A Recombinant Bacteriophage-Based Assay for the Discriminative Detection of Culturable and Viable but Nonculturable *Escherichia coli* O157:H7

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A previously green fluorescent protein (GFP)-labeled PP01 virulent bacteriophage, specific to *Escherichia coli* O157:H7, was used to construct lysozyme-inactivated GFP-labeled PP01 phage (PP01e⁻/GFP). The new recombinant phage lacked lytic activity because of the inactivation of gene *e*, which produces the lysozyme responsible for cell lysis. Gene *e* was inactivated by inserting an amber stop codon. Prolonged incubation of *E. coli* O157:H7 cells with PP01e⁻/GFP did not lead to cell lysis, while the propagation of PP01e⁻/GFP in host cells increased the intensity of green fluorescence. Retention of cell morphology and increase in fluorescence enabled the direct visualization and enumeration of *E. coli* O157:H7 cells within an hour. The PP01e⁻/GFP system, when combined with nutrient uptake analysis, further allowed the discriminative detection of culturable, viable but nonculturable (VBNC), and dead cells in the stress-induced aquatic environment. Stress-induced cells, which retained culturability, allowed phage propagation and produced bright green florescence. Nonculturable cells (VBNC and dead) allowed only phage adsorption but no proliferation and remained low fluorescent. The low-fluorescent nonculturable cells were further differentiated into VBNC and dead cells on the basis of nutrient uptake analysis. The low-fluorescent cells, which grew in size by nutrient incorporation during prolonged incubation in nutrient medium, were defined as metabolically active and in the VBNC state. The elongated VBNC cells were then easily recognizable from dead cells. The proposed assay enabled the detection and quantification of VBNC cells. Additionally, it revealed the proportion of culturable to VBNC cells within the population, as opposed to conventional techniques, which demonstrate VBNC cells as a differential value of the total viable count and the culturable cell count.

Introduction

Escherichia coli O157:H7 (*E. coli* O157:H7) is an increasingly recognized human pathogen causing severe illness including diarrhea and renal failure. *E. coli* O157:H7 may be transmitted by food or water, the latter including both recreational and drinking water (*4, 38*). *E. coli* O157:H7 persists in cattle manure (*24*) and manure-amended soil (*11*), and experiments with models have suggested that it may leach through soil, thereby contaminating aquatic systems (*8*). On the other hand, sewage discharge into estuaries, coastal waters, and open ocean may also be a possible transmitting factor in illnesses resulting from recreational waters (*10, 38*).

It is well-known that bacteria can respond to environmental stress by activating survival mechanisms (*29*). While a number of microorganisms can survive by forming resistant spores, nonsporulating microorganisms are able to persist in adverse environments as vegetative cells with low metabolic activities via the activation of the viable but nonculturable (VBNC) state (*1*). The existence of the VBNC state in Gram-negative bacteria has been demonstrated by a number of studies on the viability of fecal contamination indicators and pathogenic bacteria (*3, 32*). Indeed, the VBNC *E. coli* O157:H7 have been shown to occur widely in a natural freshwater environment in Tokyo, Japan (*13*). Conditions such as limited nutrient availability,

osmotic stress, and variation in temperature induce the VBNC state in *E. coli* O157:H7 (*18, 27, 36*).

A cell is considered to be in a VBNC state if it is metabolically active while being incapable of undergoing the sustained cellular division required to form a colony on media that are regularly used for bacterial enumeration (*23*). The inability to detect VBNC in the environment by employing routine bacteriological methods poses a potential hazard, because some VBNC pathogens retain the capacity to cause disease (*9, 26*). Long-term survival of *E. coli* O157:H7 by entering the VBNC state (*18, 27, 36*) could have implications for the transmission of disease. The demonstration of a VBNC state of *E. coli* O157:H7 in the environment, therefore, is important from a public health point of view.

Many genetic and immunological techniques have been developed for the detection of serotype O157:H7, including polymerase chain reaction (*17*), nucleic acid based assays (*33*), and immunological tests (*25*). However, while these assays are specific, sensitive, and rapid, they are often expensive and labor intensive. Moreover, they require an enrichment step to increase the target number to a detectable level. Such methods are therefore confined to the detection of culturable cells and fail to detect the cells in the VBNC state.

The VBNC state has been elucidated by techniques that assess respiration, enzymatic activity, and cellular activity, such as various direct viable count (DVC) methods (*14, 20, 28*). Such methods, however, can be applied only to axenic systems and

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not to the mixed microbial populations since these methods cannot discriminate between target cells and other bacteria. Moreover, they employ three separate systems to detect dead cells, total viable cells, and culturable cells, and even then, VBNC cells are elucidated indirectly as a differential value of the total viable count and the culturable cell count. A recent report for dealing with complex samples by using ethidium monoazide polymerase chain reaction (EMA-PCR) for DNA-based viable/dead differentiation has been introduced (*21*, *30*). However, this approach is also unable to detect and determine the proportion of the cells in the VBNC state.

The existence of bacteriophages specific for *E. coli* O157: H7 presents opportunities to develop methods for O157:H7 detection. The O157-specific T-even phage (PP01) was previously isolated from porcine faeces (*19*) and was used to construct the green fluorescent protein (GFP)-labeled bacteriophage for specific detection of *E. coli* O157:H7 (*22*). Its applications were limited because of the virulent characteristics of the phage, which resulted in bacterial lysis within 15−30 min post-infection. In the next study, we therefore used the lysozyme-inactivated T4 phage previously reported (*12*), instead of wild-type T4, to express GFP (T4e−/GFP) (*34*). Lysozyme is the product of gene *e* that degrades the peptidoglycan of the *E. coli* cells during cell lysis (*16*). Inactivation of gene *e* by insertion of an amber mutation deprives the phage of its virulent characteristics. T4e−/GFP enabled the detection and quantification of general fecal coliforms but could not detect *E. coli* O157: H7. To improve the efficiency of the previous GFP-labeled PP01 phage system, we constructed a new recombinant lysozyme-inactivated GFP-labeled PP01 (PP01e−/GFP) in this study.

E. coli cells under starvation conditions or in the VBNC state do not permit phage replication (*5*, *37*). In our previous study using the T4e−/GFP system (*34*), we observed that incubation of VBNC and dead cells with a GFP-labeled phage did not increase the fluorescence intensity because the cells allowed phage adsorption but not phage proliferation, while, in culturable cells, a time-dependent increase in fluorescence intensity due to phage propagation was observed. We therefore proposed that the difference in fluorescence intensity could serve as a marker to distinguish between nonculturable and culturable cells. The study, however, could not distinguish between VBNC and dead cells, since both types of cells allowed phage adsorption. The differentiation between VBNC and dead cells is important for the verification and demonstration of the VBNC state in the environment. The presence of the VBNC state of bacterial cells can be evidenced by detection of their metabolic activity. Specifically, although they do not undergo active cell division, VBNC cells are sufficiently metabolically active to grow to elongated filamentous structures by nutrient uptake during prolonged incubation in nutrient medium. The elongated VBNC cells can then easily be differentiated from dead cells (*14*).

The present study examines the advantages of a new recombinant phage (PP01e−/GFP) for the detection of *E. coli* O157:H7 and further combines the phage system with nutrient uptake analysis to enable the discriminative detection of culturable, VBNC, and dead *E. coli* O157:H7 in a single assay.

Materials and Methods

Bacterial Strains and Bacteriophages. The bacterial strains and phages used in this study are described in Table 1. The *E. coli* O157:H7 ATCC43888 bacterial strain was used to examine survivability in aquatic systems. This strain does not produce either Shiga toxin 1 or 2 (Stx1 and Stx2) because of the absence of genes for these toxins, yet it possesses an envelope structure similar to enterohemorrhagic *E. coli* O157:H7.

Table 1. Phages, *E. coli* Strains, and Plasmids Used in This Study

phage, strain, or plasmid	properties
phage	
PP01wt/GFP	GFP-labeled, virulent, *E. coli* O157:H7-specific (*22*)
PP01e−/GFP	lysozyme-inactivated PP01/GFP (in this study)
T4 e−	lysozyme-inactivated T4 phage (*34*)
E. coli	
O157:H7 (ATCC43888)	for culturability tests
CR63pOmpC	amber suppressor *E. coli* for propagation of PP01e−/GFP
K 12	insensitive to PP01, used as negative control
plasmids	
pUC118	general cloning vector
pT4 g e−	495bp PCR end-modified e− cloned into *Hin*dIII/*Bam*HI site of pUC118

Since PP01e−/GFP cannot cause the lysis of *E. coli* O157: H7 cells, an *E. coli* CR63pOmpC amber suppressor mutant strain, which carries the plasmid for expression of the outer membrane protein C (OmpC), a receptor protein for PP01, was used as a host cell to propagate PP01e−/GFP.

Cell concentrations were estimated by measuring the optical density of the medium at 600 nm (OD_{600}) using a Klett spectrophotometer (Bact-550, Hitachi High-Technologies Corp., Japan). For dilution and preservation of phages, samples were stored in SM buffer (10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin, and 50 mM Tris-HCl [pH 7.5]).

Construction of Lysozyme-Inactivated GFP-Labeled PP01 Phage (PP01e−/GFP). Inactivated lysozyme gene *e* was amplified by PCR from a gene *e* amber mutant T4 phage genome (T4 e−) template (*34*) using the KOD DNA polymerase. The primers used for the amplification were as follows: upper primer, 5′-CG<u>GGATCC</u>ATGAATATATTTGAAATGTTA-3′, and lower primer, 5′-CCC<u>AAGCTT</u>TTATAGATTTTTATA-CGCGTC-3′, where the underlined letters are the restriction sites. The resulting 495bp PCR product was digested with *Hin*dIII/*Bam*HI and reinserted into the *Hin*dIII/*Bam*HI site of pUC118 to produce pT4ge−. Homologous recombination was carried out by transforming *E. coli* O157:H7 with pT4ge− by electroporation. Transformant *E. coli* cells were incubated in Luria−Bertani broth (LB) supplemented with 50 mg mL^{-1} of ampicillin. At an OD_{600} of 0.1, wild-type PP01/GFP (PP01wt/ GFP) phages were added at a multiplicity of infection (MOI) of 0.01. After 80 min of incubation, chloroform was added to lyse the cells, and the culture was centrifuged to remove cell debris. The cell lysate was diluted in SM buffer to obtain the phage concentration of 10^3 PFU mL^{-1}. The diluted phage lysate was mixed with *E. coli* CR63pOmpC in 0.5% agar supplemented with isopropyl-thio-β-D-galactopyranoside (IPTG) and ampicillin and overlaid onto LB plates. The plaques obtained were then transferred one by one to both the LB plates overlaid with *E. coli* O157 and the CR63pOmpC, respectively, for the selection of GFP e− mutants. Finally, plaques that appeared on the CR63pOmpC lawn but not on *E. coli* O157 were isolated and propagated using the plate lysate method for further use.

Detection of *E. coli* O157:H7 Using PP01e−/GFP. *E. coli* O157:H7 culture in the logarithmic growth phase (termed fresh cells) was mixed with an equal volume of phage solution at an MOI of 10 and incubated at 37 °C for 100 min. Throughout the incubation, 400 μL of the mixture was withdrawn every 20 min, followed by centrifugation (10 000g, 3 min, 4 °C), washing with phosphate-buffered saline (PBS, pH 7.0), and resuspension in PBS. To examine the cells, a gel-coated glass slide was used
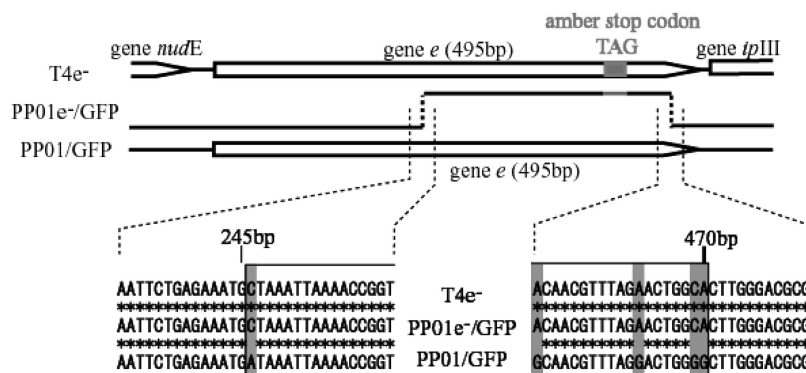
Figure 1. Outline of the homologous recombination process leading to the amber mutation (TAG) upstream of PP01/GFP gene *e*. The highlighted points indicate the recombination sites in the region between 245 and 470 bp of gene *e*.

as described previously (*34*). Luminous *E. coli* O157 cells, due to the adsorbed/infected PP01e⁻/GFP phage, were observed under an epifluorescence microscope (BX60; Olympus Co., Tokyo, Japan) equipped with an appropriate filter (U-MWIBA/ GFP, Olympus). Photographs were acquired using a cool charge-coupled device (CCD) digital camera (DP70, Olympus, Japan), under the light and fluorescent fields. Exposure times for the light field and for the fluorescent field were 1/5 and 2 s, respectively.

Establishment of the VBNC State. The cells in the late exponential phase were harvested, washed, and resuspended in sterilized distilled water (DW) and artificial seawater (SW) (SEALIFE, Marine-Tech, Japan) and held static at 4 °C for an 84-day period (termed stress-induced cells). The aliquots were periodically plated onto LB agar at 28 °C for 48 h to determine culturability. The total number of cells was determined by 4,6-diamidino-2-phenyl indole dihydrochloride (DAPI), a fluorescent dye that attaches to the AT-site in the minor groove of DNA and becomes excited at 325 nm. The cell viability was measured by carboxy-fluorescein diacetate (CFDA), a substrate that, when cleaved by the microbial esterase, produces fluorescein, thus indicating the cell's viability. The method used to stain bacterial cells with DAPI and CFDA has been described previously (*20*). The VBNC cells were defined as the differential value of the CFDA-based viable cell count and the plate count (colony forming units (CFU)).

Discriminative Detection of Culturable, VBNC, and Dead *E. coli* O157. At the end of 21 days incubation under stress (in SW and DW held at 4 °C), when three types of populations, namely, culturable, VBNC, and dead, coexisted within each microcosm, as depicted by the plate count, CFDA and DAPI, the aliquot of stress-induced cell culture was mixed with an equal volume of PP01e⁻/GFP phage solution at MOI 500, 100, and 50. The mixture was incubated in LB at 37 °C, and the cells were examined after 3, 6, 12, 18, and 24 h. Luminous *E. coli* O157:H7 cells, due to the adsorbed or proliferated PP01e⁻/GFP phage, were observed under an epifluorescence microscope, and images were captured as described before. The phage adsorption (low fluorescence) onto the cell surface and the intracellular proliferation/replication (bright green fluorescence) criteria were used to differentiate between the nonculturable and culturable cells, respectively. The relative fluorescent values for cells with adsorbed or replicating phages were determined by image analysis software (Lumina Vision Mitani-Corp., Japan) using the average green value (G-value). Theoretically, the G-value ranges from 0 to 255. To differentiate the bright green fluorescent cells from the low-fluorescent cells, the threshold of the G-value was optimized. In this study, fresh cells with replicated phages were used as the standard for fixing a threshold

value of 30. Cells with a G-value above 30 were considered bright green fluorescent and thus "culturable". The G-value for culturable cells ranged from 30 to 100 with a mean value of about 60. The rest of the cells, with a G-value ranging from 0 to 30, were considered low fluorescent and thus "nonculturable".

In the same set of experiments, the low-fluorescent nonculturable cells were further differentiated into dead and VBNC cells on the basis of nutrient uptake analysis. The low-fluorescent cells, which grew in size by taking up the nutrients during prolonged incubation in LB, were defined as metabolically active and in the VBNC state. The cell images before and after incubation in LB were subjected to image analysis software (Scion Image, Frederick, MD), and the cells that elongated to at least 2 times the average initial size were considered and enumerated as VBNC cells.

Results

Construction and Characterization of the PP01e⁻/GFP Phage. The lysozyme gene *e* of virulent PP01wt/GFP phage was inactivated by amber mutation (TAG) to produce a nonvirulent PP01e⁻/GFP phage. A protocol used to integrate the lysozyme-inactivated gene in the PP01wt/GFP phage genome is outlined in Figure 1. Inactivated lysozyme gene *e* was amplified by PCR from a gene *e* amber mutant T4 phage genome (T4 e⁻) (*34*). The resulting 495bp PCR product was digested with *Hin*dIII/*Bam*HI and then reinserted into the same site of pUC118 to produce pT4ge⁻. Homologous recombination was performed between constructed plasmid pT4ge⁻ and PP01wt/GFP phages. The frequency of recombination was found to be 1.0%. Figure 1 shows the presence of the recombination sites in the region between 245 and 470 bp of gene *e*. The turbidities of the *E. coli* O157:H7 cultures were measured after phage infection (Figure 2). After 1 h of incubation of the fresh culture of *E. coli* O157:H7, when the OD_{600} value reached 0.2, one of the two types of phages, PP01wt/GFP or PP01e⁻/GFP, was added to the cultures at an MOI of 10. Infection with PP01wt/GFP decreased the culture turbidity as a result of cell lysis after 40 min of infection, while the cell turbidity remained constant upon infection with PP01e⁻/GFP (Figure 2).

Detection of *E. coli* O157:H7 Using PP01e⁻/GFP. *E. coli* O157:H7 cells in the logarithmic phase were incubated with PP01e⁻/GFP (MOI = 10) for 100 min at 37 °C. No detectable morphological changes, depictive of cell lysis, could be observed for cells infected with PP01e⁻/GFP under an epifluorescence microscope. A time-dependent increase in the fluorescence intensity of PP01e⁻/GFP-infected *E. coli* O157:H7 cells was observed and reached a plateau at 3 h of incubation. The increase in fluorescence intensity reflected the replication of recombinant
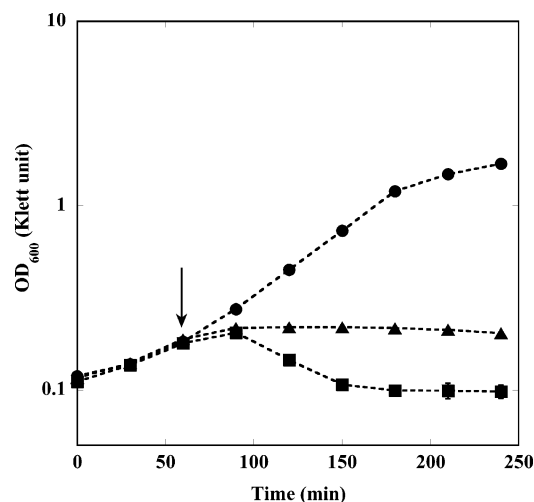
Figure 2. *E. coli* O157:H7 lysis by PP01wt/GFP (■) and PP01e⁻/GFP (▲). The control (●) was assessed in the absence of phage infection. A 50 μL *E. coli* O157:H7 overnight culture was inoculated into 5 mL of LB at time zero and incubated at 37 °C. The arrow indicates the addition of 500 μL of phage solution (MOI = 10) when the OD_{600} of the culture reached 0.2.

progeny phages in cells. Proliferation of PP01e⁻/GFP phages inside the cells and retention of cell morphology enabled the distinct and clear visualization of cells by fluorescence microscopy (Figure 4C,D).

Establishment of the VBNC State. In SW (Figure 3A), the culturable cells, as defined by plate count, reached an undetectable level after 70 days (below 10 CFU mL⁻¹), and thereafter nonculturability (less than 0.1 CFU mL⁻¹) was observed. However, there was no significant decline in the total cell number, as determined by DAPI. Only a 2 log order of magnitude decline in the number of metabolically active cells was observed as defined by CFDA. The resulting difference between the viable count and the plate count shows the loss of culturability and indicates that cells entered into the VBNC state. DW (Figure 3B), in contrast, showed a partial entry to the VBNC state and still retained culturable population around 10⁵ CFU mL⁻¹ until the end. The cells under these conditions were reduced in size and appeared as small rods.

Discriminative Detection of Culturable, VBNC, and Dead *E. coli* O157. After 21 days of incubation under stress, three types of cell populations could be predicted by the data shown in Figure 3, namely, culturable, VBNC, and dead, such that more than 99% was nonculturable, and less than 1% was culturable. To check whether the phage can adsorb to all types of cells, the stress-induced cells at the 21-day incubation stage were incubated with PP01e⁻/GFP. With an MOI of 500, the visualization of the cells under fluorescent microscopy showed the adsorption of phage to 100% of the stressed cells, similar to fresh cells, irrespective of their physiological state (Figure 4A and E). It was confirmed that phage can also adsorb to the VBNC and dead cells.

Stress-induced cells were incubated with the PP01e⁻/GFP phage at MOIs of 50 and 100. Whereas, almost 100% of the fresh cells displayed bright green fluorescence due to phage infection and proliferation (Figure 4C), in stress-induced cells, only 0.08 and 0.9% of the cells exhibited bright green fluorescence in SW and DW, respectively. Such cells were defined as culturable cells (Table 2; Figure 4G(c)), and the percentage is almost in agreement with that of the plate count (Figure 3).

Almost 99% of the cells were detected as low fluorescent and thus nonculturable. Among low-fluorescent cells, 2.8% of the cells in SW and 2.3% of the cells in DW depicted the active metabolism by growing in size and were considered in the VBNC state (Table 2). Since the average initial size of stressed cells before incubation was found to be 2.25 μm, the cells elongated to 4.5 μm and greater after 12 h incubation were considered elongated (Figure 5B,C).

The PP01e⁻/GFP phage, together with nutrient uptake analysis, therefore allowed the discriminative detection of culturable, VBNC, and dead cells in a stress-induced aquatic environment (Figure 4G(c,v)). As the clearest discrimination between the three types of cells was obtained within an 8−12 h incubation period, the results up to 12 h have been reported here.

Discussion

A large number of outbreaks of *E. coli* O157:H7 have been associated with the consumption of contaminated municipal water and well water and contact with recreational waters (*4*). Many culture-based, genetic, or immunological techniques are available for the detection of *E. coli* O157:H7, such as MacConkey agar (*15*), PCR for detecting Shiga toxin genes (*24*), and so on. Although these assays may be useful in the examination of human or animal fecal samples, their usefulness is limited in the examination of environmental samples because of the widespread presence of bacteria sharing common features, such as non-sorbitol-fermenting *E. coli* or nonpathogenic bacteria carrying Shiga toxins, and so on. Moreover, the conversion of enteropathogenic bacteria to the VBNC state also makes it difficult to detect these cells in natural environments
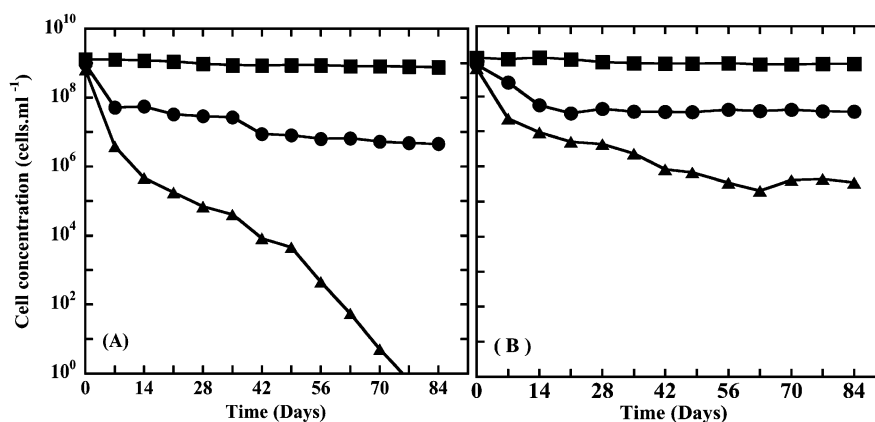

Figure 3. Survival of *E. coli* O157:H7 under various stress-induced conditions. Cells were incubated in sterilized artificial SW (A) and DW (B) at 4 °C for 84 days: (■) total cell count determined by DAPI; (●) viable count determined by CFDA; and (▲) plate count on LB agar.
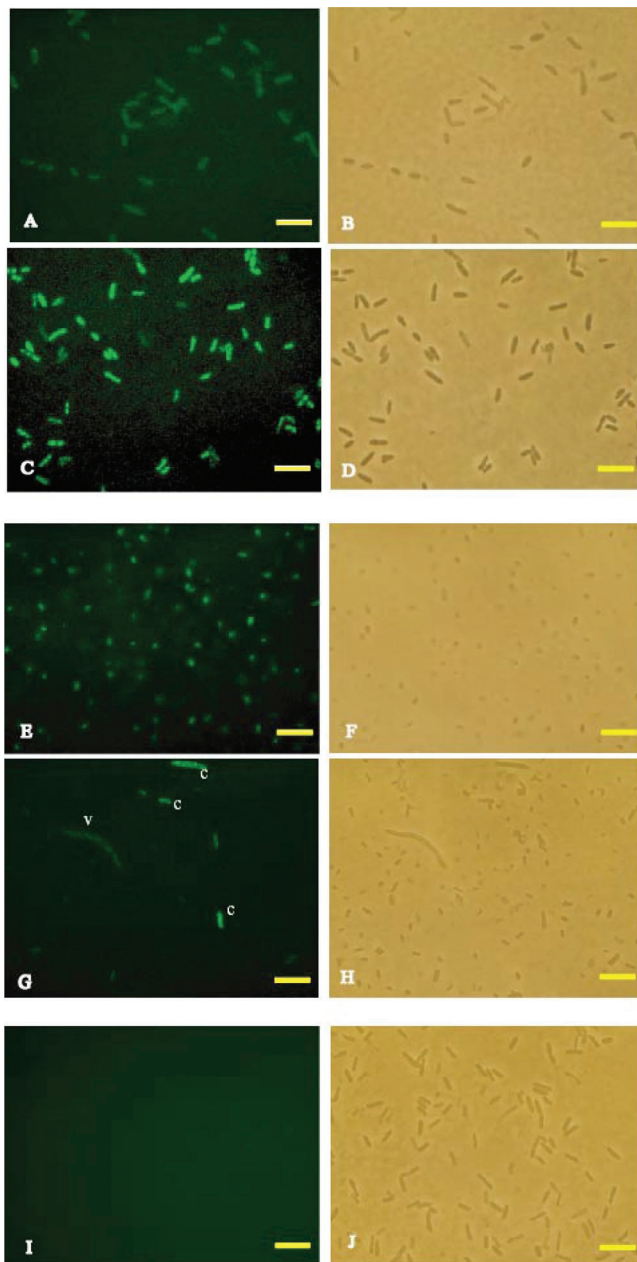
Figure 4. Detection of *E. coli* O157:H7 by the PP01e⁻/GFP phage. The fluorescent microscopic images (left-hand side) and optical images (right-hand side) of *E. coli* O157:H7 cells. Adsorption of the PP01e⁻/GFP phage on fresh cells in the logarithmic phase (A,B). Proliferation of the PP01e⁻/GFP phage in fresh cells (C,D). Adsorption of the PP01e⁻/GFP phage on stress-induced cells stored for 21 days in DW at 4 °C (E,F). Discriminative detection of culturable cells (c) and VBNC cells (v) after 12 h incubation of stressed cells (DW) with PP01e⁻/GFP phage in LB (G,H); the rest of the cells were classified as dead cells. Incubation of *E. coli* K12 with PP01e⁻/GFP (I,J). Scale bar = 10 μm.

such as rivers, lakes, or seawater. There have been swimming-associated outbreaks (*38*) where the pathogen was not recovered from water samples. This failure may be a result of the inability of routine bacteriological techniques to detect VBNCs in the environment. The development of a rapid and specific method, capable of detecting culturable as well as VBNC *E. coli* O157:H7 in the environment, has been desired.

A new recombinant PP01e⁻/GFP phage lacking lysozyme activity was constructed in this study. The lysozyme gene *e* of the previous PP01wt/GFP (*22*) was inactivated by inserting an amber mutation. PP01 phage is specific to *E. coli* O157:H7 (*19*)

Table 2. Comparison of Cell Enumeration Based on Conventional Methods versus the PP01e⁻/GFP-Based Method after a 21-Day Storage under Stress in SW and DW

	mean % (SD)					
	CFDA assay count			PP01e⁻/GFP assay count		
	culturable cells[a]	VBNC cells[b]	total viable[e]	culturable cells[c]	VBNC cells[d]	total viable[e]
SW	0.02(0.0)	3.0(0.1)	3.0	0.08(0.03)	2.8(0.1)	2.9
DW	0.6 (0.1)	2.7(0.3)	3.3	0.9 (0.3)	2.3(0.1)	3.2

[a] Percentage of cells based on the plate count method. [b] Percentage of the differential value of the CFDA-based viable count and the plate count. [c] Percentage of bright green cells. [d] Percentage of elongated cells. [e] Percentage of culturable cells + percentage of VBNC cells.
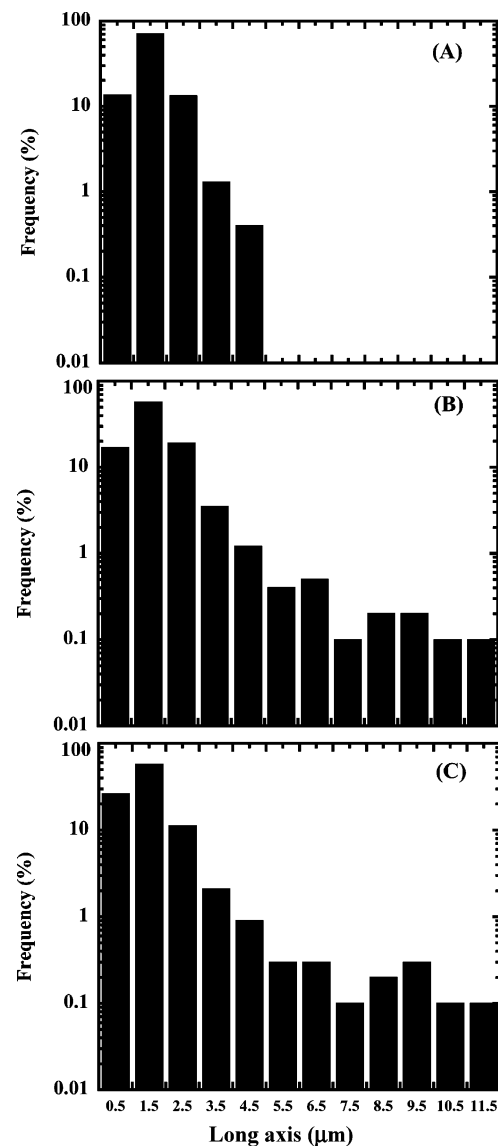

Figure 5. Detection of VBNC cells in SW (B) and DW (C). The stress-induced cells after a 21-day storage in SW and DW at 4 °C were incubated in LB for 12 h in the presence of the PP01e⁻/GFP phage. After 12 h, the images were taken and the image analysis was performed to examine the cell size distribution. The size of the fresh cells without incubation (A) was used as the standard for comparison. The number of cells counted for fresh cells, SW, and DW were 1426, 2007, and 1222, respectively. The cells that grew in size to 4.5 μm or more as a result of nutrient incorporation, following prolonged incubation in LB, were defined as VBNC cells.

and is a member of the T2 family (*22*). The finding that PP01 is a member of the T-even phage family enables us to apply genetic information on T-even phages to the genetic manipula-

tion of PP01. With this background, we used the homologous recombination of an amber mutant of the T4 lysis gene *e* to replace the cognate gene in PP01wt/GFP in the region between 245 and 470 bp of gene *e*, (Figure 1). The inactivation of lysozyme gene *e* deprived the phage of its virulent characteristics without interfering with phage infectivity and the fertility of the host cell.

PP01wt/GFP expresses GFP as a fusion to the small outer capsid (SOC) protein (*22*), the primary structural protein of the phage capsid. The burst size or phage yield of PP01 is 14 within 15−30 min of infection (*7*). One phage particle displays 840 SOC molecules (*22*). The total number of SOC−GFPs produced in a single cell is therefore estimated to be about 1.17×10^4 molecules. When visualized under a microscope, PP01wt/GFP increased the fluorescence intensity of infected *E. coli* O157: H7 cells during the initial stages of infection, but led to host cell disruption in the final stages, making it difficult to identify the phage-infected cells by fluorescent microscopy (data not shown). In contrast, PP01e⁻/GFP infection did not lead to cell lysis, and cell turbidity also remained constant (Figure 2). Following 1 h incubation with PP01e⁻/GFP, the fluorescence intensity of the cells increased gradually. The increase in fluorescence intensity reflected the replication of PP01e⁻/GFP progeny phages and the subsequent increase in the number of SOC−GFP molecules in the cells. Fluorescence of PP01e⁻/GFP progeny phages and retention of cell morphology allowed distinct visualization of *E. coli* O157:H7 cells under an epifluorescence microscope within 1 h (Figure 4C). The cell morphology in optical images was also clearer, more compact, and sharper (Figure 4D). In contrast, *E. coli* K12, which is not sensitive to PP01, was not found to be adsorbed or infected by PP01e⁻/GFP and did not generate detectable fluorescence (Figure 4I). Lysis from without (LO) is peculiar to the T4 phage (*35*). LO is lysis due to adsorption of a large number of T4 phage particles on the cell wall and occurs at MOI > 20. However, the LO of PP01e⁻/GFP was not observed up to an MOI of 500 (Figure 4A,E).

The efficiency of PP01e⁻/GFP phage system to detect *E. coli* O157:H7 in stress-induced conditions was evaluated. The cells were stored in DW and SW at 4 °C for an 84-day period, the conditions shown to induce the VBNC state in *E. coli* O157: H7 (*18*, *27*). However, a 21-day incubation stage was considered best to show the capability of the PP01e⁻/GFP phage system to differentiate between different types of cell populations. At this stage, culturable, VBNC, and dead cells coexisted in detectable proportion. Before 21 days, the VBNC population was undetectable, and, after 21 days, the culturable population was difficult to detect (Figure 3). During the incubation of stressed cells with PP01e⁻/GFP, phage adsorption was found, independent of the physiological state of the cells, and 100% of the stressed cells were adsorbed by the phage (Figure 4E,F). However the phage infection depended on the physiological state of the cells. Less than 1% of the stressed cells allowed the phage propagation and produced bright green fluorescence. Such cells were therefore classified as culturable cells (Figure 4G(c)) and were easily distinguishable from nonculturable cells (VBNC and dead). Nonculturable cells (dead and VBNC) remained low-fluorescent cells due to the inability of the phage to propagate. However, verification of the VBNC state needs further differentiation between VBNC and dead cells.

VBNC cells are not prone to active cell division, yet they possess metabolic activities such that the nutrient uptake during prolonged incubation in a nutrient medium results in cell growth to elongated filamentous structures (*14*). Differentiation between elongated cells and normal cells is crucial. Singh et al. (*31*) found that a 1.5-fold elongation with respect to the length at

the initial time was a suitable measurement to determine the viability of bacteria. In the present study, low-fluorescent cells, which grew in size to 4.5 μm or more (which is at least a 2-fold increase with respect to the average initial size of 2.25 μm) as a result of nutrient incorporation, following prolonged incubation in LB, were therefore defined as VBNC cells (Figure 5B,C). Such elongated cells were then easily recognizable from the dead cells (Figure 4G(v)). The cell size distribution of fresh cells without incubation was used as a reference, which depicted a maximum size up to 4.5 μm. Cells longer than 4.5 μm were not detected for fresh cells (Figure 5A).While, in SW and DW, cells ranging in size from 4.5 to 11.5 μm were detected (Figure 5B,C), which confirmed the presence of VBNC cells. However, almost 98% of the cell population in SW and DW did not show significant increase in size (less than 4.5 μm) and were considered dead. To confirm that the elongated cells were in fact *E. coli* O157:H7 and not the result of autofluorescence of some contaminating bacteria, *E. coli* K12 was incubated with PP01e⁻/GFP. The autofluorescence of *E. coli* K12 was found to be negligible (Figure 4I). Similarly, the autofluorescence of heat-treated *E. coli* O157:H7 cells was also found to be negligible (data not shown).

Appropriate MOI and optimum incubation time were the key parameters for the detection of the maximum number of culturable and VBNC cells in the present assay. Using an MOI of 10 in the present study, 100% of the cells were found to be infected (Figure 4C). An MOI of 100, on the other hand, allowed good contrast between the phage-adsorbed cells (VBNC and dead) and the phage-infected cells (culturable) (Figure 4G). Regarding incubation time, the best results were obtained after 12 h, whereas, during the initial 6 h, no difference in fluorescence or cell size could be detected.

The percentage of elongated VBNC cells was higher in SW relative to that in DW, which indicates that the degree of entrance to the VBNC state was higher in SW (Table 2). On the other hand, the culturable population was found to be higher in DW relative to that in SW. A similar trend of culturability and entry to the VBNC state was observed using the conventional techniques (Figure 3). The cell enumeration using the conventional techniques and that using the new assay were found to be compatible (Table 2). However, the phage-based assay allowed for the direct and independent enumeration of culturable, VBNC, and total cells in a single assay. Using conventional techniques, the VBNC cells are enumerated as a differential value of the viable count and the plate count (*2*, *6*, *30*). The VBNC count is therefore dependent upon the viable count and the plate count, and the rate of recovery of viable and culturable cells in turn affects the VBNC cells' enumeration. The total cell counts, using different nucleic acid stains, such as acridine orange, DAPI, and so on, vary from each other, and the variation in cell count has been attributed to the culture conditions, pH, and the physiological state of the cells (*2*). In the present study, the total cell number was counted precisely and conveniently from optical microscopic fields (Figure 4D,H) without using any stain.

In the phage-based assay, the bright green fluorescence of culturable cells and the size of elongated VBNC cells made them readily detectable from the rest of the cells and enabled the enumeration of even less than 0.08% culturable cells or 2.3% VBNC cells present in the microcosm (Table 2). In the present study, a maximum of 2000 cells was counted for each incubation condition. However, the sensitivity and detection range can further be increased by scanning a larger number of microscopic fields and counting a larger number of cells.

The recombinant phage PP01e⁻/GFP system, when used alone, allows the rapid detection and quantification of culturable

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E. coli O157:H7 within 40−60 min. When combined with nutrient uptake analysis, the PP01e⁻/GFP assay enables the detection and enumeration of both culturable and VBNC cells of *E. coli* O157:H7 within 12 h. The proposed assay may find application in the detection of *E. coli* O157:H7 in aquatic environments (i.e., verification of the VBNC state and helping deepen the understanding of the ecology of the pathogen in this environment). However, in the future, it will be necessary to determine whether appropriate pretreatment of the sample will be required for application of this technique to the quantization of *E. coli* O157:H7 in environments such as sewage samples, rivers, or seawater. Of special concern is the presence of autofluorescing or light-quenching particles in environmental samples, which may hinder the detection and must be removed prior to analysis through stepwise centrifugation and filtration.

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