Journal of Environmental Biotechnology
Vol. 12, No. 1, 33–38, 2012

Original paper (regular paper)

Expression of the *gyrB* gene as an indicator of growth activity of *Escherichia coli*

Katsuji Tani[2]*, Takeshi Kobayashi[1], Akiko Sakotani[2], Takehiko Kenzaka[2] and Masao Nasu[1]

[1] *Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan*

[2] *Environmental Science and Microbiology, Faculty of Pharmacy, Osaka Ohtani University, 3–11–1 Nishikiori-kita, Tondabayashi, Osaka 584–8540, Japan*

* Tel & Fax: +81–721–9742

* E-mail: tanika@osaka-ohtani.ac.jp

(Received; 16 November, 2011/Accepted; 7 May, 2012)

To determine whether the DNA gyrase gene (*gyrB*) can be used as an indicator of bacterial growth activity, its expression levels were estimated in *Escherichia coli* under different growth conditions. *E. coli* was cultured in liquid medium at various temperatures, and its nucleic acid was extracted and purified. The copy numbers of *gyrB* mRNA in the log, log-stationary, and stationary phase cultures in Luria-Bertani broth at 37°C were 4.7, 0.015, and $<10^{-6}$ (below the detection limit), respectively. When the generation times of *E. coli* were 0.42, 1.5, 5.3, and 9.9 h, the respective ratios of *gyrB* mRNA to DNA were 4.7, 1.5, 0.15, and 0.09. *gyrB* mRNA was expressed at high levels in growing *E. coli* cells. Nucleic acids were extracted from surface river water and river sediment samples. The ratio of *gyrB* mRNA to DNA for the *E. coli-Shigella* group was 1.3 in the river sediment sample and 0.008 in the surface river water sample. These results suggest that *gyrB* mRNA expression can be used as an indicator of bacterial growth activity and that the river sediment is a potential natural reservoir for the *E. coli-Shigella* group.

Key words: *gyrB* gene expression, growth activity, *Escherichia coli*, river water

1. Introduction

Physiologically active bacteria are only a part of the diverse bacterial populations present in natural environments[1,3,16,27]. Determining the bacterial growth activity in natural environments is important for understanding bacterial habitats, survival, and growth conditions. Respiration, enzymatic activity, and incorporation of specific substances have been used as indicators of bacterial physiology or growth activity[12,19,22,25,29]. The rate of incorporation of thymidine, leucine, or their analogs is widely used for the measurement of bacterial growth rate in environments[7,8]. However, these methods have some limitations when determining the growth activity of a specific bacterial species in a natural environment, because samples need to be incubated, thus causing changes in bacterial activity. *gyrB* is a housekeeping gene that encodes the B subunit of bacterial DNA gyrase, which plays an essential role in DNA replication. Regulation of *gyrB* transcription is associated with the cell cycle in each cell[18], and hence, *gyrB* mRNA expression may be used as an indicator of bacterial growth. To investigate the relationship between bacterial growth and *gyrB* mRNA expression, we quantified the copy number of *gyrB* mRNA in *Escherichia coli* at each growth phase using real-time reverse transcription (RT)-PCR. Furthermore, we evaluated the utility of measuring *gyrB* gene expression by comparing it with the conventional direct viable count (DVC) method[14].

Consumption of water contaminated by human or animal feces is an important cause of intestinal and systemic illnesses. Estimates of the total coliform count are routinely used to monitor fecal contamination in water, but the US Environmental Protection Agency has stated that *E. coli* counts provide a better indicator of water quality[28]. Furthermore, a previous study showed that amount of this bacterium can be used as a major indicator of fecal contamination in the river environment[2], while other reports have suggested that *E. coli* can grow in soil[6,24]. However, studies have shown that *E. coli* is non-culturable under adverse conditions, such as low nutrient availability and/or low temperature[20,21]. The present study investigated *gyrB* expression in *E. coli* to better understand the habitat of this bacterium in natural river environments.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Experiments were performed using *E. coli* K-12 W3110, *Shigella sonnei* IID 969, *Salmonella enteritidis* IID 604, *Salmonella enterica* serovar Choleraesuis IID1682, and *Vibrio vulnificus* ATCC 27562. *E. coli* was cultured in Luria-

Bertani (LB) broth (0.5% NaCl, 1% tryptone, 0.5% yeast extract) at 37°C or M9 medium (0.5% glucose, 100 μM $CaCl_2$, 200 mM $MgSO_4$, 1.36% Na_2HPO_4, 0.6% KH_2PO_4, 0.1% NaCl, and 0.2% NH_4Cl) at 20, 22, or 25°C. *Shi. sonnei*, *Sal. enteritidis*, and *Sal. enterica* serovar Choleraesuis were cultured in LB broth at 37°C. *V. vulnificus* was cultured in LB broth (3% NaCl) at 37°C.

2.2. Simultaneous recovery of RNA and DNA

RNA and DNA were recovered from *E. coli* cultures using the Qiagen RNA/DNA System Midi Kit (Qiagen. K. K., Japan) according to the manufacturer's instructions. RNA and DNA were simultaneously recovered from the surface river water and river sediment samples using the method described by Hurt *et al.*[10] with modified concentrations of sodium dodecyl sulfate and cetyltrimethylammonium bromide in the extraction buffer[23]. The extracted DNA and RNA were dissolved in 100 μL of TE buffer (pH 8.0) and 80 μL of Tris-HCl (50 mM, pH 7.5), respectively. Twenty units of DNase I (Roche Diagnostics, Indianapolis, IN), 10 μL of 60 mM $MgCl_2$, and 10 μL of 20 mM $CaCl_2$ were added to the RNA sample, and the mixture was incubated for 30 min at 37°C. After incubation, RNA was precipitated with isopropanol and resuspended in 100 μL of TE buffer (pH 7.5). Recovered RNA and DNA samples were stored at –80°C and –20°C, respectively.

2.3. Preparation of standard DNA and RNA

E. coli was cultured in LB broth at 37°C. Stationary phase cultures were sampled and nucleic acids were extracted and purified as described by Tsai and Olson[26]. In brief, nucleic acids were incubated with 100 μg mL^{-1} ribonuclease A (Sigma-Aldrich Co., St. Louis, MO) for 30 min at 37°C to digest RNA, then purified using Tris-EDTA-buffered phenol-chloroform-isoamyl alcohol (25 : 24 : 1, pH 8.0) and ethanol precipitation as described by Iwamoto *et al.*[11], and dissolved in TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. Extracted DNA was analyzed by electrophoresis (100 V, 30 min) on ethidium bromide-stained agarose gels (1%, wt/vol) to confirm the purity of chromosomal DNA, i.e., without any fragmentation or contamination with RNA. DNA concentration was determined by PicoGreen (Molecular Probes Inc., Eugene, OR) DNA staining, and a standard curve was constructed using a series of lambda phage DNA (Takara Bio Inc., Japan) solutions (serial dilutions, 10–1,000 ng mL^{-1}) in TE buffer. The lambda phage DNA and bacterial DNA samples were diluted 100- or 1,000-fold with TE buffer and stained with 1/400 volume of PicoGreen in a 96-well microplate. Fluorescence intensity (emission at 530 nm) was measured using a fluorescence multi-well microplate reader (CytoFluor II; PreSpetive Biosystems Inc., Framingham, MA) at an excitation wavelength of 485 nm. The copy number of genomic DNA was calculated using the measured DNA concentration and the molecular weight of each *E. coli* W3110 chromosomal DNA (5.0×10^{-18} g $molecule^{-1}$).

A plasmid (pGEM T-Easy Vector; Promega Co., Madison, WI) containing the *gyrB* insert cloned from *E. coli* W3110 was constructed.The gyrB insert was amplified by PCR with the primers ESgyrB171F and ESgyrB448R (Table 1). The plasmid DNA was linearized using NdeI (Molecular Biosystems Inc., San Diego, CA). *In vitro* transcription was performed using SP6/T7 transcription kit (Roche Diagnostics, Indianapolis, IN). To eliminate DNA contamination, RNA was digested with DNase I (Roche Diagnostics, Indianapolis, IN).

Measurement of RNA concentration was performed using the RiboGreen RNA quantification kit (Molecular Probes Inc., Eugene, OR). The standard curve was generated with a series of 16S rRNA solutions (serial dilutions, 10–1,000 ng mL^{-1}) using the 16S rRNA standard solution included in the kit. 16S rRNA and *in vitro* transcripts of *E. coli* W3110 *gyrB* clone were stained with 1/400 volume of RiboGreen in a 96-well microplate. The fluorescence intensity (emission at 530 nm) was measured using a fluorescence multi-well microplate reader (CytoFluor II; PreSpetive Biosystems Inc., Framingham, MA) at an excitation wavelength of 485 nm.

2.4. Real-time PCR and real-time RT-PCR of *E. coli gyrB* DNA and mRNA

The LightCycler system (Roche Diagnostics, Indianapolis, IN) was used for real-time PCR and real-time reverse transcription (RT)-PCR of *E. coli gyrB* DNA and mRNA. To quantify the *gyrB* DNA from *E. coli*, LightCycler DNA Master Hybridization Probes (Roche Diagnostics, Indianapolis, IN) were used with 5 mM Mg^{2+}, 0.5 μM of each primer (ESgyrB171F and ESgyrB448R), and 0.2 μM of each FRET probe [ESgyrB376 (3'-FITC) and ESgyrB404 (5'-LightCycler-Red640)] (Table 1). After an initial denaturation at 95°C for 10 min, 50 PCR cycles at 95°C for 15 s, with annealing at 66°C for 5 s and an extension at 72°C for 25 s, were conducted. A standard DNA template of 5 to 5×10^6 copies per reaction of *E. coli* W3110 genomic DNA was used to construct a standard curve for real-time PCR quantification. To quantify *gyrB* mRNA of *E. coli*, LightCycler FastStart RNA Master Hybridization Probes (Roche Diagnostics, Indianapolis, IN) were used with 3.25 mM Mn^{2+}, primers (ESgyrB171F and ESgyrB448R), and FRET probes [ESgyrB376 (3'-FITC) and ESgyrB404 (5'-LightCycler-LCRed640)]. After reverse transcription for 15 min at 61°C for 15 min, 50 PCR denaturation cycles at 95°C for 1 s, with annealing at 64°C for 10 s and an extension at 72°C for 15 s, were conducted. A standard RNA template of 5 to 5×10^7 copies per reaction of *in vitro* transcripts of the *E. coli* W3110 *gyrB* clone was used to construct a standard curve for real-time RT-PCR quantification.

LightCycler data were analyzed using LightCycler software version 3.5 (Roche Diagnostics, Indianapolis, IN). The concentrations and crossing points were calculated, which represented the threshold cycle numbers where the fluorescence signal first exceeded the level of the background noise. These were obtained using the second derivative maximum function of LightCycler software, together with propor-

Table 1. Primers and probes for real-time PCR

Primer and probe	Target gene	Target position	Sequence
ESgyrB171F (forward primer)	*gyrB* gene of *E. coli*	171–194[d]	5'-aga aat tat cgt cac cat tca cgc-3'
ESgyrB448R (reverse primer)	*gyrB* gene of *E. coli*	427–448[d]	5'-gta cac cgt gtt cgt aga tct g-3'
ESgyrB376[a] (probe 1 for FRET[c])	*gyrB* gene of *E. coli*	376–401[d]	5'-ctg tcg caa aaa ctg gag ctg gtt at-3'
ESgyrB404[b] (probe 2 for FRET)	*gyrB* gene of *E. coli*	404–426[d]	5'-agc gcg agg gta aaa ttc acc gt-3'
pgL1908f (forward primer)	Luciferase gene	1908–1927[e]	5'-agg aag ctt tcc atg gaa ga-3'
Luc175r (reverse primer)	Luciferase gene	2063–2082[e]	5'-cag cgt aag tga tgt cca cc-3'
n-LucHP1[a] (probe 1 for FRET)	Luciferase gene	2008–2029[e]	5'-tga aga gat acg ccc tgg ttc c-3'
n-LucHP2[b] (probe 2 for FRET)	Luciferase gene	2030–2058[e]	5'-gga aca att gct ttt aca gat gca cat a-3'

[a] 3' FITC labeled
[b] 5' LCRed640 labeled
[c] Fluorescence resonance energy transfer
[d] Numbering of bases is *E. coli* numbering
[e] Numbering bases of pGeneGRIP™-Luc

tional and arithmetic baseline adjustments that were made according to the manufacturer's instructions. The standard curve was generated by linear regression of the crossing point versus the logarithms of the concentrations for each standard sample.

2.5. Recovery rate of DNA and RNA

To determine the DNA and RNA recovery rates, internal standards of DNA and RNA were added to each sample and the internal standards were quantified by real-time PCR and real-time RT-PCR, respectively, after recovery. The luciferase gene was used as the internal standard because it was absent from samples used in the present study. A 1.7-kb PCR amplicon of the luciferase gene was used as the internal DNA standard and a 1.8-kb *in vitro* transcript of the luciferase gene clone was used as the internal RNA standard. Real-time PCR and real-time RT-PCR of the luciferase gene and *in vitro* transcripts used pgL1908f (forward primer), Luc175r (reverse primer), n-LucHP1 (probe 1 for FRET), and n-LucHP2 (probe 2 for FRET) (Table 1). The luciferase gene was quantified using LightCycler DNA Master Hybridization Probes (Roche Diagnostics, Indianapolis, IN) with 5 mM Mg^{2+}, 0.5 μM of each primer, and 0.2 μM of each FRET probe. After an initial denaturation at 95°C for 10 min, 40 PCR denaturation cycles at 95°C for 15 min, with annealing at 60°C for 10 s and an extension at 72°C for 15 s, were conducted. Luciferase transcripts were quantified using LightCycler-FastStart RNA Master Hybridization Probes (Roche Diagnostics, Indianapolis, IN) with 3.25 mM Mn^{2+}. After reverse transcription at 61°C for 20 min, 40 PCR denaturation cycles at 95°C for 1 s, with annealing at 60°C for 10 s and an extension at 72°C for 15 s, were conducted.

2.6. Sampling of *E. coli* culture

The relationship between the copy number of *gyrB* mRNA (*gyrB* mRNA expression) and the growth rate was determined using *E. coli* W3110 cultured in LB broth at 37°C, which was measured at a wavelength of 610 nm. Log, log-stationary (end of log phase), and stationary phase cultures were sampled. To determine the relationship between *gyrB* mRNA expression and growth rate, *E. coli* W3110 was cultured in M9 medium (containing 0.5% glucose) at 20, 22, or 25°C. The log phase culture was sampled and 10^9 cells were collected on a 0.2-μm pore size polycarbonate membrane filter (Advantec, Japan). The generation time was calculated using the following formula:

Generation time$=\log 2/\mu$

μ=specific growth rate

The specific growth rate was calculated from OD_{610} measurements ($\mu=\Delta \ln OD_{610}/\Delta t$, where t is time).

2.7. Enumeration of growing bacterial cells

The number of growing bacterial cells was enumerated using the method of Kogre *et al.*[14] with minor modifications. *E. coli* was incubated in LB broth at 37°C. Based on the growth curve of *E. coli*, sampling time points were selected to obtain cultures for total direct count (TDC) and DVC. The sampling time points were 1.5 h (log phase), 3.5 h (log-stationary phase), and 24 h (stationary phase). Samples for TDC were fixed with 2% formaldehyde neutral buffer solution (2%; Nacalai Tesque, Japan). Samples for DVC were incubated in LB broth containing 20 μg mL^{-1} nalidixic acid (Wako Pure Chemical Industries Ltd., Japan) at 37°C. After 1 h, they were fixed with formaldehyde neutral buffer solution. Each fixed sample was stained with 4',6-diamidino-2-phenylindole (DAPI) and counted using an epifluorescence microscope (Eclipse 80i; Nikon Co., Japan).

2.8. Sampling of surface river water and river sediment

Surface river water and river sediment was sampled at Kuwazu from the Inagawa River in Hyogo, Japan. Surface river water was collected in a sterilized polyethylene terephthalate copolyester bottle, which was returned to the lab at 4°C in the dark. River sediment sampling was performed in triplicate. Sediment samples were transported to the lab in an ethanol/dry-ice bath. The surface river water sample was filtered through a 0.2-μm pore size polycarbonate membrane filter and at least 10^9 bacterial cells were collected on the filter. RNA and DNA were recovered from the filter or 2 g (wet weight) of the river sediment. The sediment was

Table 2. Expression of *gyrB* gene in *E. coli* cells[a] in different growth phase

Growth phase	Copy number of *gyrB* mRNA/cell
Log	4.7±2.3
Log-stationary[b]	0.015±0.002
Stationary	$<10^{-6}$

(n=3)

[a] *E. coli* cells were cultured in LB broth at 37°C
[b] During the transition from log phase to stationary phase

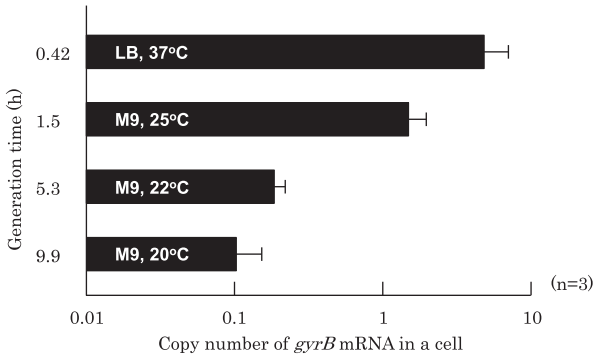

Fig. 1. Relation between copy number of *gyrB* mRNA and generation time in *E. coli*. *E. coli* cells were cultured in LB broth at 37°C or in M9 medium containing 0.5% glucose at 20°C, 22°C or 25°C, and were collected at mid log phase. *gyrB* mRNA were quantified by real-time RT-PCR. Each blotted dot shows the mean of quantified mRNA copy number (n=3), and error bar shows the standard deviation.

suspended in sterilized deionized water, mixed vigorously with a vortex mixer for 30 s, and then sonicated using an ultrasonic cleaner (Model B3210J; Branson, Danbury, CT) for 10 min at 120 W. Bacterial cells were trapped on the polycarbonate filter and enumerated using epifluorescence microscopy (E400; Nikon Co., Japan) with UV excitation after being stained with 1 μg mL^{-1} of DAPI.

3. Results

The specificity of PCR primers and FRET probes was experimentally examined *in silico*. The *gyrB* gene sequences of *Salmonella* spp. were most similar to those of *E. coli* and *Shigella*. The sequences of ESgyrB171F, ESgyrB448R, and ESgyrB404 were dissimilar to the corresponding sequences of *Salmonella*. ESgyrB376 (3'-labeled FRET probe) matched perfectly with 8 of the 28 *Salmonella* DNA gyrase sequences. ESgyrB404 (5'-labeled FRET probe) had more than two mismatched bases with all *Salmonella* DNA gyrase sequences. PCR using ESgyrB171F and ESgyrB448R was able to amplify the DNA gyrase gene of *E. coli* and *Shi. sonnei* but not that of *Sal. enteritidis*, *Sal. enterica* serovar Choleraesuis, or *V. vulnificus*. The copy numbers of *gyrB* mRNA in an *E. coli* cell at the log, log-stationary, and stationary phases in LB broth at 37°C were 4.7±2.3, 0.015±0.002, and $<10^{-6}$ (below the detection limit, n=3), respectively (Table 2). The copy number of *gyrB* mRNA in a log phase cell was 300 times higher than that in a log-stationary phase cell. The copy number was below the detection limit in stationary phase cells. Thus, *gyrB* mRNA expression levels were high in growing *E. coli* W3110 cells, but they declined rapidly after the cells reached the log phase. When the generation times were 0.42, 1.5, 5.3, and 9.9 h, the ratios of *gyrB* mRNA to DNA were 4.7, 1.5, 0.15, and 0.09, respectively, in *E. coli* (Fig. 1).

Table 3. Growing cells in *E. coli* cells[a] in different growth phase

	Growth phase		
	Log	Log-stationary[b]	Stationary
TDC[c]	$6.6\times10^{7}\pm9.3\times10^{6}$	$1.2\times10^{9}\pm1.2\times10^{8}$	$7.0\times10^{9}\pm1.2\times10^{9}$
DVC[d]	98±0.007%	1.0±0.005%	0.64±0.005%

(n=3)

[a] *E. coli* cells were cultured in LB broth at 37°C
[b] During the transition from log phase to stationary phase
[c] Total direct count (cells/mL)
[d] Direct viable count (percentage of elongated cells per total cells)

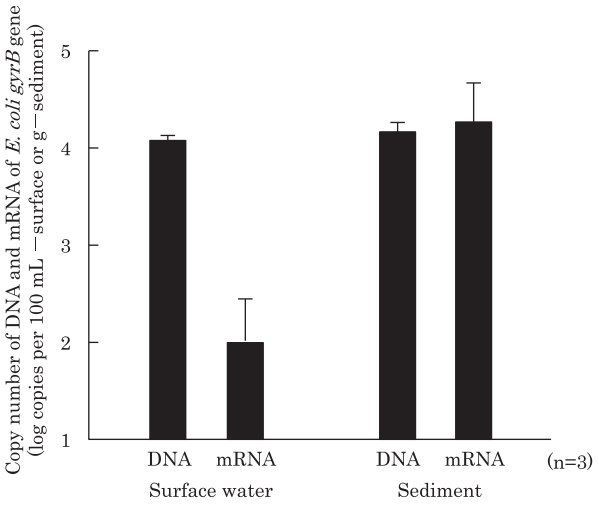

Fig. 2. Expression of *gyrB* mRNA of *E. coli* in river surface water and sediment. Each error bar indicates the standard deviation derived from triplicate samples.

The ratios of growing bacterial cells to total cells were enumerated by the DVC method. The ratios of growing *E. coli* cells at the log, log-stationary, and stationary phases were 98±0.007%, 1.0±0.005%, and 0.64±0.005%, respectively (Table 3, n=3). These results agreed with those of *gyrB* gene expression.

Following the experiments with standard strain, we evaluated *gyrB* gene expression in the environment. The number of bacteria in the surface river water and river sediment was 2.7×10^{7} cells mL^{-1} and 1.9×10^{9} cells g^{-1}, respectively. The copy numbers of *gyrB* for the *E. coli-Shigella* group were 1.1×10^{4} copies 100 mL^{-1} for the surface river water sample and 1.1×10^{4} copies g^{-1} for the river sediment. The ratio of mRNA to DNA was 1.3 in the river sediment sample and 0.008 in the surface river water sample. The ratio in the sediment was more than 100 times greater than that found in the surface water (Fig. 2).

4. Discussion

We found that *gyrB* mRNA was expressed at high levels in growing *E. coli* cells, in which a higher growth rate corresponded to a higher *gyrB* expression level. DVC and the ratio of mRNA to DNA were also correlated. These results suggest that *gyrB* mRNA expression can be used as an indicator of bacterial growth activity. The *gyrB* gene is a housekeeping gene that encodes for subunit B of bacterial DNA gyrase, and hence, it has an essential role in DNA replication in all bacterial species[18]. Thus, *gyrB* can be used to determine growth activity of bacterial species other than *E. coli* in natural environments. Only a single copy of the *gyrB* gene exists in the genomes of most bacteria. Therefore, the copy number of the *gyrB* gene represents the number of bacterial genomes, which indicates the number of bacteria if the generation time is more than 1 h. However, multiple copies of genomic DNA can be found in each cell in more rapidly growing cultures.

The rate of incorporation of a specific compound, such as thymidine, leucine, or their analogs, is widely used to determine bacterial growth activity[7,8]. However, this approach requires samples to be incubated with the appropriate substance in a closed environment (i.e., a vial), where the bacterial growth activity could change during incubation. *gyrB* mRNA expression can be measured without incubation; hence, it can provide a more accurate indicator of *in situ* bacterial growth activity. The cellular rRNA content of bacteria reportedly correlates with the bacterial growth rate[5,17]. However, the half-life of rRNA in an inactive cell can be up to several days in natural environments[13]. The bacterial rRNA content may indicate the growth rate over the preceding hours or even over several days, and thus, *gyrB* mRNA expression is a more appropriate indicator for determining *in situ* bacterial growth activity in a rapidly changing environment. The frequency of cell division may accurately indicate the *in situ* growth activity and growth rate in environments[9,15] because incubation is not required for the enumeration of dividing cells. Furthermore, samples used for counting can be fixed immediately after sampling, which can preserve the bacterial population in a state that is representative of its state in the original environment. However, counting bacteria microscopically is often made difficult by the autofluorescence of mineral materials and/or the overlapping of cells in aggregates, biofilms, and micro-colonies. In contrast, *gyrB* mRNA can be measured in various samples if RNA can be recovered from the sample, including biofilms, soils, and sediments. The recovery rate of DNA and RNA must be determined to accurately measure the copy numbers of *gyrB* DNA and mRNA. In the present study, the recovery rates were determined by quantifying added DNA (PCR amplicons of the luciferase gene) and RNA (*in vitro* transcripts of the luciferase clone), which were used as internal standards. Neither the luciferase gene nor its transcript was detected in the river samples (data not shown), and hence, recovery rates could be accurately determined for each sample. The growth activity of *E. coli-Shigella* in surface river water and river sediment samples was evaluated by measuring *gyrB* mRNA expression. The copy numbers of *gyrB* for the *E. coli-Shigella* group were 1.1×10^4 copies 100 mL^{-1} in the surface river water sample and 1.1×10^4 copies g^{-1} in the sediment sample. The ratio of mRNA to DNA was 1.3 in the river sediment sample and 0.008 in the river surface water sample. The ratio in the sediment sample was more than 100 times higher than that in the surface water sample. Thus, river sediment may be reservoir for the *E. coli-Shigella* group in natural environments. Microenvironments provided by particulate matter, biofilms, and sediments are reportedly rich in organic substances and nutrients, and *E. coli* can grow and survive well in such environments[4,23]. However, there has never been any evidence that *E. coli* grows in aquatic environments. The present study showed that *E. coli* grew in surface river water and river sediments and that the growth activity of *E. coli* in the river sediment was much higher than that in the surface river water. These findings suggest that river sediment is a potential source of *E. coli* in surface river water, which is important for understanding the fate of *E. coli* in aquatic environments. *gyrB* is a bacterial housekeeping gene, and more than 16,000 sequence data entries can now be found for this gene in DNA databases, which is much more than other genes related to bacterial growth activity. *gyrB* is also useful in the phylogenetic analysis of bacteria[18]. Phylogenetic inferences based on *gyrB* sequences are available; thus, this novel method of measuring *gyrB* mRNA expression for determining bacterial growth activity in natural environments could also be applied to bacterial species other than *E. coli* and the *Shigella* group.

References

1) Artursson, V. and J.K. Jansson. 2003. Use of bromodeoxyuridine immunocapture to identify active bacteria associated with arbuscular mycorrhizal hyphae. Appl. Environ. Microbiol. 69: 6208–6215.
2) Baudisova, D. 1997. Evaluation of *Escherichia coli* as the main indicator of faecal pollution. Water Sci. Technol. 35: 333–336.
3) Bernard, L., C. Courties, C. Duperray, H. Schäfer, G. Muyzer, and P. Lebaron. 2001. A new approach to determine the genetic diversity of viable and active bacteria in aquatic ecosystems. Cytometry 43: 314–321.
4) Davies, C.M., J.A.H. Long, M. Donald, and N.J. Ashbolt. 1995. Survival of fecal microorganisms in marine and freshwater sediments. Appl. Environ. Microbiol. 61: 1888–1896.
5) DeLong, E.F., G.S. Wickham, and N.R. Pace. 1989. Phylogenetic stains: ribosomal RNA-based probes for the cells. Science 243: 1360–1363.
6) Desmarais, T.R., H.M. Solo-Gabriele, and C.J. Palmer. 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Appl. Envir. Microbiol. 68: 1165–1172.
7) Fuhrman, J.A. and F. Azam. 1980. Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl. Environ. Microbiol. 39: 1085–1095.
8) Fuhrman, J.A. and F. Azam. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. 66: 109–120.

9) Hagström, Å., U. Larsson, P. Hörstedt, and S. Normark. 1979. Frequency of dividing cells, a new approach to the determination of bacterial growth rates in aquatic environments. Appl. Environ. Microbiol. 37: 805–812.
10) Hurt, R.A., X. Qui, L. Wu, Y. Roh, A.V. Palumbo, J.M. Tiedje, and J. Zhou. 2001. Simultaneous recovery of RNA and DNA from soils and sediments. Appl. Environ. Microbiol. 67: 4495–4503.
11) Iwamoto, T., K. Tani, K. Nakamura, Y. Suzuki, M. Kitagawa, M. Eguchi, and M. Nasu. 2000. Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol. Ecol. 32: 129–141.
12) Kawai, M., N. Yamaguchi, and M. Nasu. 1999. Rapid enumeration of physiologically active bacteria in purified water used in the pharmaceutical manufacturing process. J. Appl. Microbiol. 86: 496–504.
13) Kemp, P.F., S. Lee, and J. LaRoche. 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl. Environ. Microbiol. 59: 2594–2601.
14) Kogre, K., U. Shimidu, N. Taga, and R.R. Colwell. 1987. Correlation of direct counts with heterotrophic activity for marine bacteria. Appl. Environ. Microbiol. 53: 2332–2337.
15) Møller, S., C.S. Kristensen, L.K. Poulsen, J.M. Carstensen, and S. Molin. 1995. Bacterial growth on surfaces: automated image analysis for quantification of growth rate-related parameters. Appl. Environ. Microbiol. 61: 741–748.
16) Pernthaler, P., J. Pernthaler, M. Schattenhofer, and R. Amann. 2002. Identification of DNA-synthesizing bacterial cells in Coastal north sea plankton. Appl. Environ. Microbiol. 68: 5728–5736.
17) Poulsen, L.K., G. Ballard, and D.A. Stahl. 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. 59: 1354–1360.
18) Roberts, R.C. and L. Shapiro. 1997. Transcription of genes encoding DNA replication proteins is coincident with cell cycle control of DNA replication in Caulobacter crescentus. J. Bacteriol. 179: 2319–2330.
19) Rodriguez, G.G., D. Phipps, K. Ishiguro, and H.F. Ridgway. 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. App. Environ. Microbiol. 58: 1801–1808.
20) Rozen, Y. and S. Belkin. 2001. Survival of enteric bacteria in seawater. FEMS Microbiol. Rev. 25: 513–529.
21) Savageau, M.A. 1983. *Escherichia coli* habitats, cell types, and molecular mechanisms of gene control. Am. Nat. 122: 732–744.
22) Schupp, D.C. and S.L. Erlandsen. 1987. A new method to determine Giardia cyst viability: correlation of fluorescein diacetate and propidium iodide staining with animal infectivity. Appl. Environ. Microbiol. 53: 704–707.
23) Sibille, I., T. Sime-Ngando, L. Mathieu, and J.C. Block. 1998. Protozoan bacterivory and *Escherichia coli* survival in drinking water distribution systems. Appl. Environ. Microbiol. 64: 197–202.
24) Solo-Gabriele, H.M., M.A. Wolfert, T.R. Desmarais, and C.J. Palmer. 2000. Sources of *Escherichia coli* in a coastal subtropical environment. Appl. Envir. Microbiol. 66: 230–237.
25) Tanaka, Y., N. Yamaguchi, and M. Nasu. 2000. Viability of *Escherichia coli* O157:H7 in natural river water determined by the use of flow cytometry. J. Appl. Microbiol. 88: 228–236.
26) Tsai, Y.-L. and B.H. Olson. 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. 57: 1070–1074.
27) Urbach, E., K.L. Vergin, and S.J. Giovannoni. 1999. Immunochemical detection and isolation of DNA from metabolically active bacteria. Appl. Environ. Microbiol. 65: 1207–1213.
28) USEPA. 1986. USEPA, Ambient water quality criteria for bacteria-1986. EPA440-584-002. US EPA, Washington, DC.
29) Yamaguchi, N. and M. Nasu. 1997. Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. J. Appl. Microbiol. 83: 43–52.