ELSEVIER

FEMS Microbiology Letters 170 (1999) 257–264

FEMS MICROBIOLOGY LETTERS

Viable, but non-culturable, state of a green fluorescence protein-tagged environmental isolate of *Salmonella typhi* in groundwater and pond water

Jang-Cheon Cho, Sang-Jong Kim *

Department of Microbiology, College of Natural Sciences, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, South Korea

Received 30 June 1998; received in revised form 1 October 1998; accepted 15 October 1998

Abstract

An environmental isolate of *Salmonella typhi* was chromosomally marked with a *gfp* gene encoding green fluorescence protein (GFP) isolated from *Aequorea victoria*. The hybrid transposon mini-Tn5 *gfp* was transconjugated from *E. coli* to *S. typhi*, resulting in constitutive GFP production. The survival of *S. typhi* GFP155 introduced into groundwater and pond water microcosms was examined by GFP-based plate counts, total cell counts, and direct viable counts. A comparison between GFP-based direct viable counts and plate counts was a good method for verifying the viable, but non-culturable (VBNC), state of *S. typhi*. The entry into a VBNC state of *S. typhi* was shown in all microcosms. *S. typhi* survived longer in groundwater than in pond water as both a culturable and a VBNC state.

Keywords: *Salmonella typhi*; Green fluorescence protein-based direct viable count method; *gfp*; Viable but non-culturable state; Groundwater

1. Introduction

Salmonella typhi is an etiologic agent of typhoid fever often detected in sewage, fresh water, estuarine water, and groundwater [1]. *Salmonella* spp. may enter a viable, but non-culturable, state (VBNC) after lengthy exposure to oligotrophic fresh and seawater under ambient temperature [2]. The VBNC state has now been shown for at least 30 bacterial species [3]. zz

Although the *Salmonella* species *Salmonella enteritidis* [4], *Salmonella typhimurium* [5], and *Salmonella salamae* [6] enter the VBNC state under conditions of starvation, entry into the VBNC state of *S. typhi*, to our knowledge, has not yet been reported. It has been reported that species, such as S*almonella typhimurium*, *Shigella dysenteriae*, enteropathogenic *Escherichia coli*, *Vibrio cholerae*, *Vibrio vulnificus*, and *Legionella pneumophila*, which were initially VBNC, retain the capacity to cause disease and are therefore still active [7]. The above reports suggest that the VBNC cells of *Salmonella typhi* in environment may be the potential etiologic agents. The demon-

* Corresponding author. Tel.: +82 (2) 880-6704; Fax: +82 (2) 889-9474; E-mail: sjkimm@plaza.snu.ac.kr

0378-1097/99/$19.00
PII: S0378-1097(98)00554-0

stration of a VBNC state of *S. typhi* in environment, therefore, is important for the public health.

The VBNC state has been elucidated by techniques that assess cellular activity, such as various direct viable count (DVC) methods [8–10]. These methods, however, can be applied only in axenic culture, and not in natural environments where mixed bacterial populations exist. Recently, 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 [11,12]. However, in spite of their proficiency in detecting culturable bacteria in natural environments, techniques using luminometry to measure in situ activity and potential activity of introduced microorganisms are limited in their ability to confirm the VBNC state [11]. The reason for this is 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 [13].

We used a green fluorescence protein (GFP) from *Aequorea victoria* [14,15] as a marker to overcome the above shortcomings, and to verify the VBNC state of *S. typhi* isolated from the environment. GFP is a very good marker system that can be easily detected by the conventional antibiotic resistance, fluorescence colony counting, and measuring direct fluorescence with spectrofluorometry or epifluorescence microscopy [16,17]. In addition, the green fluorescent phenotypes are detectable in all growth phases even under starved conditions [18].

The purpose of this study was to verify the VBNC state of *S. typhi* and to estimate the survival rate of *S. typhi* in groundwater. To confirm the VBNC state of *S. typhi* in natural groundwater, the survival rate of a GFP-tagged *S. typhi* was tested in laboratory and in in situ groundwater microcosms by using conventional culture techniques and a developed GFP-based DVC method.

2. Materials and methods

2.1. Bacterial strains and culture

S. typhi 38-7 was isolated from the NakDong River in Korea. This strain was identified from fatty acid profiles by the Microbial Identification system (MIDI, Delaware, USA) and confirmed by PCR and Southern hybridization [19]. Rifampicin-resistant *S. typhi* 38-7 was generated by spontaneous mutation from the wild-type. *E. coli* S17-1 (λ pir) was electroporated with pUT*gfp* (derived from *E. coli* CC118 (λ pir) pUT*gfp*; pUT*gfp* was a gift from Eberl [15]), and selected on Luria–Bertani (LB) agar (10 g peptone, 5 g yeast extract, 10 g sodium chloride, 15 g agar per liter) supplemented with 50 μg ml^{-1} kanamycin. *S. typhi* 38-7 *gfp* (Rif^R Km^R) was obtained by mating *E. coli* S17-1 (λ pir) pUT*gfp* (Km^R) with *S. typhi* 38-7 (Rif^R) [20]. Exconjugants chromosomally marked with green fluorescence protein cassette, transposon mini-Tn5 *gfp*, were selected on LB medium supplemented with 50 μg ml^{-1} of rifampicin and kanamycin. A stably strong green fluorescence-emitting clone, *S. typhi* GFP155, was selected from among 6000 colonies of exconjugants by illuminating plates on a UV transilluminator. All cells were cultured at 30°C.

2.2. Groundwater and pond water microcosms

To prepare the laboratory groundwater microcosms, groundwater samples were collected from a deep aquifer (depth, 65 m) contaminated with livestock wastewater in the WonJu agricultural area, Korea, using a suction-lift pump. The concentration of total organic carbon was 5.5 mg l^{-1}, and the water temperature was 14.5°C. 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. In addition, pond water samples from JaHaYeon, an eutrophic pond located in Seoul National University, were collected for the purpose of studying the effect of predation on bacteria. In the pond samples, the concentration of total organic carbon was 15.5 mg l^{-1}, and the water temperature was 16.7°C. Five-hundred-milliliter aliquots of either 0.22-μm filter-sterilized, 3.0-μm-filtered, or untreated whole pond water, were dispensed into 1-l Erlenmeyer flasks in triplicate. Microcosms without addition of *S. typhi* GFP 155 were used as controls.

Heterotrophic nanoflagellates (HNF) were counted at the start of the experiment. Untreated

whole groundwater and untreated and 3.0-μm-filtered pond water samples fixed with 2% formalin were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) filtered through 0.4-μm black Nuclepore polycarbonate filters [21]. HNFs were enumerated by epifluorescence microscopy at a magnification of ×1000, and each individual was checked for the presence of autofluorescence.

An overnight culture of *S. typhi* GFP155 grown in LB broth amended with 50 μg ml^{-1} of rifampicin and kanamycin at 30°C was harvested by centrifugation at 12 000×*g* for 15 min and resuspended to a cell density of approximately 4×10^9 cells ml^{-1} (adjustments were made by direct epifluorescence microscopy). One-milliliter portions of cell suspensions were inoculated into microcosms to achieve an initial density of approximately 8×10^6 cells ml^{-1}. All samples were incubated in the dark at 15°C and shaken with orbital shaker at 50 rpm. The microcosms were sampled on days 0, 1, 3, 6, 9, 12, 16, 20, and 27.

For the in situ groundwater microcosms, groundwater samples from the surface layer and 25-m depth layer were collected in order to compare the survival rates of *S. typhi* GFP155 from surface layer and bottom layer microcosms. Five-hundred-milliliter aliquots of either 0.22-μm-filtered, 3.0-μm-filtered, or untreated whole groundwater samples, were dispensed into dialysis bags (SPECTRA/POR 2 membranes, Spectrum Medical Industries, LA) which were sterilized by UV irradiation for 2 h. The dialysis bags were used for the free penetration of groundwater and dissolved nutrient while maintaining an isolated microbial community. After introducing an overnight culture of *S. typhi* GFP155 into the dialysis bags (approximately final concentrations of 1.5×10^7 cells ml^{-1}), the dialysis bags were sealed and fixed to a stainless-steel frame. The bags were immersed in boreholes at the surface layer and at depth of 25 m. The dialysis bags were retrieved, sampled, and resealed on days 0, 1, 4, 7, 11, 14, 18, 22 and 29.

2.3. Culturable cells, total cells and viable cells

To determine whether *S. typhi* GFP155 maintained antibiotic resistance under starvation conditions, the 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 50 μg ml^{-1} of rifampicin and kanamycin. After incubation of agar plates for 48 h at 30°C, the colonies were enumerated.

The culturability of cells in a microcosm was determined in triplicate by spread plating on LB agar supplemented with 50 μg ml^{-1} of rifampicin and

Table 1
The decline slopes of introduced *S. typhi* GFP155 in laboratory groundwater and pond water microcosms

Laboratory groundwater microcosms				Laboratory pond water microcosms			
Treatment	Cell counts	Decline slope (λ)[a]	r^2 (n)[b]	Treatment	Cell counts	Decline slope (λ)	r^2 (n)
Autoclaved	TC[c]	−0.10	0.98 (9)	0.22-μm-filtered	TC	−0.11	0.93 (9)
	DVC[d]	−0.13	0.97 (9)		DVC	−0.14	0.97 (9)
	HPC[e]	−0.39	0.98 (9)		HPC	−0.33	0.98 (9)
0.22-μm-filtered	TC	−0.17	0.97 (9)	3.0-μm-filtered	TC	−0.26	0.98 (9)
	DVC	−0.21	0.99 (9)		DVC	−0.32	0.97 (9)
	HPC	−0.44	0.97 (9)		HPC	−0.96	0.99 (7)
Untreated	TC	−0.25	0.98 (9)	Untreated	TC	−0.35	0.98 (9)
	DVC	−0.28	0.97 (9)		DVC	−0.38	0.96 (9)
	HPC	−0.75	0.99 (8)		HPC	−1.30	0.99 (6)

[a]Decline slopes were calculated [$C_t = C_o \cdot \exp(\lambda\,[t-t_o])$, where λ is decline rate of cell count, C_t is cell concentration at time t, C_o is cell concentration at time 0, by linear regression analysis at the 5% level of significance over time.
[b]r^2 represents correlation coefficients and n represents the number of samples.
[c]TC represents GFP-based total microscopic counts.
[d]DVC represents GFP-based direct viable counts.
[e]HPC represents GFP-based heterotrophic plate counts.

kanamycin. Plates were incubated for 48 h at 30°C, and illuminated at 360 nm on a UV transilluminator. Green fluorescence-emitting 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, Maidstone, UK) 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 ×1600. Cell viability was determined by the GFP-based DVC method [9]. 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.

2.4. Calculation of cell decline rate and statistical analysis

Slopes for total, viable, and culturable cells with each treatment were calculated [$C_t = C_o \cdot \exp(\lambda\,[t - t_o])$, where λ is decline rate of cell count, C_t is cell concentration at time t, C_o 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. Survival of GFP-tagged S. typhi in laboratory groundwater and pond water microcosms

Culturable *S. typhi* GFP155 declined significantly ($P < 0.05$, ANOVA) more rapidly than viable cells and total cells in all samples of laboratory groundwater microcosms (Fig. 1, Table 1). The slope of log plate counts in untreated whole groundwater samples (-0.75 day^{-1}) is steeper than in 0.22-μm-filtered groundwater (-0.44 day^{-1}) and autoclaved groundwater samples (-0.39 day^{-1}). No green fluorescent

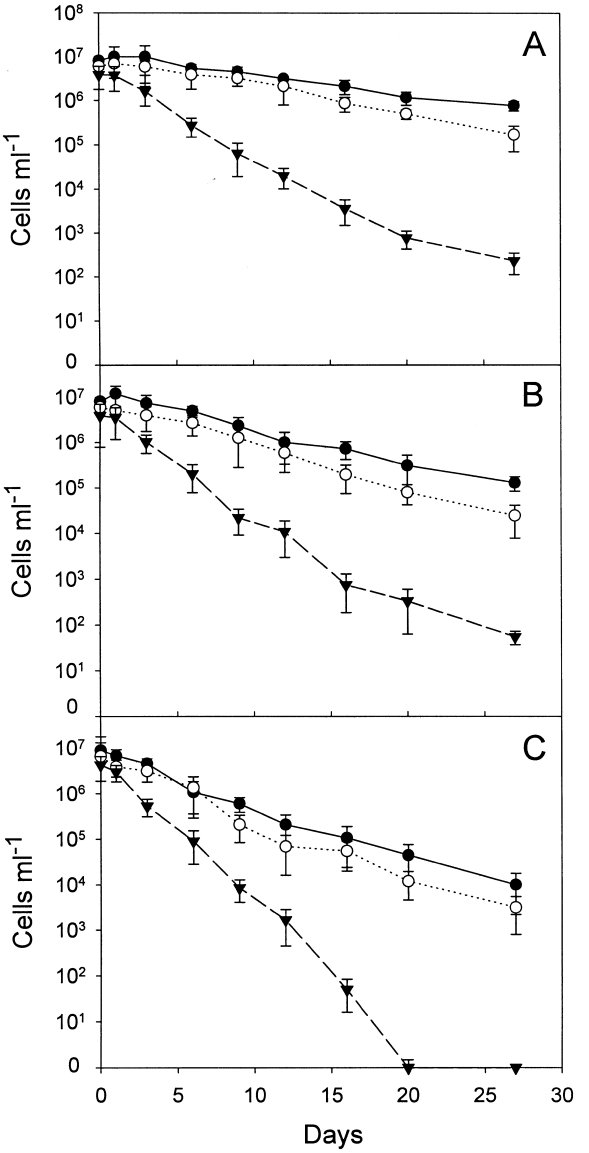

Fig. 1. Survival of *S. typhi* GFP155 in the laboratory groundwater microcosms. Culturable cells (▼) were measured on LB agar supplemented with rifampicin and kanamycin, and confirmed by their green fluorescence. Total cells (●) were measured by epifluorescence microscopy with a Zeiss no. 9 filter set. Viable cells (○) were measured by GFP-based DVC method. Each test was performed in triplicate in each microcosm. Error bar represents standard deviation ($n = 9$). (A) Autoclaved groundwater. (B) Groundwater filtered with 0.22-μm-pore-size membrane. (C) Untreated whole groundwater.

Table 2
The decline slopes of introduced *S. typhi* GFP155 in in situ groundwater microcosms

Treatment	Cell counts	In situ surface groundwater microcosm		In situ a depth of 25-m groundwater microcosms	
		Decline slope (λ)[a]	r^2 (n)[b]	Decline slope (λ)	r^2 (n)
0.22-μm-filtered	TC[c]	−0.13	0.93 (9)	−0.14	0.96 (9)
	DVC[d]	−0.21	0.95 (9)	−0.24	0.98 (9)
	HPC[e]	−0.48	0.98 (9)	−0.53	0.98 (9)
3.0-μm-filtered	TC	−0.16	0.97 (9)	−0.16	0.99 (9)
	DVC	−0.21	0.97 (9)	−0.23	0.98 (9)
	HPC	−0.62	0.98 (9)	−0.74	0.99 (8)
Untreated	TC	−0.24	0.97 (9)	−0.25	0.98 (9)
	DVC	−0.29	0.93 (9)	−0.31	0.97 (9)
	HPC	−0.90	0.99 (7)	−0.91	0.97 (7)

[a]Decline slopes were calculated [$C_t = C_o \cdot \exp(\lambda\ [t - t_o])$], where λ is decline rate of cell counts, C_t is cell concentration at time t, C_o is cell concentration at time 0, by linear regression analysis at the 5% level of significance over time.
[b]r^2 represents correlation coefficients and n represents the number of samples.
[c]TC represents GFP-based total microscopic counts.
[d]DVC represents GFP-based direct viable counts.
[e]HPC represents GFP-based heterotrophic plate counts.

cells were detected in the control samples (without addition of GFP155). The concentration of *S. typhi* GFP155 starved in 0.22-μm-filtered groundwater for 20 days was determined to be 7.76×10^2 CFU ml^{-1} when grown on LB agar supplemented with rifampicin and kanamycin and 7.85×10^2 CFU ml^{-1} on LB agar without addition of antibiotics. As no significant differences between plate counts on LB without addition of antibiotics and those on LB supplemented with rifampicin and kanamycin were observed ($P > 0.4$, ANOVA), it was demonstrated that *S. typhi* GFP155 maintained antibiotic resistance even under starvation conditions.

Viable cells, which could be easily detected by examining the swollen morphology of green cells based on the DVC method, 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 experiment. The number of cells in the VBNC state was highest when the direct viable counts and heterotrophic plate counts were significantly different. 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.

The decline slopes of *S. typhi* GFP155 in the pond water microcosms are represented in Table 1. These experimental sets were designed to evaluate the effects of biotic factors (mainly predation) and to compare survival rates of *S. typhi* in groundwater and pond water. The number of HNFs in untreated whole pond water and 3.0-μm-filtered pond water was 4.5×10^4 individuals ml^{-1} and 6.9×10^3 individuals ml^{-1}, respectively. HNFs, however, were not detected in 100-ml samples of untreated whole groundwater. Plate counts decreased rapidly in whole pond water (−1.30 day^{-1}) and 3.0-μm-filtered pond water (−0.96 day^{-1}), but rather slowly in 0.22-μm-filtered pond water (−0.33 day^{-1}). The direct viable counts and total microscopic counts also decreased rapidly in whole pond water (−0.38 and −0.35 day^{-1}, respectively), but more slowly in 0.22 μm (−0.14 and −0.11 day^{-1}, respectively) and 3.0-μm-filtered pond water (−0.32 and −0.26 day^{-1}, respectively). The slopes of plate counts, direct viable counts, and total microscopic counts in whole pond water microcosms were steeper than in whole groundwater microcosms ($P < 0.05$, ANOVA). In the whole pond water microcosm, the introduced

cells were no longer detectable by heterotrophic plating after 12 days, while the cells in the 3.0-μm-filtered pond water were not detectable after 16 days. The highest number of VBNC cells in the whole pond water microcosms and 3.0-μm-filtered pond water microcosms was 5.4×10^4 cells ml^{-1} on 12 days, 3.8×10^4 cells ml^{-1} on 16 days, respectively.

3.2. Survival of GFP-tagged S. typhi in in situ groundwater microcosms

The decline slopes of *S. typhi* GFP155 at the surface and at a depth of 25 m layer are represented in Table 2. The patterns of survival curves at the surface were very similar to those at a depth of 25 m. The decline slopes of plate counts, direct viable counts, and total microscopic counts at a depth of 25 m were steeper than those in surface groundwater, respectively, but the differences were statistically insignificant (all $P>0.5$, ANOVA). As in laboratory microcosms, plate counts declined more rapidly than viable cells and total cells in all treated samples of in situ groundwater microcosms ($P<0.05$, ANOVA). In all microcosm sets, the VBNC state of introduced cells was also observed.

4. Discussion

Use of the GFP gene allowed rapid and precise detection of introduced *S. typhi* GFP155 into the groundwater by plating and microscopic analysis. As no green fluorescent cells in control microcosms (without addition of *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 regarded as introduced *S. typhi* GFP155.

Viable cells in microcosms could be easily detected by direct green fluorescent cell counting methods using epifluorescence microscopy based on DVC method. The numbers of green fluorescent viable elongated cells were counted and easily differentiated from the same non-viable cells. This method provided information regarding the proportion of viable cells within a population. By marking a bacterium with the bioluminescence phenotype, it is now possible to detect viable recombinant bacteria and determine their viability in the environment [22,23]. However, the metabolic activity of *lux*-marked cells cannot directly represent DVCs. By using GFP-based DVC method that overcame the above shortcomings, we were able to confirm the VBNC state of *S. typhi* in the groundwater and the pond water based on the differences between viable cells and culturable cells.

Our study used the chromosomally GFP-marked *S. typhi* based on the mini-Tn5 system of Herrero et al. [20]. 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 [24] have used a plasmid-borne green fluorescence protein gene as a marker for monitoring survival of genetically engineered microorganisms (GEMs). However, as during starvation, plasmid loss has been reported [25,26]. Thus the experiments of Leff and Leff [24] may not precisely reveal the survival of introduced GEMs. As the Tn5-based system does not rely upon plasmids, it is a good system to monitor pathogenic bacteria and GEMs introduced into the natural environments.

The survival of *S. typhi* in sterile and untreated whole groundwater and pond water was investigated using laboratory flask microcosms and dialysis membrane chambers. 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 experiment. In the whole groundwater microcosms at the end of experiment (day 27), all the introduced cells were undetectable by heterotrophic plate counting and a minority of introduced cells remained in the VBNC state. This indicates that a non-culturable population of *S. typhi* that can survive longer than culturable cells in groundwater and pond water was present and could be a potential etiologic agent.

The decline slopes of culturable cells (-1.30 day^{-1}) in whole pond water were steeper than those in whole groundwater (-0.75 day^{-1}). Because the decline slopes of culturable cells in 0.22-μm-filtered groundwater (-0.44 day^{-1}) and 0.22-μm-filtered pond water (-0.33 day^{-1}) showed no significant differences ($P>0.3$, ARDRA), the differences in decline slopes between whole groundwater and whole pond water were due to the significant differences of protozoan abundances between two microcosms.

Grazing by protozoa has been regarded as one of the main factors that remove allochthonous bacteria from natural environments [27,28]. Therefore, *S. typhi* can survive relatively longer in groundwater, where grazing pressure is very low, than in stream, lake, and pond water.

In conclusion, the GFP-based DVC method described here can provide a direct and precise estimation of viable cells of introduced bacteria into natural environments. By using these methods, the VBNC state of *S. typhi* was demonstrated for the first time. The significance of a VBNC population of *S. typhi* in the spread of typhoid fever in natural environments needs further investigation.

Acknowledgments

This work was supported by the G-7 Projects Grant (9-2-4) from the Ministry of Environment of the Republic of Korea. We thank the National Institute of Environmental Research of the Republic of Korea for leading the projects. We would like to thank Dr. Leo Eberl (Germany) for the gifts of *gfp* vectors and strains.

References

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

[2] Roszak, D.B. and Colwell, R.R. (1987) Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51, 365–379.

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

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

[5] Jimenez, L., Muniz, I., Toranzos, G.A. and Hazen T.C. (1989) Survival and activity of *Salmonella typhimurium* and *Escherichia coli* in tropical freshwater. J. Appl. Bacteriol. 67, 61–69.

[6] Monfort, P. and 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.

[7] McDougald, D., Rice, S.A., Weichart, D. and Kjelleberg, S. (1998) Nonculturability: adaptation or deblitation? FEMS Microbiol. Ecol. 25, 1–9.

[8] Barcina, I., Arana, I., Santorum, P., Iriberri, J. and 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.

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

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

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

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

[13] Meikle, A., Glover, L.A., Killham, K. and 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.

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

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

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

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

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

[19] Kim, S.J., Park, J.W., Lee, D.H., Jahng, D.J., Lee, K.H. and Kim, M.W. (1997) Development of rapid detection methods of hazardous materials in water, pp. 74–91. Korean Ministry of Environment, Seoul.

[20] Herrero, M., de Lorenzo, V. and 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.

[21] Porter, K.G. and Feig, Y.S. (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25, 943–948.

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

[23] Rattray, E.A.S., Prosser, J.I., Killham, K. and Glover, L.A. (1990) Luminescence based non-extractive techniques for in situ detection of *Escherichia coli* in soil. Appl. Environ. Microbiol. 56, 3368–3374.

[24] Leff, L.G. and 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.

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

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

[27] Christoffersen, K., Ahl, T. and Nybroe, O. (1995) Grazing of nonindigenous bacteria by nano-sized protozoa in a natural coastal system. Microb. Ecol. 30, 67–78.

[28] Iriberri, J., Azua, I., Labirua-Iturburu, A., Artolozaga, I. and Barcina, I. (1994) Differential elimination of enteric bacteria by protist in a fresh water system. J. Appl. Bacteriol. 77, 476–483.