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Green fluorescent protein-based direct viable count to verify a viable but non-culturable state of *Salmonella typhi* in environmental samples

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Abstract

The *gfp*-tagging method and *lux*-tagging method were compared to select a better method for verifying a viable but nonculturable (VBNC) state of bacteria in the environment. An environmental isolate of *Salmonella typhi* was chromosomally marked with a *gfp* gene encoding green fluorescent protein (GFP). The hybrid transposon mini-Tn5 *gfp* was transconjugated from *E. coli* to *S. typhi*. Using the same method, *S. typhi* was chromosomally marked with *luxAB* genes encoding luciferase. The survival of *gfp*-tagged *S. typhi* introduced into groundwater microcosms was examined by GFP-based plate count, total cell count, and a direct viable count method. In microcosms containing *lux*-tagged *S. typhi*, luminescence-based plate count and the measurement of bioluminescence of each microcosm sample were performed. In microcosms containing *lux*-tagged *S. typhi*, viable but nonculturable cells could not be detected by using luminometry. As no distinguishable luminescence signals from the background signals were found in samples containing no culturable cells, a VBNC state of *S. typhi* could not be verified in *lux*-based systems. However, comparison between GFP-based direct viable counts and plate counts was a good method for verifying the VBNC state of *S. typhi*. Because GFP-based direct viable count method provided a direct and precise estimation of viable cells of introduced bacteria into natural environments, it can be used for verifying the VBNC state of bacteria in environmental samples.

Keywords: *gfp*; GFP-based direct viable count method; *luxAB*; *Salmonella typhi*; Viable but nonculturable state

1. Introduction

The existence of a 'viable but nonculturable' (VBNC) state in Gram-negative bacteria has been demonstrated by the intensive studies on the viability of fecal contamination indicators and pathogenic bacteria (Byrd et al., 1991; Smith et al., 1994; Barcina et al., 1997). It has been reported that some VBNC pathogens retain the capacity to cause diseases (Grimes and Colwell, 1986; Rahman et al., 1994; McDougald et al., 1998). The above reports are important from a public health point of view because VBNC pathogens may be potential etiologic agents. The demonstration of a VBNC state of pathogenic bacteria in the environment, therefore, is important from a public health point of view.

Bacterial survivability in an axenic culture system can be determined by the comparison of the number

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of culturable cells, viable cells and total cells. The VBNC state of bacteria has been elucidated by techniques that assess respiration, enzymatic activity, and cellular activity such as various direct viable count (DVC) methods (Kogure et al., 1979; Roszak and Colwell, 1987; Barcina et al., 1995). These methods, however, can be applied only to populations released in axenic systems, and not in the natural environment where mixed bacterial populations exist since these methods cannot discriminate between introduced cells and other bacteria. Molecular marker systems have been used as an alternative strategy for tracing microorganisms in the environment to overcome the above shortcomings (Bale et al., 1987; Winstanley et al., 1989, Leung et al., 1995). Among the various methods, the prokaryotic *lux* genes encoding luciferase have been used as markers to examine VBNC state as well as the survival rate of allochthonous bacteria in the environment (Flemming et al., 1994; Duncan et al., 1994; Oliver et al., 1995; Ferguson et al., 1995). However, in spite of their proficiency in detecting culturable bacteria in natural environments, techniques using luminometry to measure *in situ* activity of introduced microorganisms have been thought to have limitations in their ability to estimate viability of the cells (Duncan et al., 1994).

Recently, a green fluorescent protein (GFP) from *Aequorea victoria* (Chalfie et al., 1994) has been used as a marker for ecological studies (Burlage et al., 1996; Leff and Leff, 1996, Eberl et al., 1997; Tresse et al., 1998). GFP is a good marker system that can be detected easily by conventional antibiotic resistance, fluorescence colony counting, and direct fluorescence measured with spectrofluorometry or epifluorescence microscopy (Valdivia et al., 1996). The green fluorescent phenotypes are also detectable in all growth phases even under starved conditions (Tombolini et al., 1997). The GFP-tagged method has advantages over *lux*-based systems for directly counting introduced allochthonous bacteria. For measuring total cells in environmental samples including *lux*-tagged cells, it is necessary to use an exposure greater than 30 min with a nitrogen-cooled CCD in a light-tight box (Silcock et al., 1992). GFP detection, however, does not require addition of substrate and long-term exposure with a CCD (Chalfie et al., 1994; Lindow, 1995), and GFP can easily be detected by epifluorescence microscopy using only an optimal fluorescence filter set in even formalin fixed samples (Eberl et al., 1997; Tombolini et al., 1997). Because of these characteristics of a GFP, direct green fluorescent cell counting by epifluorescence microscopy based on DVC method can be applied to counting viable cells in natural environments.

In this study, *Salmonella typhi* was chosen as a model pathogenic bacterium for the elucidation of VBNC state by tagging with *lux* and *gfp* genes. *S. typhi* is an etiologic agent of typhoid fever often detected in sewage, fresh water, estuarine water, and groundwater (Thong et al., 1996). Although the *Salmonella* species *S. enteritidis* (Morinigo et al., 1990), *S. typhimurium* (Joux et al., 1997) and *S. salamae* (Monfort and Baleux, 1994) enter the VBNC state under conditions of starvation, entry into the VBNC state of *S. typhi*, to our knowledge, has not yet been reported.

The purposes of this study are to show the superiority of *gfp*-tagging method over *lux*-tagging method for the estimation of VBNC state and to verify the VBNC state of *S. typhi*. In this study, an environmental isolate of *S. typhi* was chromosomally marked with a *gfp* gene only. In addition, the same strain was marked with a *luxAB* gene only. The survival rates of *luxAB* or *gfp*-tagged *S. typhi* were tested in groundwater microcosms by using conventional culture techniques, luminometry, and a developed GFP-based direct viable count method.

2. Materials and methods

2.1. Generation of S. typhi LUX38-75 and S. typhi GFP155

S. typhi 38-7 was isolated from the NakDong River in Korea (Kim et al., 1997). Rifampicin-resistant *S. typhi* 38-7 was generated by spontaneous mutation from the wild type. For the generation of *lux*-tagged *S. typhi* 38-7, *E. coli* S17-1 (λpir) was electroporated with pUT*luxAB* (mini-Tn5 harboring plasmid derived from *E. coli* CC118 (λpir) pUT*luxAB;* a gift from Timmis (de Lorenzo et al., 1990), and selected on Luria-Bertani (LB) agar supplemented with 15 μg ml^{-1} tetracycline. *S. typhi* 38-7 *luxAB* (Rif^R Tc^R) was obtained by mating *E. coli* S17-1 (λpir) pUT*luxAB* (Tc^R) with *S. typhi* 38-7

(Rif^R) using the filter conjugation method (Herrero et al., 1990). Exconjugants, chromosomally marked with *luxAB* by having the hybrid transposon mini-Tn5 *luxAB* inserted, were obtained by spreading the cell suspension on LB medium supplemented with 50 μg ml^{-1} and 15 μg ml^{-1} of rifampicin and tetracycline, respectively. A clone showing the highest relative light unit (RLU), *S. typhi* LUX38-75, was chosen among the exconjugants and used throughout the study.

For the generation of *gfp*-tagged *S. typhi* 38-7, *E. coli* S17-1 (λpir) was electroporated with pUT*gfp* (a gift from Eberl (Eberl et al., 1997), and selected on LB agar plate supplemented with 50 μg ml^{-1} kanamycin. *S. typhi* 38-7 *gfp* (Rif^R Km^R) was obtained by mating *E. coli* S17-1 (λpir) pUT*gfp* (Km^R) with *S. typhi* 38-7 (Rif^R). Exconjugants chromosomally marked with green fluorescent protein cassette, transposon mini-Tn5 *gfp*, were selected on LB medium supplemented with 50 μg ml^{-1} each of rifampicin and kanamycin. A strongly green fluorescence-emitting clone, *S. typhi* GFP155, was selected from among 6000 colonies of exconjugants by illuminating plates on a UV transilluminator.

To determine whether *S. typhi* LUX38-75 and *S. typhi* GFP155 maintained antibiotic resistance under starvation conditions, starved cells maintained in 0.22 μm filtered groundwater for 20 days were spread in triplicate onto LB agar without addition of antibiotics and LB agar supplemented with rifampicin and tetracycline (in case of LUX38-75) and rifampicin and kanamycin (in case of GFP155). After incubation of agar plates for 48 h at 30°C, the colonies were enumerated, and compared.

2.2. Groundwater microcosms

To prepare the groundwater microcosms, groundwater samples were collected from a deep aquifer (depth: 65 m) in the WonJu agricultural area, Korea, using a suction-lift pump. Five hundred milliliter aliquots of either 0.22 μm filter-sterilized, autoclaved, or untreated whole groundwater samples, were dispensed into 1 l Erlenmeyer flasks in triplicate. Microcosms without addition of *S. typhi* LUX38-75 and *S. typhi* GFP155 were used as controls. Overnight cultures of *S. typhi* LUX38-75 and *S. typhi* GFP155 grown in LB broth at 30°C were harvested by centrifugation at 12 000 × *g* for 15 min and resuspended. One milliliter portions of cell suspensions were inoculated into microcosms to achieve initial densities of approximately 7×10^6 cells ml^{-1} of *S. typhi* LUX38-75 and 8×10^6 cells ml^{-1} of *S. typhi* GFP155. All microcosms were incubated in the dark at 15°C and shaken on an orbital shaker at 50 rpm. Samples were taken on days 0, 1, 3, 6, 9, 12, 16, 20, and 27.

2.3. Culturable cells, total cells, and bioluminescence in the microcosms containing S. typhi LUX38-75

The culturability of *S. typhi* LUX38-75 in the microcosms was determined in triplicate by spread plating on LB agar supplemented with 50 μg ml^{-1} of rifampicin and 15 μg ml^{-1} of tetracycline. Plates were incubated for 48 h at 30°C. After incubation, 100 μl of *n*-decyl aldehyde were added onto the plate lid followed by incubation for 30 min at 30°C. The plates were observed directly by using an Eagle Eye™ II digital imaging system (Stratagene, La Jolla, CA, USA) in the dark. The luminescent colonies were taken to represent *S. typhi* LUX38-75. Total cells only in autoclaved samples were measured by acridine orange direct count method (AODC, Watson et al., 1977). Luminescence was measured in 250 μl samples from each microcosm using an Microlumat LB96P luminometer (EG&G Berthold, Wildbad, Germany). As the *S. typhi* LUX38-75 lacks the genes for synthesis of the luciferase substrate, luminescence was measured after incubation for 20 min at room temperature in the presence of 0.2% of *n*-decyl aldehyde. Luminescence measurements were carried out in triplicate for each sample, and were expressed as RLU.

2.4. Culturable cells, total cells, and viable cells in the microcosms containing S. typhi GFP155

The culturability of cells in a microcosm was determined in triplicate by spread plating on LB agar supplemented with 50 μg ml^{-1} each of rifampicin and kanamycin. Plates were incubated for 48 h at 30°C, and illuminated at 360 nm on a UV transilluminator. Green fluorescent colonies were enumerated and taken to represent *S. typhi* GFP155. For microscopic enumeration, unstained and formaldehyde-fixed microcosm samples were filtered through

a Anodisc 0.2 μm-pore size filter (Whatman International Ltd., Maidstone, England) and mounted with type FF immersion oil. Green fluorescent cells were enumerated with a Zeiss epifluorescent microscope and a Zeiss No.9 filter set (excitation: 450–490 nm, emission 525 nm) at a magnification of $\times 1600$. Cell viability was determined by the GFP-based DVC method. Yeast extract (0.025% final concentration) and nalidixic acid (0.002% final concentration) were added to the sample. After incubation for 8 h, bacteria were recovered by filtration on 0.2 μm-pore size Anodisc filters. The filters were examined by epifluorescence microscopy as described above. The cells were considered viable when they had obtained a length at least 2.5 times the average length of control cells. Control cells were cells from the same water sample that had not been subjected to the DVC procedure. Direct fluorescence of subsamples was measured by the SLM Aminco 48 000™S Spectrofluorometer (SLM instruments, Urbana, IL, USA). The fluorescence intensities were measured at 395 nm excitation wavelength and 509 nm emission wavelength.

2.5. Calculation of cell decline rates and statistical analysis.

Slopes for total, viable, and culturable cells with each treatment were calculated [$C_t = C_0 \times \exp(\lambda[t - t_0])$] where λ is decline rate of cell count, C_t is cell concentration at time t, C_0 is cell concentration at time 0) by linear regression analysis (EXCEL, ver 7.0) at the 5% level of significance over time. The slopes, which measured the average decline in each of the response variable, were analyzed by one-way analysis of variance (SAS system, release ver 6.08) at the 5% level of significance.

3. Results

3.1. Generation of chromosomally lux or gfp-tagged S. typhi

Both the pUT*luxAB* and pUT*gfp* vectors were used for incorporation of the Tn5-*luxAB* and Tn5-*gfp* cassettes into the chromosome of *S. typhi* 38-7. In the experiment for the incorporation of Tn5-*luxAB* cassette, about 300 *luxAB*-tagged exconjugants were obtained by the mini-Tn5-based filter conjugation method. When substrate for luciferase, *n*-decyl aldehyde, was added onto the plate lid, luminescence was observed in 126 exconjugants by the naked eye in a dark room. To select the brightest clone among the luminescent *S. typhi* clones, their maximum luminescence was measured by using luminometer and the strongest luminescent strain, *S.typhi* LUX38-75 was selected. In the experiment for the incorporation of the Tn5-*gfp* cassette, the *gfp*-tagged clones could be easily differentiated from other clones by their bright green fluorescence. The strongest fluorescent clone, *S. typhi* GFP155 was selected from several GFP-expressing mutants by the naked eye and measuring direct fluorescence of cells using spectrofluorometry.

The colony shape and size of LUX38-75 and GFP155 did not differ significantly from the wild type and the μ_{max}, as evaluated from the slope of exponential phase of growth, was nearly the same as that of the wild type (data not shown). The concentrations of *S. typhi* LUX38-75 and GFP155 starved in 0.22 μm filtered groundwater for 20 days were determined to be 5.36×10^2 CFU ml^{-1} and 7.76×10^2 CFU ml^{-1} on LB agar supplemented with appropriate antibiotics and 5.51×10^2 CFU ml^{-1} and 7.85×10^2 CFU ml^{-1} on LB agar without addition of antibiotics, respectively. As there were no significant differences between the plate counts on LB without the addition of antibiotics and those on LB supplemented with antibiotics (all, $p > 0.4$, ANOVA), it was demonstrated that *S. typhi* LUX38-75 and GFP155 maintained antibiotic resistance even under starvation conditions.

3.2. Survival of lux-marked S. typhi LUX38-75 in groundwater

Microcosm experiments were performed to evaluate the survival rates of *S. typhi* LUX38-75 in groundwater. Measurements of culturable cells by luminescence-based plate counting and the estimation of direct luminescence in each sample were performed. There were no differences between the CFUs counted under visible light and the luminescent colonies counted in the dark after the addition of *n*-decyl aldehyde. Therefore, CFUs on LB plates amended with rifampicin and tetracycline could be

regarded as *S. typhi* LUX38-75. However, in the survival experiments, only luminescent bacteria in dark condition were counted as *S. typhi* LUX38-75 in all microcosms in order to minimize false positive *S. typhi* LUX38-75.

The survival curves and the decline rates of *S. typhi* LUX38-75 in groundwater microcosms are represented in Fig. 1 and Table 1. The slope of log plate counts in untreated whole groundwater samples (-0.73 d^{-1}) was significantly ($p<0.01$, ANOVA) steeper than in 0.22 μm filtered groundwater (-0.40 d^{-1}) and autoclaved groundwater samples (-0.35 d^{-1}). Acridine orange direct counts in the autoclaved samples remained nearly constant throughout the 20-day period, and slightly declined after 20-day period. The slope of log total counts in autoclaved samples (-0.07 d^{-1}) was significantly ($p<0.001$, ANOVA) gentler than those of culturable cells in all microcosms. No luminescent cells were detected in the control samples (microcosms without *S. typhi* LUX38-75).

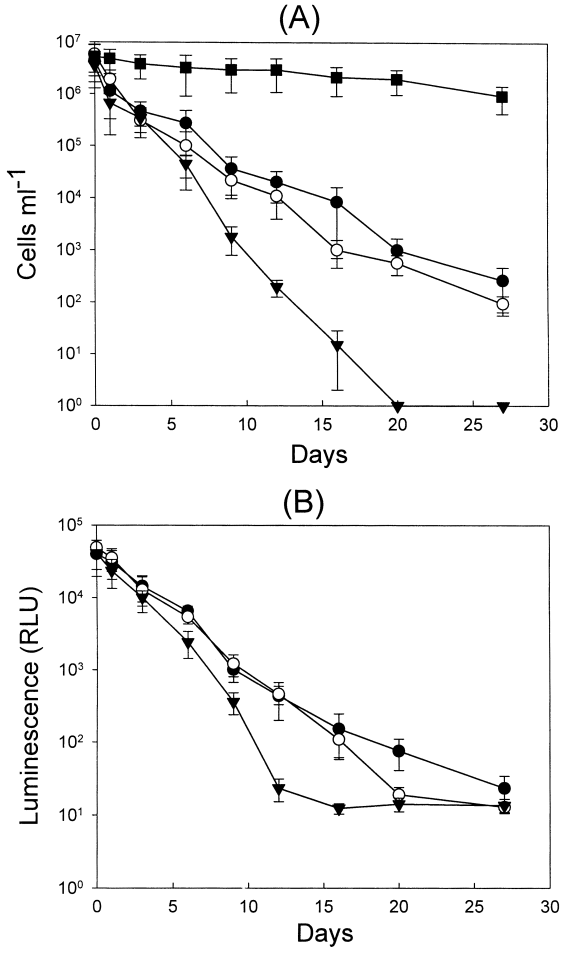

Fig. 1. Changes in the total cells and luminescence-based plate counts (A) and the bioluminescence (B) of *S. typhi* LUX38-75 in groundwater microcosms. Symbols, ■; total cells by the acridine orange direct count in autoclaved samples, ●; luminescence-based plate counts and bioluminescence in autoclaved samples, ○; luminescence-based plate counts and bioluminescence in 0.2 μm filtered samples, ▼; luminescence-based plate counts and bioluminescence in untreated whole groundwater samples. Error bar represents standard deviation.

When luminescence produced by metabolically active *S. typhi* LUX38-75 was measured in microcosms, RLU decreased similar to the pattern observed with the plate counts until RLU fell to background signals (Fig. 1). Bioluminescence signals were nearly at background levels when the number of culturable cells were approximately below two orders of magnitude. No detectable luminescence above background signals was found in samples containing no culturable cells. When the plate counts and RLU were plotted on XY planes, RLU showed a correlation ($r^2=0.92$, $p<0.01$) with luminescence-based plate counts (Fig. 2). From these results, the VBNC state of *S. typhi* could not be verified by the *lux*-based systems.

3.3. Survival of gfp-tagged S. typhi in groundwater microcosms.

Culturable *S. typhi* GFP155 declined significantly ($p<0.05$, ANOVA) more rapidly than viable cells and total cells in all samples of groundwater microcosms (Fig. 3, Table 1). The slope of log plate counts in untreated whole groundwater samples (-0.75 d^{-1}) is steeper than in 0.22 μm filtered groundwater (-0.44 d^{-1}) and autoclaved groundwater samples (-0.39 d^{-1}).

Viable cells could be easily detected by examining the morphology of green cells based on the DVC method. Because cell elongation occurs only if cells are viable, cells were regarded as viable if they were at least 2.5 times the length of those not treated with nalidixic acid. For one example, the average length of cells not treated with nalidixic acid in 0.2 μm

Table 1
The decline slopes of introduced *S. typhi* GFP155 and *S. typhi* LUX38-75 in groundwater microcosms

Microcosms with *S. typhi* GFP155			Microcosms with *S. typhi* LUX38-75		
Treatment	Cell counts	Decline slope (λ)[a]	Treatment	Cell counts	Decline slope (λ)
Autoclaved	GFP-TC[b]	−0.10	Autoclaved	AODC-TC[e]	−0.07
	GFP-DVC[c]	−0.13		LUX-HPC[f]	−0.35
	GFP-HPC[d]	−0.39			
0.22 μm filtered	GFP-TC	−0.17	0.22 μm filtered	LUX-HPC	−0.40
	GFP-DVC	−0.21			
	GFP-HPC	−0.44			
Untreated	GFP-TC	−0.25	Untreated	LUX-HPC	−0.73
	GFP-DVC	−0.28			
	GFP-HPC	−0.75			

[a] Decline slopes were calculated using the following equation($[C_t = C_0 \times exp(\lambda[t - t_0])]$where λ is the decline rate of cell count, C_t is the cell concentration at time t, C_0 is the cell concentration at time 0) by linear regression analysis at the 5% level of significance over time.
[b] GFP-TC, GFP-based total microscopic counts.
[c] GFP-DVC, GFP-based direct viable counts.
[d] GFP-HPC, GFP-based heterotrophic plate counts.
[e] AODC-TC, total microscopic counts by acridine orange direct count method.
[f] LUX-HPC, luminescence-based heterotrophic plate counts.

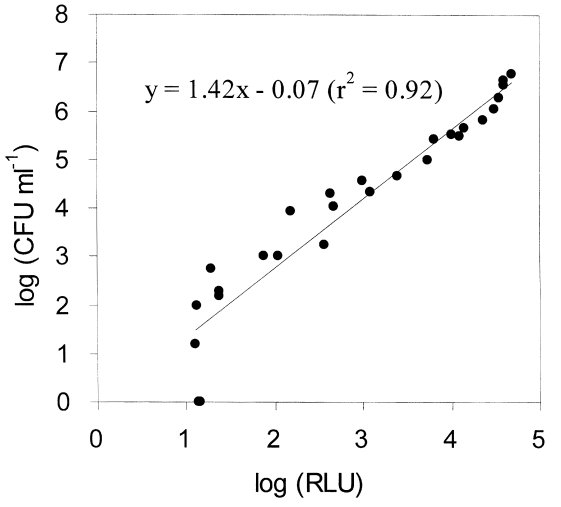

Fig. 2. The linear regression analysis between luminescence-based plate counts and bioluminescence production of *S. typhi* LUX38-75 in groundwater microcosms. All experimental data of plate counts and relative light units(RLU) were plotted. The equation from the regression result is shown in the figure.

filtered groundwater (at 0 day sample) was 2.76 μm, while that of elongated viable cells treated with nalidixic acid in the same sample was 9.83 μm. Viable cells declined more rapidly than total microscopic counts. The entry into a VBNC state of *S. typhi* during starvation in groundwater was demonstrated by the differences between direct viable counts and plate counts. In the whole groundwater microcosm, the introduced cells were no longer detectable by heterotrophic plating after 20 days, while the cells in the 0.22 μm filtered and autoclaved microcosms were detectable throughout the experiment. The entrance of some cells into the VBNC state had occurred from the start of the experiment. The highest number of VBNC cells in the whole groundwater microcosms throughout the study was 2.5×10^4 cells ml^{-1} on 20 days. Fluorescence intensity measured by spectrofluorometry showed a good correlation with total microscopic counts (Fig. 4). Correlation coefficients between fluorescence intensity and total cells in autoclaved, 0.22 μm filtered, and untreated whole groundwater samples were 0.94 ($p < 0.01$), 0.95 ($p < 0.01$), and 0.99 ($p < 0.01$), respectively.

4. Discussion

In this study, it was shown that GFP can be a more stable and useful marker than luciferase. It allows for easy, rapid and inexpensive detection of a VBNC state of *S. typhi* among groundwater microflora. For the detection of culturable cells, use of both *gfp* and *luxAB* reporter genes allowed rapid and

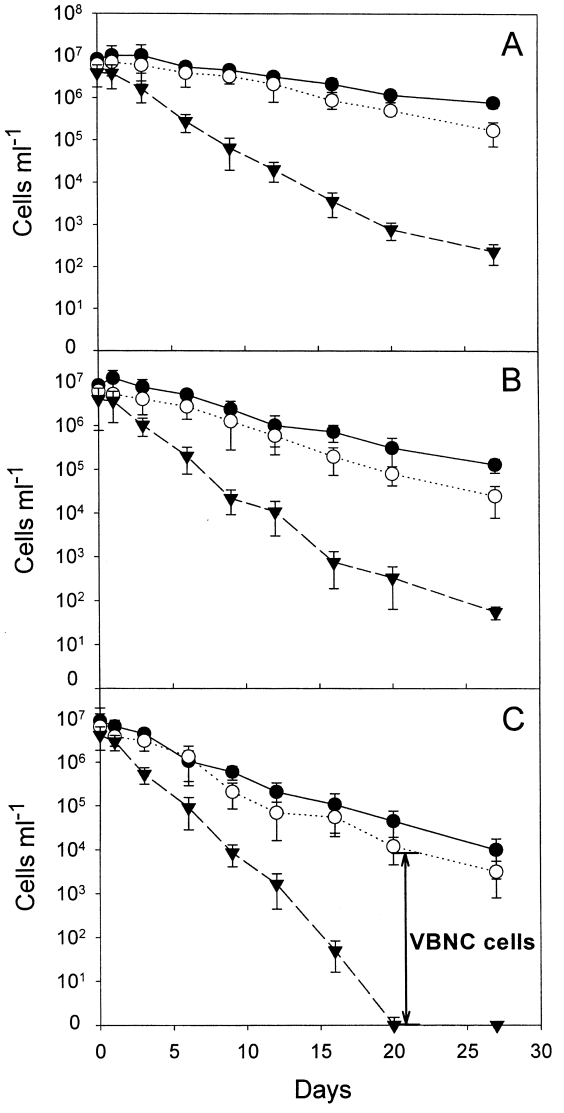

Fig. 3. Survival of *S. typhi* GFP155 in the groundwater microcosms. Culturable cells (▼) were measured on LB agar supplemented with rifampicin and kanamycin, and confirmed by their green fluorescence. Total cells (●) were measured by epifluorescence microscopy with a Zeiss No.9 filter set. Viable cells (○) were measured by GFP-based DVC method. Panels; A, autoclaved groundwater; B, groundwater filtered with 0.22 μm-pore size membrane; C, untreated whole groundwater. Error bar represents standard deviation.

precise detection of introduced *S. typhi* LUX38-75 and *S. typhi* GFP155 into the groundwater by selective plating. The incubation with an additional substrate (*n*-decyl aldehyde) and dark box were

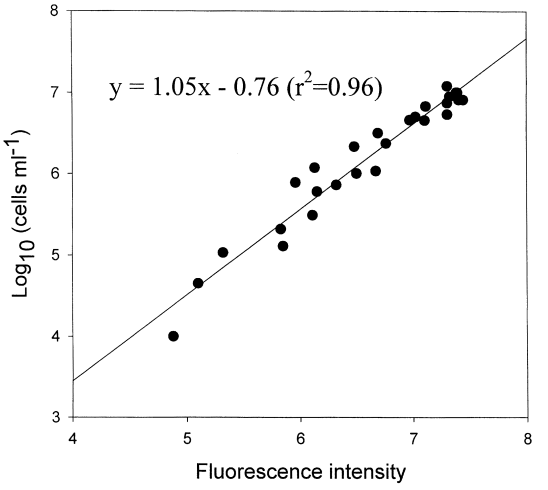

Fig. 4. The linear regression analysis between fluorescence intensities and GFP-based total counts of *S. typhi* GFP155 in groundwater microcosms. All experimental data of fluorescence intensities and GFP-based total counts were plotted. The equation from the regression result is shown in the figure.

necessary to estimate culturable cells in *lux*-based system, however, only a UV transilluminator or a hand-held UV lamp was necessary in *gfp*-based system. Accordingly, the *gfp*-based method was simpler and more economical than the *lux*-based one to detect culturable fractions of introduced *S. typhi*.

Viable cells in microcosms containing *S. typhi* GFP155 could easily be detected by direct green fluorescent cell counting method using epifluorescence microscopy based on DVC method. Elongated cells, which were at least 2.5 times the normal length, were counted as viable cells to prevent confusion between dead and viable cells. The numbers of green fluorescent viable elongated cells were counted and easily differentiated from the nonviable cells. The cells in a VBNC state could be easily estimated by comparing the number of culturable cells and direct viable counts. When nonculturable cells were formed in microcosms containing *S. typhi* LUX38-75, culturable cell concentrations decreased in parallel with luminescence, suggesting that a decrease in metabolic activity may lead to nonculturable cell formation. However, existence of VBNC cells was not observed by using luminometry. There was no detectable luminescence above background signals in samples containing zero culturable cells.

The reason for this may be that *in situ* detection of *lux*-encoded luciferase activity is dependent on energy reserves within cells, but energy reserves of bacteria in the environment may be too low to allow *in situ* detection of high energy requiring enzyme systems (Meikle et al., 1994). The results that no luminescence signals were found in samples containing VBNC cells indicate the limitations of *lux*-tagging methods for verifying the VBNC state of *S. typhi*.

Developed GFP-based DVC method provided information regarding the proportion of responsive cells to substrate within a population. However, measuring the bioluminescence activity by luminometry in *lux*-based systems provides information on the bulk activity of a population, rather than the proportion of viable cells within a population (Duncan et al., 1994). For these reasons, the metabolic activity of *lux*-tagged cells cannot represent DVCs. Our study used the chromosomally GFP-tagged *S. typhi* based on the mini-Tn5 system of Herrero *et al*. (1990). In this system, the Tn5 does not carry its own transposition functions; its features prevent secondary transposition in Tn5 *gfp* tagged cells. Leff and Leff (1996) have used a plasmid-borne green fluorescent protein gene as a marker for monitoring survival of genetically engineered microorganisms (GEMs). However, as during starvation, plasmid loss has been reported (Caldwell et al., 1989; Lowcock and Edwards, 1994). Thus the experiments of Leff and Leff (1996) may not precisely reveal the survival of introduced GEMs. By using GFP-based DVC method, which overcame the above shortcomings, the VBNC state of *S. typhi* in the groundwater could be confirmed.

The survival of *S. typhi* in sterile and untreated whole groundwater was investigated using laboratory flask microcosms. The decline rates between culturable *S. typhi* GFP155 and *S. typhi* LUX38-75 and μ_{max} between wild type and *lux* or *gfp*-tagged mutant were not significantly different. This indicated that *lux* and *gfp*-tagging did not affect the physiology of the wild type. By examining the differences between the number of plate counts and direct viable counts, it was shown that some cells had entered the VBNC state from the start of the experiment. In the whole groundwater microcosms at the end of experiment (day 27), all the introduced cells were undetectable by heterotrophic plate counting and that some cells remained in the VBNC state.

We were able to visualize individual *S. typhi* GFP155 cells and measure the cell length by epifluorescence microscopy due to their green fluorescence. As no green fluorescent cells in control microcosms (without *S. typhi* GFP155) were observed, and capability of gene transfer did not exist because of the chromosomally tagged GFP gene, all green fluorescent cells were taken to represent introduced *S. typhi* GFP155. Direct measurement of fluorescence intensity by spectrofluorometry in formalin-fixed samples showed excellent correlation with GFP-based total counts. Therefore, direct measurement of fluorescence intensity by spectrofluorometry could be applied for counting *gfp*-tagged total cells, and could reduce labor and time.

In conclusion, *gfp*-tagged *S. typhi* could be monitored more easily and precisely than *lux*-tagged *S. typhi*. Particularly, GFP-based DVC method described here can provide a direct and precise estimation of viable cells of introduced bacteria into natural environments. It is expected that the VBNC state of numerous bacteria in the environment can be verified by using *gfp*-tagging and GFP-based DVC method.

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References

Bale, M.J., Fry, J.C., Day, M.J., 1987. Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones. J. Gen. Microbiol. 133, 3099–3107.

Barcina, I., Arana, I., Santorum, P., Iriberri, J., Egea, L., 1995. Direct viable count of Gram-positive and Gram-negative bacteria using ciprofloxacin as inhibitor of cellular division. J. Microbiol. Methods 22, 139–150.

Barcina, I., Lebaron, P., Vives-Rego, J., 1997. Survival of allochthonous bacteria in aquatic systems: a biological approach. FEMS Microbiol. Ecol. 23, 1–9.

Burlage, R.S., Yang, Z.K., Mehlhorn, T., 1996. A transposon for green fluorescent protein transcriptional fusions: application for bacterial transport experiments. Gene 173, 53–58.

Byrd, J.J., Xu, H.S., Colwell, R.R., 1991. Viable but nonculturable

bacteria in drinking water. Appl. Environ. Microbiol. 57, 875–878.

Caldwell, B.A., Ye, C., Griffiths, R.P., Moyer, C.L., Morita, R.Y., 1989. Plasmid expression and maintenance during long-term starvation-survival of bacteria in well water. Appl. Environ. Microbiol. 55, 1860–1864.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. Science 263, 802–805.

de Lorenzo, V., Herrero, M., Jakubzik, U., Timmis, K.N., 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. 172, 6568–6572.

Duncan, S., Glover, L.A., Killham, K., Prosser, J.I., 1994. Luminescence-based detection of activity of starved and viable but nonculturable bacteria. Appl. Environ. Microbiol. 60, 1308–1316.

Eberl, L., Schulze, R., Ammendola, A., Geisenberger, O., Erhart, R., Stenberg, C., Molin, S., Amann, R., 1997. Use of green fluorescent protein as a marker for ecological studies of activated sludge communities. FEMS Microbiol. Lett. 149, 77–83.

Ferguson, Y., Glover, L.A., McGillivray, D.M., Prosser, J.I., 1995. Survival and activity of *lux*-marked *Aeromonas salmonicida* in seawater. Appl. Environ. Microbiol. 61, 3494–3498.

Flemming, C.A., Lee, H., Trevors, J.T., 1994. Bioluminescent most-probable-number method to enumerate *lux*-marked *Pseudomonas aeruginosa* UG2Lr in soil. Appl. Environ. Microbiol. 60, 3458–3461.

Grimes, D.J., Colwell, R.R., 1986. Viability and virulence of *Escherichiacoli* suspended by membrane chamber in semi-tropical ocean water. FEMS Microbiol. Lett. 34, 161–165.

Herrero, M., de Lorenzo, V., Timmis, K.N., 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. 172, 6557–6567.

Joux, F., Lebaron, P., Troussellier, M., 1997. Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. FEMS Microbiol. Ecol. 22, 65–76.

Kim, S.J., Park, J.W., Lee, D.H., Jahng, D.J., Lee, K.H., Kim, M.W., 1997. In: Development of rapid detection methods of hazardous materials in water, Korean Ministry of Environment, Seoul, pp. 74–91.

Kogure, K., Simidu, U., Taga, N., 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25, 415–420.

Leff, L.G., Leff, A.A., 1996. Use of green fluorescent protein to monitor survival of genetically engineered bacteria in aquatic environments. Appl. Environ. Microbiol. 62, 3486–3488.

Leung, K., Trevors, J.T., Lee, H., 1995. Survival of and *lacZ* expression in recombinant *Pseudomonas* strains introduced into river water microcosms. Can. J. Microbiol. 41, 461–469.

Lindow, S.E., 1995. The use of reporter genes in the study of microbial ecology. Mol. Ecol. 4, 555–566.

Lowcock, D., Edwards, C., 1994. Survival of genetically-marked *Aeromonas hydrophila* in water. Lett. Appl. Microbiol. 19, 121–123.

McDougald, D., Rice, S.A., Weichart, D., Kjelleberg, S., 1998. Nonculturability: adaptation or debilitation? FEMS Microbiol. Ecol. 25, 1–9.

Meikle, A., Glover, L.A., Killham, K., Prosser, J.I., 1994. Potential luminescence as an indicator of activation of genetically-modified *Pseudomonas fluorescens* in liquid culture and in soil. Soil Biol. Biochem. 26, 747–755.

Monfort, P., Baleux, B., 1994. Effects of environmental factors present in the St Lawrence Estuary (Quebec, Canada) on experimental survival of *Salmonella salamae* as determined by flow cytometry. Can. J. Microbiol. 40, 712–719.

Morinigo, M.A., Cornax, R., Castro, D., Martinez-Manzanares, E., Borrego, J.J., 1990. Viability of *Salmonella* spp. and indicator microorganisms in seawater using membrane diffusion chambers. Antonie Van Leeuwenhoek 57, 109–117.

Oliver, J.D., McDougald, D., Barrett, T., Glover, L.A., Prosser, J.I., 1995. Effect of temperature and plasmid carriage on nonculturability in organisms targeted for release. FEMS Microbiol. Ecol. 17, 229–238.

Rahman, I., Shahamat, M., Kirchman, P.A., Russek-Cohen, E., Colwell, R.R., 1994. Methionine uptake and cytopathogenicity of viable but nonculturable *Shigella dysenteriae* 1. Appl. Environ. Microbiol. 60, 3573–3578.

Roszak, D.B., Colwell, R.R., 1987. Metabolic activity of bacterial cells enumerated by direct viable count. Appl. Environ. Microbiol. 53, 2889–2893.

Silcock, D.J., Waterhouse, R.N., Glover L.A Prosser, J.I., Killham, K., 1992. Detection of a single genetically modified bacterial cell in soil by using charge coupled device-enhanced microscopy. Appl. Environ. Microbiol. 58, 2444–2448.

Smith, J.J., Howington, J.P., McFeters, G.A., 1994. Survival, physiological response, and recovery of enteric bacteria exposed to a polar marine environment. Appl. Environ. Microbiol. 60, 2977–2984.

Thong, K.L., Cordano, A.M., Yassin, R.M., Pang, T., 1996. Molecular analysis of environmental and human isolates of *Salmonella typhi*. Appl. Environ. Microbiol. 62, 271–274.

Tombolini, R., Unge, A., Davey, M.E., de Bruijn, F.J., Jansson, J.K., 1997. Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. FEMS Microbiol. Ecol. 22, 17–28.

Tresse, O., Errampalli, D., Kostrzynska, M., Leung, K.T., Lee, H., Trevors, J.T., van Elsas, J.D., 1998. Green fluorescent protein as a visual marker in a *p*-nitrophenol degrading *Moraxella* sp. FEMS Microbiol. Lett. 164, 187–193.

Valdivia, R.H., Hromockyj, A.E., Monack, D., Ramakrishnan, L., Falkow, S., 1996. Applications for green fluorescent protein(GFP) in the study of host-pathogen interactions. Gene 173, 47–52.

Watson, S.W., Novitsky, T.J., Quinby, H.L., Valois, F.W., 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. 33, 940–946.

Winstanley, C., Morgan, J.A.W., Pickup, R.W., Jones, J.G., Saunders, J.R., 1989. Differential regulation of lambda pL and pR promoters by a cI repressor in a broad-host-range thermoregulated plasmid marker system. Appl. Environ. Microbiol. 55, 770–777.