are shown in dark gray, and partially conserved regions are shown in light gray.

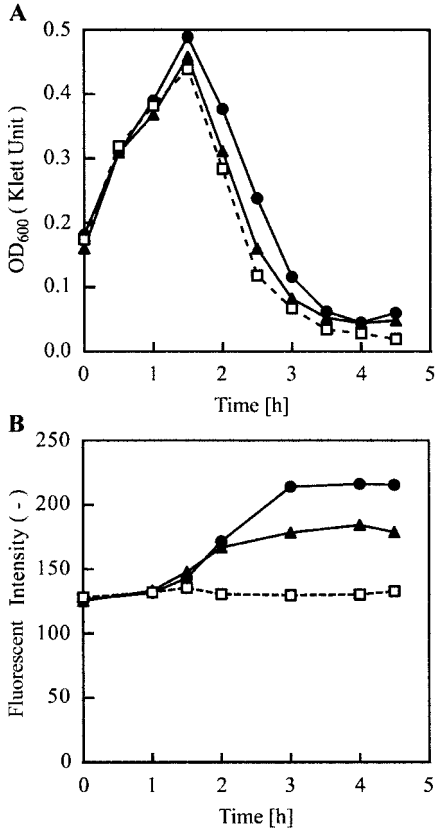

FIG. 3. (A) *E. coli* O157:H7 lysis by PP01-wt (squares), PP01-GFP/SOC (circles), and PP01-SOC/GFP (triangles). (B) Phage-induced fluorescence of the culture. A 200-μl *E. coli* O157:H7 overnight culture was inoculated into 20 ml of LB medium at time zero and incubated at 28°C. When the OD_{600} of the culture reached 0.2, 200 μl of phage containing PBS was added at an MOI of 0.04.

most constant. The maximum fluorescence intensity of the PP01-GFP/SOC culture was higher than that of PP01-SOC/GFP culture.

PP01 formed relatively large (0.5 to 1.0 mm) and clear plaques on a cell lawn of *E. coli* O157:H7 but did not form any plaques on a lawn of *E. coli* K-12 strains and other related bacteria (16). The host range of the three phages was examined by using seven *E. coli* strains and *Pseudomonas aeruginosa* PAO1 (Table 3). No difference in the host range was observed.

To investigate the effect of GFP fusion to SOC on phage binding to the host cell, a phage adsorption assay was conducted. Free phage (P_{free}, in PFU per milliliter) adsorption on the host cell (B_{free}, in CFU per milliliter) surface proceeded by an initial reversible interaction, followed by a second irreversible interaction. The overall adsorption reaction and its kinetics can be described as follows.

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

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

TABLE 3. Host range of GFP-labeled PP01 phage

Host serotype or strain	Susceptibility to phage[a]		
	wt-PP01	PP01-GFP/SOC	PP01-SOC/GFP
E. coli			
K-12 (W3110)	−	−	−
O157:H37 (CE237)	−	−	−
O157:H19 (A2)	+	+	+
O157:H7 (CR3)	+	+	+
O157:H7 (ATCC 43888)	+	+	+
BE	−	−	−
HfrH	−	−	−
P. aeruginosa PAO1	−	−	−

[a] +, susceptible; −, not susceptible.

TABLE 4. Phage adsorption assay results

Phage	k_a $(B_0)_{O157}$ $(min^{-1})^a$	k_a $(10^{-9}$ ml CFU^{-1} min$^{-1})$
PP01-wt	0.0555 ± 0.0032	1.58
PP01-GFP/SOC	0.0779 ± 0.0010	2.21
PP01-SOC/GFP	0.0846 ± 0.0012	2.40

[a] $(B_0)_{O157}$, $3.52 \times 10^7 \pm 1.51 \times 10^7$ CFU/ml; results are means ± standard deviations.

The k_a value (milliliters per CFU per minute) is an adsorption rate constant. Under low-MOI (<0.01) conditions, 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 the free phage concentration (P_{free}) in the culture provided $k_a(B_0)$ and k_a, which represents phage adsorption affinity on the host cell. An *E. coli* O157:H7 cell culture (10^7 CFU/ml) in the early logarithmic growth phase was mixed with the same amount of one of the three phage solutions (10^5 PFU/ml) at 25°C. P_{free} in the mixture was analyzed and plotted against the incubation time to estimate the $k_a(B_0)$ and k_a values (Table 4). The k_a value of PP01-wt was smaller than those of PP01-GFP/SOC and PP01-SOC/GFP, indicating that the GFP fusion to SOC enhanced the phage binding affinity for the host cells.

Since the T4 phage SOC deletion mutant has reduced stability under high-pH conditions (8), the effect of GFP fusion to SOC on phage stability was analyzed under high-pH conditions (Fig. 4). PP01-wt was relatively stable at a high pH. The viability of PP01-wt was approximately 70% during a 2-h incubation. A 2-min incubation of PP01-GFP/SOC and PP01-SOC/GFP at pH 10.6 reduced their viabilities to 47 ± 6% (PP01-GFP/SOC) and 37 ± 7% (PP01-SOC/GFP). However, a longer incubation period did not considerably reduce the phage viability. GFP-labeled phages PP01-GFP/SOC and PP01-SOC/GFP were more sensitive than PP01-wt at pH 10.6. GFP fusion to SOC reduced the phage stability in an alkaline solution.

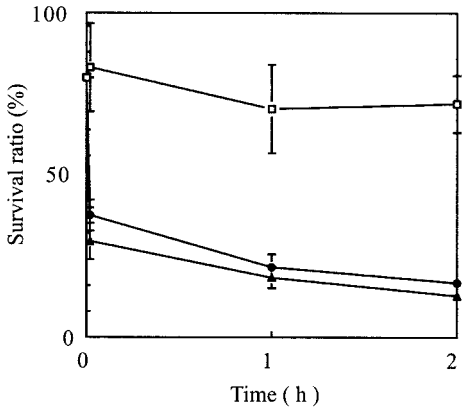

FIG. 4. Phage stability in alkaline solution. Three phages, PP01-wt (squares), PP01-GFP/SOC (circles), and PP01-SOC/GFP (triangles), were incubated in SM buffer (pH 10.6) at 37°C, without shaking. Survival ratios were estimated by comparing phage titers in the alkaline SM buffer with those in neutral SM buffer (pH 7.5).

Specific detection of *E. coli* O157:H7 by using GFP-labeled PP01. *E. coli* O157:H7 and *E. coli* K-12 (W3110) were incubated with PP01-wt, PP01-GFP/SOC, or PP01-SOC/GFP for 10 min at 25°C. After being washed with PBS, *E. coli* cells infected with the three phages were observed under an epifluorescence microscope (Fig. 5). *E. coli* O157:H7 infected with PP01-GFP/SOC and PP01-SOC/GFP generated fluorescence. On the other hand, *E. coli* K-12 (W3110) was not observed to be infected with the two phages. Fluorescence intensities of *E. coli* O157:H7 cells labeled with PP01-GFP/SOC and PP01-SOC/GFP were almost the same.

A cell mixture of *E. coli* O157:H7 and *E. coli* K-12 (W3110) cells was incubated with PP01-GFP/SOC for 10 min at 25°C. Cells were counted under optical and fluorescence microscopes. The count obtained by use of the optical microscope reflects the number of *E. coli* cells of strains O157:H7 and K-12 (W3110). In contrast, the count obtained by use of the fluorescence microscope reflects the number of *E. coli* O157:H7 cells. The percentage of *E. coli* O157:H7 cells in the *E. coli* mixture was plotted against the calculated percentage (Fig. 6). Both percentages showed a linear relationship ($y = 0.951x - 6.777$; $r^2 = 0.964$), indicating that the fluorescence count of the cells reflects the number of *E. coli* O157:H7 cells in the *E. coli* mixture.

Since most of the *E. coli* cells in different environments, such as wastewater, groundwater, and river water, are in the VBNC state, detection of VBNC *E. coli* O157:H7 by using PP01-SOC/GFP was investigated. The VBNC state was created by allowing *E. coli* O157:H7 to incubate in PBS buffer at 4°C for 1 week. After the 1-week incubation, the CFU count of cells decreased to 0.1. In contrast, the visible count of cells through the optical microscope did not change. The morphology of VBNC cells was relatively small and round. Ninety percent of the cells that did not form colonies were assumed to be in the VBNC state. Gentle pasteurization without damaging the cell surface was performed by incubating cells at 64°C for 5 min. The viability of *E. coli* cells was reduced to 0.6% after this pasteurization. The adsorption rate constant k_a (in milliliters per CFU per min) of PP01-SOC/GFP for VBNC and pasteurized *E. coli* O157:H7 cells was the same, that is, 1.3×10^9 ml/CFU/min. The k_a values for VBNC and pasteurized *E. coli* O157:H7 cells were lower than that (2.4×10^9 ml/CFU/min) for cells in the logarithmic growth phase. However, VBNC and pasteurized *E. coli* O157:H7 cells could be visualized by incubation with PP01-SOC/GFP (Fig. 5).

DISCUSSION

In the summer of 1996 in Japan, large outbreaks of EHEC O157:H7 infection occurred, particularly in Osaka City. After that, sporadic prevalent EHEC infections have been reported, and many school children and elderly people have been killed every year by this disease in Japan. In most of the cases, the serotype of EHEC was O157:H7. The conventional method of detection of *E. coli* O157:H7 is based on the cultivation of a sample on a cefixime-tellurite-sorbitol-MacConkey agar plate in combination with additional enzymological tests. These conventional methods are time consuming and require professional skill. The conversion of enteropathogenic bacteria to the VBNC state also makes it difficult to detect these cells in

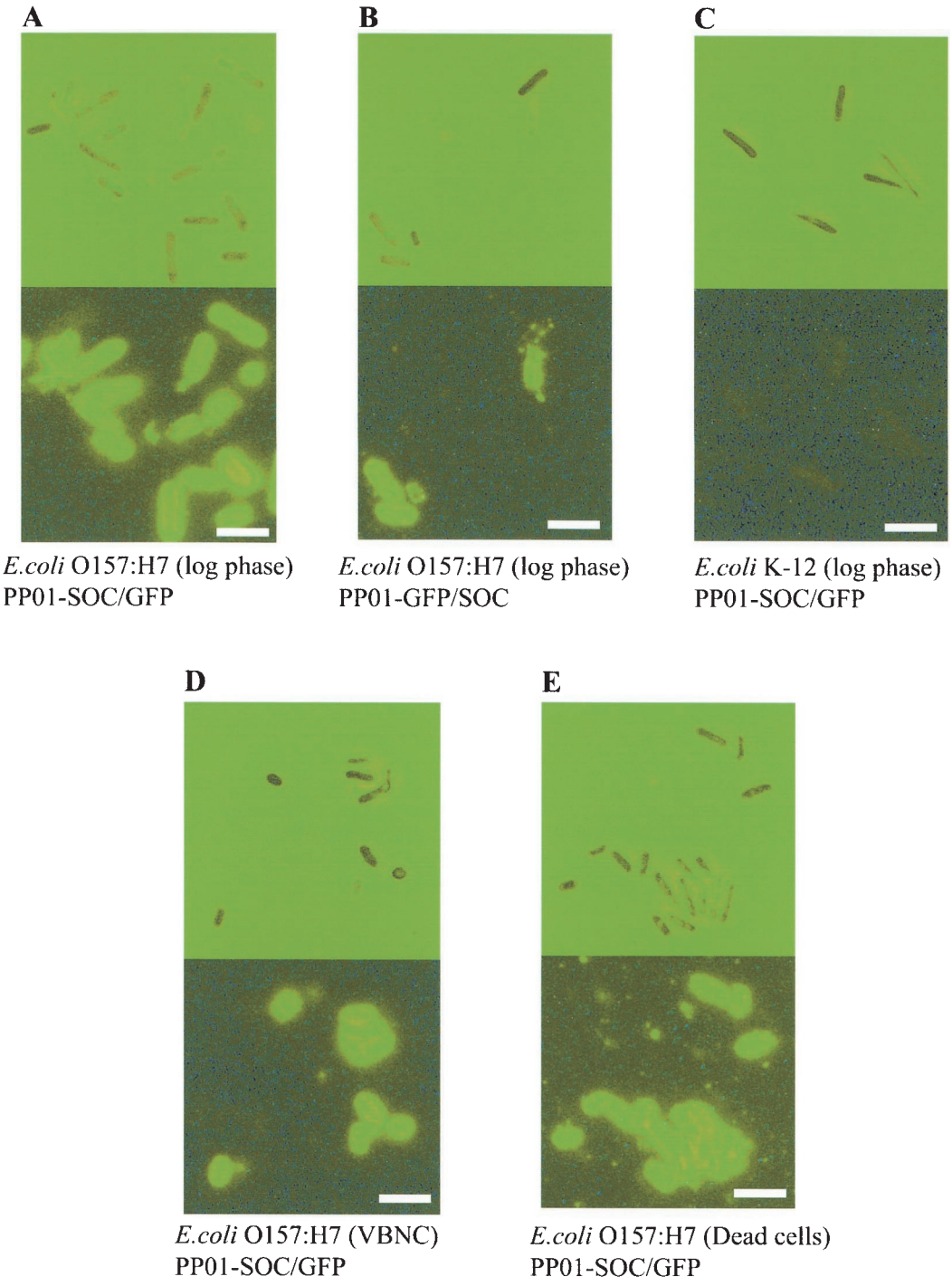

FIG. 5. Optical microscope images (upper panels) and fluorescence microscope images (lower panels) of *E. coli* O157:H7 (A, B, D, and E) and *E. coli* K-12 (W3119) (C). The bacteria (10^7 CFU/ml) were incubated at 25°C for 10 min with PP01-SOC/GFP (A, C, D, and E) or PP01-GFP/SOC (B) (10^{10} PFU/ml). The *E. coli* cell states were logarithmic growth phase (A to C), VBNC (D), and dead (E). Scale bar, 2.5 μm.

natural environments, such as river water and food. The development of rapid and reliable methods for *E. coli* O157:H7 detection is needed.

The PP01 phage used in this study was isolated from a swine stool sample. The finding that PP01 phage is a member of the T-even phage family enables us to apply genetic information on T-even phages to gene manipulation of PP01. T4 SOC is used as the platform for T4 phage display (10, 18). Since the high amino acid homology between PP01 SOC and T4 SOC was identified, PP01 SOC was also assumed to be available for PP01 phage display. Since the binding site of the SOC protein for the phage capsid protein is not located in the N or C

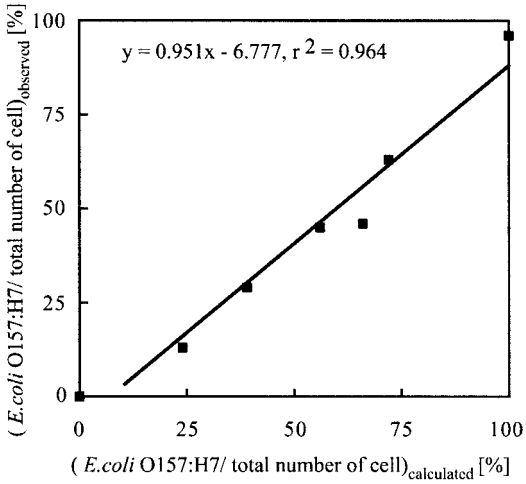

FIG. 6. Correlation of *E. coli* O157:H7 percentage in cell mixture. A mixture of *E. coli* O157:H7 and *E. coli* K-12 (W3110) cells was incubated with PP01-GFP/SOC for 10 min at 25°C. The observed percentages were based on microscope counts and were plotted against calculated percentages from the initial concentrations of *E. coli* O157:H7 and K-12 cells.

terminus of SOC, foreign protein fusion to SOC does not disrupt the interaction between SOC and the phage capsid (10, 18). Gene alignments around *soc* for T-even phages are as follows: for T4, *g56-g69-soc*; for T2, *g56-soc.2-soc.1-soc*; and for RB15, *g56-soc* (17). The similarity of gene alignment around *soc* in PP01 and T2 also supported the finding that PP01 is a member of the T2 family. Even though PP01 is closely related to T2, the host cell specificities of these two phages are discriminative. The host range of PP01 is limited to *E. coli* serotype O157:H7 (16). Since the phage capsid is not involved in host cell recognition, modification of SOC did not change the host range of PP01. The adsorption rate constants, or k_a values, of the recombinant phages, PP01-SOC/GFP and PP01-GFP/SOC, were larger than that of wild-type PP01. The number of SOC molecules present on the capsid was estimated to be 840 (9). Fusion of GFP to SOC enlarged the surface area of the capsid and may increase the collision probability for the recombinant phage and its host. A precise investigation of phage stabilities under various conditions, such as different temperatures and ionic strengths, was necessary for the preservation of the phage.

Infection of culturable *E. coli* O157:H7 cells by GFP-labeled phages increased the fluorescence intensity of the culture. The initial fluorescence was derived from the added phage and from auto-fluorescence of *E. coli* O157:H7 and the LB medium. Following a 1-h incubation with the recombinant phage, the fluorescence intensity of the culture increased gradually and reached a plateau at 3 h of incubation. The increase in fluorescence intensity reflected replication of recombinant progeny phage in cells. On the other hand, when the recombinant phages were added to VBNC or pasteurized *E. coli* O157:H7, no increase in fluorescence intensity was observed (data not shown). It is obvious that the host cell function is indispensable for phage replication in cells. Recombinant phages recognized both VBNC and pasteurized *E. coli* O157:H7 cells (Fig. 5). Based on these observations, discrimination of culturable cells from VBNC or dead cells may be possible by monitoring the change in the culture fluorescence intensity or by modifying the MOI. Under low-MOI conditions, the fluorescence intensity of phage-infected cells may be low. However, a 1-h incubation enables phage replication in the culturable cells, which are countable under a fluorescence microscope. On the other hand, the fluorescence intensity of VBNC or pasteurized *E. coli* O157:H7 cells infected by recombinant phages may remain constant. For the detection of both culturable and VBNC *E. coli* O157:H7 cells, a high MOI is desirable to enable strong visualization of the cells. Lysis from without (LO) is peculiar to the T4 phage (20). LO is lysis due to adsorption of a large number of T4 particles on the cell wall and occurs at MOIs of >20. However, LO of PP01 was not observed up to an MOI of 1,000.

Attempts to detect bacteria by use of specific phages have been reported previously (3, 5, 13, 23). However, the basic principles of these trials were based on the expression of fluorescence marker proteins, such as GFP and luciferase, in the host cells by integration of genes encoding the marker protein into the phage genome. Since the production of a marker protein depends on host cell activities, these methods fail to detect bacteria in the VBNC state, which is the most common state for bacteria in natural environments. GFP-labeled PP01 phage enables us to detect *E. coli* O157:H7 in both culturable and VBNC states. For the detection of both culturable and VBNC cells, a high MOI and a short incubation were used. On the other hand, for the detection of culturable cells alone, a low MOI and a long incubation time were needed. By changing the experimental conditions, we could distinguish culturable and VBNC cells.

The utilization of flow cytometry or image analysis might enhance the reliability of the method and shorten the detection time. For the application of this method for food enrichment or water samples, further studies, such as the assignment of a positive versus negative cutoff point, will be necessary.

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