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ORIGINAL ARTICLE

High-frequency phage-mediated gene transfer in freshwater environments determined at single-cell level

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Lateral gene transfer by phages has contributed significantly to the genetic diversity of bacteria. To accurately determine the frequency and range of phage-mediated gene transfer, it is important to understand the movement of DNA among microbes. Using an *in situ* DNA amplification technique (cycling primed *in situ* amplification-fluorescent *in situ* hybridization; CPRINS-FISH), we examined the propensity for phage-mediated gene transfer in freshwater environments at the single-cell level. Phage P1, T4 and isolated *Escherichia coli* phage EC10 were used as vectors. All *E. coli* phages mediated gene transfer from *E. coli* to both plaque-forming and non-plaque-forming *Enterobacteriaceae* strains at frequencies of 0.3–8 $\times 10^{-3}$ per plaque-forming unit (PFU), whereas culture methods using selective agar media could not detect transductants in non-plaque-forming strains. The DNA transfer frequencies through phage EC10 ranged from undetectable to 9×10^{-2} per PFU (undetectable to 2×10^{-3} per total direct count) when natural bacterial communities were recipients. Direct viable counting combined with CPRINS-FISH revealed that more than 20% of the cells carrying the transferred gene retained their viability in most cases. These results indicate that the exchange of DNA sequences among bacteria occurs frequently and in a wide range of bacteria, and may promote rapid evolution of the prokaryotic genome in freshwater environments.
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Introduction

Bacteriophages are abundant and ubiquitous in the natural environment. They induce bacterial mortality, contribute to the carbon cycle and also affect host diversity (Fuhrman, 1999). Moreover, they mediate gene transfer between prokaryotes. Recent whole-genome analyses suggest that lateral gene transfer by phages has contributed significantly to the acquisition of new genetic traits, the ability of bacteria to exploit new environments and the genetic diversity of many bacteria (Ochman *et al.*, 2000; Brüssow *et al.*, 2004; Bordenstein and Reznikoff, 2005; Pallen and Wren, 2007). In gene transfer by phages, phage particles accidentally incorporate a piece of the bacterial DNA into a phage head in place of phage DNA during the propagation. As phage capsids prevent nuclease digestion, phages may serve as reservoirs for foreign genes. Potential gene transfer via phages has been documented in soil, freshwater and marine water environments (Saye *et al.*, 1987; Zeph *et al.*, 1988; Jiang and Paul, 1998). In these studies, researchers inoculated well-known or isolated phages packed with indicator genes into environmental samples and performed transduction assays using natural bacterial communities as recipients. They have suggested that transduction could be an important mechanism for lateral gene transfer in natural environments.

Culture methods using selective agar media have a leading role in the study of transduction (Zinder and Lederberg, 1952; Ogunseitan, 2008). However, many environmental bacteria are resistant to culture on conventional media (Amann *et al.*, 1995). In addition, the genetic characteristics used as an indicator for transduction are found in indigenous bacteria. For instance, there are antibiotic-resistant bacteria in the natural environment; their occurrence makes it difficult to distinguish indigenous antibiotic-resistant bacteria from transductants (Teuber, 2001). Transfer of foreign DNA molecules (DNA entry) into a recipient bacterium is an important first step in genetic diversification through lateral gene transfer,

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but the expression level of the transferred gene and cell growth on media may differ for each recipient cell. Culture methods have a limited ability to quantify the genetic material introduced into individual cells at the DNA level. Thus, our current knowledge of gene transfer via phages in the environment is rather limited because of such methodological constraints.

Detection of single-copy genes in individual cells is necessary to determine the frequency and range of DNA transfer in environmental bacteria, and to understand gene flow among microorganisms. *In situ* DNA amplification methods allow the visualization of specific DNA sequences inside bacterial cells (Kenzaka *et al.*, 2005; Maruyama *et al.*, 2005), clarifying the movement of a specific gene among *Escherichia coli* cells at the single-cell level (Kenzaka *et al.*, 2007). The purpose of this study was to investigate the propensity for DNA transfer via phages in natural freshwater habitats at the single-cell level. We used an *in situ* DNA amplification technique (cycling primed *in situ* amplification-fluorescent *in situ* hybridization; CPRINS-FISH; Kenzaka *et al.*, 2005), and both frequency and possible range of DNA transfer via three *E. coli* phages (P1, T4 and isolated phage) were first examined at the single-cell level using both plaque-forming and non-plaque-forming *Enterobacteriaceae* strains as recipients. To explore the viability of cells that acquired the gene from phage, direct viable counting (DVC) was carried out after DNA was transferred by phages (Kogure *et al.*, 1979). The propensity of DNA transfer obtained by this method was compared with values determined by conventional methods. Green fluorescent protein gene (*gfp*) was used as an indicator of gene transfer because the freshwater samples used in this study did not contain the *gfp* gene sequence.

Materials and methods

Bacterial strains

Bacterial strains used in this study are described as follows: *Citrobacter freundii* IFO 12681, *Enterobacter aerogenes* BM 2688, *E. coli* C600 RK2, *E. coli* NBRC 12713 KEN1, which carries transposon *Tn1* (4951 bp) including an ampicillin resistance gene (beta lactamase gene; *bla*) on the chromosome, *E. coli* NBRC 12713 with or without RK2::*gfp*, *E. coli* W3110 with or without plasmid RK2::*gfp*, *Pseudomonas putida* ATCC 12633, *Proteus mirabilis* clinical isolate, *Salmonella enteritidis* IID 640, *Serratia marcescens* clinical isolate, *Yersinia enterocolitica* IID 981. The *gfp*-tagged broad host-range plasmid RK2 (RK2::*gfp*) was constructed using RK2 and pGFPuv (Clontech Laboratories, Palo Alto, CA, USA) as described by Jorquera *et al.* (2006). *E. coli* strains were grown in Luria–Bertain (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37 °C. Strains with the *bla* gene were cultured in LB broth containing 50 μg ml^{-1} ampicillin. Other strains were grown in LB medium at 30 °C. The absence of *bla* gene and *gfp* gene in the genomic DNA of all recipient strains was confirmed by PCR targeting the genes before transduction and DNA transfer experiments were performed.

Bacteriophage

Phage P1*kc* NBRC 20008, a derivative of phage P1 was obtained from National Institute of Technology and Evaluation, Japan (Enomoto and Stocker, 1974). Phage T4GT7, a derivative of phage T4 was obtained from National Institute of Genetics, Japan (Wilson *et al.*, 1979). Transducing *E. coli* phage EC10 was isolated from eutrophic river (Kenzaka *et al.*, 2007). Phages were propagated with appropriate donor *E. coli* strains (NBRC 12713 KEN1 for P1*kc* and T4GT7, *E. coli* W3110 RK2::*gfp* or *E. coli* NBRC 12713 RK2::*gfp* for EC10) in 1 l LB broth containing 0.2% $MgSO_4$ and 10 mM $CaCl_2$ overnight at 37 °C. Purification of phages by ultracentrifugation was performed as described by Kenzaka *et al.* (2007). DNase treatment was performed before phages were purified by ultracentrifugation to prevent transformation. Before transduction and DNA transfer experiments, DNA was extracted from each phage (P1, T4 and EC10) infecting *E. coli* strains lacking the *bla* and *gfp* genes using the Wizard Lambda Preps DNA Purification System (Promega, Madison, WI, USA), and the absence of the *bla* and *gfp* genes was confirmed by PCR.

Environmental samples

Surface river water samples were taken from Kitahashi in the Neyagawa River, Juhachijo in the Kanzakigawa River, Kuwazu in the Inagawa River and Takiue in the Minohgawa River, in the northern part of Osaka, Japan (Figure 1). At the sites, water samples were collected twice in early spring of 2005 (February–March) and three times in autumn of 2009 (October–November). Kitahashi is located in a commercial area, Osaka Business Park. Kuwazu is located in an industrial area. These sites were considered to be polluted by organic carbon (Tani *et al.*, 1996; Yamaguchi and Nasu, 1997; Kenzaka *et al.*, 2001). Takiue is surrounded by forest and is an oligotrophic site. At the site, the river is narrow and shallow, and the water is not exposed to domestic or industrial effluents (Tani *et al.*, 1996; Kenzaka *et al.*, 1998, 2001). The water samples were collected in sterile 500-ml glass bottles and carried to the laboratory on ice. The absence of *gfp* gene in environmental samples was confirmed by PCR before DNA transfer experiments were performed.

Plaque assays

A volume of 100 μl of stationary-phase cultures of bacterial strains was incubated with 10 μl of diluted phage (10^{-1}–10^{-12}) in 90 μl of SM buffer (50 mmol l^{-1}

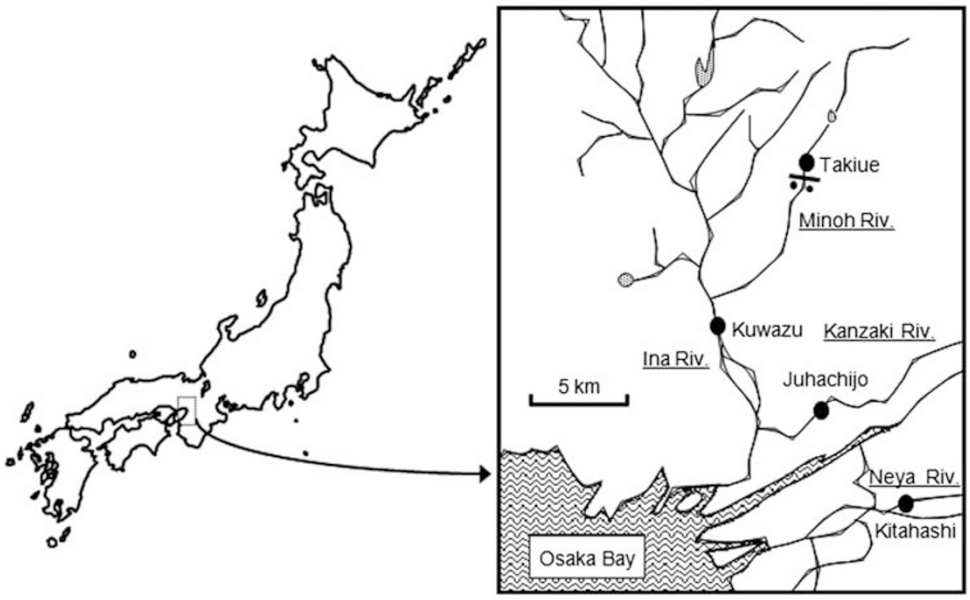

Figure 1 Map of river water sampling point. The sampling sites are marked by black circles. Takiue is located upstream of the Minoh River. Kuwazu, Juhachijo and Kitahashi are located downstream of the Ina River, the Kanzaki River and the Neya River, respectively. These rivers flow into Osaka Bay.

Tris–HCl (pH7.5), 100 mmol l^{-1} NaCl, 8 mmol l^{-1} $MgSO_4$ and 0.01% gelatin) at 30 °C for 10 min. After incubation, samples were poured with LB soft agar (0.8% agar) on LB plate and incubated at 30 °C overnight to determine the plaque formation.

Transduction assays

For cultured recipients, 200 ml of stationary-phase cultures were collected by centrifugation at 8000 *g* for 10 min at 4 °C and suspended in 10 ml of LB broth. A volume of 3 ml of the suspended cultures was incubated with phages in 3 ml of SM buffer at 37 °C for 10 min at multiplicities of infection ranging from 0.2 to 2. Each control contained an equal volume of the recipient cell culture and SM buffer. After a 10-min adsorption period at 37 °C for *E. coli* and at 30 °C for other strains, cells were placed onto selective LB plates containing 50 μg ml^{-1} ampicillin and incubated at 30 °C for 2 days. The transducing phage lysate (containing no recipient) and recipient (containing no transducing phage) were also plated onto selective plates as a control. The frequencies of transduction were represented as the number of colonies on the selective LB plates per initial number of colonies on the non-selective LB plates (colony-forming unit; CFU) of recipient or per initial plaque-forming unit (PFU) of phage. Results presented are averages of three transduction experiments.

DNA transfer experiments

A volume of 200 μl of stationary-phase culture was incubated with 200 μl of SM buffer containing each phage at 37 °C for 10 min at multiplicities of infection ranging from 0.2 to 2. The concentration of bacterial cells was adjusted to approximately 1×10^9 cells per ml. For other strains used as recipients, cultures were incubated with phages at 30 °C for 10 min. For gene transfer assays with recipients at low concentration, concentration of *E. coli* was adjusted to approximately 1×10^5, 10^6, 10^7 and 10^8 cells per ml, and 1% of *E. coli* was added to *P. putida* at the concentration ranging from 4×10^5 to 10^7 cells per ml. The diluted cultures were incubated with phage EC10 at 25 °C for 10 min at the multiplicity of infection 2. For gene transfer experiments in which indigenous river bacterial communities were used as recipients, 10–100-ml portions of river water samples were incubated with phage EC10 at 25 °C for 20 min at mixing ratio ranging from 2 to 200. The mixing ratio was calculated on the basis of the number of bacteria stained by SYBR Gold-labeled phage EC10 in river water samples as described below.

After the mixtures of recipients and phage were incubated under the conditions, as mentioned above, samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (0.13 mol l^{-1} NaCl, 7 mmol l^{-1} Na_2HPO_4 and 3 mmol l^{-1} NaH_2PO_4 (pH 7.2)) at 4 °C for 16 h. After fixation, 1 μl–30-ml portions were filtered through gelatin (0.1% gelatin, 0.01% $CrK(SO_4)_2$)-coated polycarbonate white filters (0.2 μm pore size, 25 mm diameter, ADVANTEC, Tokyo, Japan) and rinsed twice with filtered deionized water. Then samples were stored at −20 °C.

Viability of recipient cells determined by DVC

To explore the viability of the recipient cells carrying the transferred gene, DVC was carried out.

After recipient cell culture was mixed with each phage under the conditions, as mentioned above, 20 μl of the mixture was added to 180 μl of LB broth containing an antibiotic cocktail (final concentration: 20 μg ml^{-1} nalidixic acid, 10 μg ml^{-1} piromidic acid, 10 μg ml^{-1} pipemidic acid, 10 μg ml^{-1} cephaloxin and 0.1 μg ml^{-1} ciprofloxacin; Joux and Lebaron, 1997) and incubated at 37 °C for *E. coli* or 30 °C for other bacteria for 3 h. For river water samples, after the samples were mixed with the phage EC10 under conditions mentioned above, antibiotic cocktail was added to the mixture at the above final concentrations, and incubated at 25 °C for 18 h (30 h for sample from oligotrophic site, Takiue).

After incubation for DVC, samples were fixed with 4% paraformaldehyde in phosphate-buffered saline at 4 °C for 16 h. After fixation, samples were filtered and were stored at −20 °C as described above.

Oligonucleotides and polynucleotides

Oligonucleotide primers and polynucleotide probes for CPRINS-FISH used in this study were described as follows. AmpR840r primer and three probes for the *bla* gene were reported in the study by Kenzaka *et al*. (2005). The nucleotide sequences of *gfp* gene were obtained from GenBank (Release 138.0), and gfp717r primer (5′-TTATTTGTAGAGCTCATCCA-3′) and probes (5′-GATGGTGATGTTAATGGGCACAAA TTTTCTGTCAGTGGAGAGGGTGAAGG-3′; 5′-GAC TTTTTCAAGAGTGCCATGCCCGAAGGTTATGTAC AGGAACGC-3′; 5′-GATGACGGGAACTACAAGACG CGTGCTGAAGTCAAGTTTGAAGG-3′; 5′-ATTGGC GATGGCCCTGTCCTTTTACCAGACAACCATTACC TGTCCA-3′; 5′-CGAAAGATCCCAACGAAAAGCGT GACCACATGGTCCTTCTTGAG-3′) for the *gfp* gene were designed in this study. The specificities of the primer and probe sequences were verified against National Center for Biotechnology Information nucleotide databases using the Basic Local Alignment Search Tool program (Altschul *et al*., 1997). All primers and polynucleotide probes were purchased from Texas Genomics (Tokyo, Japan), and probes were labeled with Alexa Fluor 546 at guanine using ULYSIS Alexa Fluor 546 Nucleic Acid Labeling Kit (Invitrogen, Tokyo, Japan) and the manufacturer's recommended procedures.

CPRINS-FISH

To detect cells carrying the *bla* or *gfp* gene transferred by phage, CPRINS-FISH was performed. Permeabilization for CPRINS-FISH was carried out as described in the study by Kenzaka *et al*. (2005). Filters with bacterial cells were coated in gelatin to avoid cell loss during extensive cell wall permeabilization. After lysozyme treatment, each filter was cut into 16 sections and subjected to CPRINS-FISH.

A one-sixteenth section of the filter was transferred to a microtube (volume: 0.2 ml) and immersed in 100 μl of the CPRINS buffer (Kenzaka *et al*., 2007). Cycling primed *in situ* amplification cycles consisted of a hot start at 95 °C for 9 min, denaturation at 94 °C for 1 min, annealing at 62 °C for 30 s and 72 °C for 1.5-min extension for AmpR840r primer or annealing at 58 °C for 30 s and 72 °C for 2-min extension for gfp717r primer. Amplification was repeated for 30 cycles with a thermal cycler (PTC-200, Bio-Rad Laboratories, Hercules, CA, USA). After amplification, filters were rinsed with 0.1% Nonidet P40 and sterile deionized water, dehydrated in 99% ethanol and vacuum dried. Hybridization, washing and 4′,6-diamidino-2-phenylindole (DAPI) staining were performed as described in the study by Kenzaka *et al*. (2007). To exclude the possibility of nonspecific probe binding to cell structures other than target DNA in the target cells, (i) FISH using laboratory strains and environmental samples without amplification of target DNA, (ii) CPRINS-FISH targeting the *bla* or *gfp* gene using *E. coli* strains that did not carry the genes, and (iii) CPRINS-FISH targeting chloramphenicol acetyltransferase gene using laboratory strains that did not carry the chloramphenicol acetyltransferase gene (Kenzaka *et al*., 2005), were performed.

Epifluorescence microscopy

Filters were observed under an epifluorescence microscope (E-400; Nikon, Tokyo, Japan) with the Nikon filter sets UV-2A (EX300-350, DM400 and BA420) for DAPI, B-2A (EX450/490, DM505 and BA520) for SYBR Gold and HQ-CY3 (G535/50, FT565 and BP610/75) for Alexa Fluor 546, respectively. Images were acquired by a cooled charge-coupled device camera (Cool Snap; Roper Photometrics, Tuscon, AZ, USA) and stored as digital files. Exposure time was 0.1 s for DAPI, 0.5 s for SYBR Gold and 1 s for Alexa Fluor 546, respectively. A total of 3000–30 000 DAPI-stained objects were counted per sample. Frequencies of gene transfer determined by CPRINS-FISH were represented as the number of CPRINS-FISH-positive cells per initial total direct counts (TDCs) of recipient or per initial PFU of phage. Frequencies were determined in triplicate for each sample. Differences between means of frequencies were tested by the Student's *t*-test with Microsoft Excel XP software (Microsoft, Redmond, WA, USA).

Fluorescent bacteriophage assays

Fluorescently stained bacteriophages were produced by using a modification of procedure described by Maniatis *et al*. (1982). The phage, EC10, was propagated with *E. coli* C600 RK2 in 250 ml LB broth containing 0.2% $MgSO_4$ overnight at 37 °C. The cell culture was centrifuged at 8000 *g*, 10 min, 4 °C, and the supernatant was withdrawn. After nuclease treatment with DNase and RNase at 37 °C for 1 h, 0.2 μl ml^{-1} SYBR Gold (10 000 ×, Invitrogen) was added and stained at 37 °C for 1 h.

The stained sample was subjected to polyethylene glycol precipitation. Stained phage particles were further purified by ultracentrifugation.

A volume of 10 µl of both log-phase and stationary-phase cultures were incubated with 10 µl of the purified SYBR Gold-labeled phage at 37 °C for 20 min. For estimating the bacterial population to which phage EC10 could inject their DNA in river water samples, 1 ml of water samples were mixed with 20 µl of the SYBR Gold-labeled phage at 30 °C for 20 min. After incubation, samples were fixed in 4% paraformaldehyde in phosphate-buffered saline at room temperature (ca. 20 °C) for 10 min. After fixation, samples were stained with 1 µg ml^{-1} DAPI for 5 min, and filtered through a polycarbonate filter (pore size: 0.2 µm; ADVANTEC). Filters were mounted in immersion oil for observation by epifluorescence microscopy.

Results

Gene transfer via phage P1 and T4

Phage P1 and T4GT7 are commonly used, generalized transducing phages for *E. coli*. They have the ability to mediate gene transfer from *E. coli* to not only *E. coli* but to also some plaque-forming *Enterobacteriaceae* strains (Goldberg *et al.*, 1974; Tetart *et al.*, 1996). The frequency and possible range of transfer of the *bla* gene on the NBRC 12713 KEN1 chromosome of *E. coli* via the two phages was first examined by both a culture-based method using selective medium containing ampicillin and a culture-independent method with CPRINS-FISH targeting the *bla* gene. Plaque-forming strains and non-forming *Enterobacteriaceae* strains were used as recipients.

Transduction was observed in *E. aerogenes* and *E. coli*, which were plaque-forming strains, and the transduction frequencies for the *bla* gene on selective medium were $0.6–2 \times 10^{-6}$ transductants per PFU for P1*kc* (Table 1), and undetectable to 2×10^{-8} transductants per PFU for T4GT7 (Table 2) at a multiplicity of infection 1. Although non-plaque-forming strains did not have the *bla* gene, they could grow on selective medium containing ampicillin perhaps due to overproduction of multidrug-resistant efflux pump when they were plated at high density (ca. $>10^7$ CFUs per ml). Thus it was hard to determine the transduction frequency in the non-plaque-forming strains by conventional methods.

The frequencies of DNA transfer determined by CPRINS-FISH were $3–8 \times 10^{-3}$ per PFU for P1*kc* (Table 1) and $0.3–2 \times 10^{-3}$ per PFU for T4GT7 (Table 2) in plaque-forming strains. The CPRINS-FISH analysis revealed that both P1*kc* and T4GT7 transferred the *bla* gene to non-plaque-forming strains. Phage P1*kc* transferred the *bla* gene to *C. freundii* and *S. enteritidis* at similar frequencies ($0.8–4 \times 10^{-3}$ per PFU), and phage T4GT7 transferred the *bla* gene to *C. freundii*, *P. mirabilis*, *S. enteritidis* and *Y. enterocolitica* at similar frequencies ($0.5–2 \times 10^{-3}$ per PFU).

Viable bacteria carrying transferred genes may be infected by other phages and the transferred gene could be further transferred to other bacteria. They may also maintain the transferred gene. Thus, DVC and CPRINS-FISH were combined to estimate viable cells carrying the transferred *bla* gene. Representative photographs of viable *C. freundii* cells to which the *bla* gene was transferred by phage P1*kc* are shown in Figures 2a and b. Direct viable counting combined with CPRINS-FISH visualized the target viable recipients as elongated cells that

Table 1 Infection range of phage P1*kc* and DNA transfer frequencies of *bla* gene with *Enterobacteriaceae* strains as recipient[a]

Recipient	*Plaque*[b]	*Transductant frequency per indicated unit*[c]		*DNA transfer frequency per indicated unit as determined by indicated method*			
				CPRINS[d]		*DVC-CPRINS*[e]	
		Per CFU	*Per PFU*	*Per TDC*	*Per PFU*	*Per TDC*	*Per PFU*
C. freundii	−	ND[f]	ND	$(4 \pm 1) \times 10^{-3}$	$(4 \pm 1) \times 10^{-3}$	$(4 \pm 2) \times 10^{-3}$	$(4 \pm 2) \times 10^{-3}$
E. aerogenes	+	$(6 \pm 0.5) \times 10^{-7}$	$(6 \pm 0.5) \times 10^{-7}$	$(8 \pm 2) \times 10^{-3}$	$(8 \pm 2) \times 10^{-3}$	$(4 \pm 2) \times 10^{-3}$	$(4 \pm 2) \times 10^{-3}$
E. coli	+	$(2 \pm 0.5) \times 10^{-6}$	$(2 \pm 0.5) \times 10^{-6}$	$(3 \pm 1) \times 10^{-3}$	$(3 \pm 1) \times 10^{-3}$	$(2 \pm 0.8) \times 10^{-3}$	$(2 \pm 0.8) \times 10^{-3}$
P. mirabilis	−	ND	ND	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$	$<7 \times 10^{-5}$	$<7 \times 10^{-5}$
S. enteritidis	−	ND	ND	$(8 \pm 3) \times 10^{-4}$	$(8 \pm 3) \times 10^{-4}$	$(1 \pm 0.3) \times 10^{-3}$	$(1 \pm 0.3) \times 10^{-3}$
Y. enterocolitica	−	ND	ND	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$	$<1 \times 10^{-5}$	$<1 \times 10^{-5}$

Abbreviations: CFU, colony-forming unit; CPRINS-FISH, cycling primed *in situ* amplification-fluorescent *in situ* hybridization; DVC, direct viable count; LB, Luria–Bertain medium; MOI, multiplicity of infection; ND, not detectable; PFU, plaque-forming unit; TDC, total direct count.
[a]Frequencies are shown per CFU, PFU or TDC. Values indicate means ± s.d. values for triplicate samples.
[b]Infection range was determined by plaque assay. +, positive; −, negative.
[c]Transductants were incubated on LB agar medium containing ampicillin for 2 days.
[d]Frequencies were determined by CPRINS-FISH at MOI of 1.
[e]Viable cells carrying *gfp* gene were detected by combined DVC and CPRINS-FISH.
[f]ND, not detectable. When plating recipient cells at high cell density ($>10^7$ CFUs per ml), they could grow on LB agar medium containing ampicillin. Thus, it was hard to detect transductant.

Table 2 Infection range of phage T4GT7 and DNA-transfer frequencies of *bla* gene with *Enterobacteriaceae* strains as recipient[a]

Recipient	*Plaque*[b]	*Transductant frequency per indicated unit*[c]		*DN- transfer frequency per indicated unit as determined by indicated method*			
		Per CFU	*Per PFU*	*CPRINS*[d]		*DVC-CPRINS*[e]	
				Per TDC	*Per PFU*	*Per TDC*	*Per PFU*
C. freundii	−	ND[f]	ND	$(2 \pm 0.7) \times 10^{-3}$	$(2 \pm 0.7) \times 10^{-3}$	$(1 \pm 0.2) \times 10^{-3}$	$(1 \pm 0.2) \times 10^{-3}$
E. aerogenes	+	$< 2 \times 10^{-9}$	$< 2 \times 10^{-9}$	$(3 \pm 4) \times 10^{-4}$	$(3 \pm 4) \times 10^{-4}$	$(1 \pm 0.5) \times 10^{-3}$	$(1 \pm 0.5) \times 10^{-3}$
E. coli	+	$(2 \pm 0) \times 10^{-8}$	$(2 \pm 0) \times 10^{-8}$	$(2 \pm 0.8) \times 10^{-3}$	$(2 \pm 0.8) \times 10^{-3}$	$(6 \pm 5) \times 10^{-4}$	$(6 \pm 5) \times 10^{-4}$
P. mirabilis	−	ND	ND	$(5 \pm 1) \times 10^{-4}$	$(5 \pm 1) \times 10^{-4}$	$(1 \pm 1) \times 10^{-3}$	$(1 \pm 1) \times 10^{-3}$
S. enteritidis	−	ND	ND	$(5 \pm 4) \times 10^{-4}$	$(5 \pm 4) \times 10^{-4}$	$(8 \pm 2) \times 10^{-4}$	$(8 \pm 2) \times 10^{-4}$
Y. enterocolitica	−	ND	ND	$(2 \pm 0.2) \times 10^{-3}$	$(2 \pm 0.2) \times 10^{-3}$	$(2 \pm 0.8) \times 10^{-3}$	$(2 \pm 0.8) \times 10^{-3}$

Abbreviations: CFU, colony-forming unit; CPRINS-FISH, cycling primed *in situ* amplification-fluorescent *in situ* hybridization; DVC, direct viable count; LB, Luria–Bertain medium; MOI, multiplicity of infection; ND, not detectable; PFU, plaque-forming unit; TDC, total direct count.
[a]Frequencies are shown per CFU, PFU or TDC. Values indicate means ± s.d. values for triplicate samples.
[b]Infection range was determined by plaque assay. +, positive; −, negative.
[c]Transductants were incubated on LB agar medium containing ampicillin for 2 days.
[d]Frequencies were determined by CPRINS-FISH at MOI of 1.
[e]Viable cells carrying *gfp* gene were detected by combined DVC and CPRINS-FISH.
[f]ND, not detectable. When plating recipient cells at high cell density ($>10^7$ CFUs per ml), they could grow on LB agar medium containing ampicillin. Thus, it was hard to detect transductant.

showed bright Alexa Fluor 546 fluorescence under green excitation (Figure 2b). More than 30% of the remaining cells carrying the *bla* gene became elongated and/or fattened, that is, possessed protein synthesis activity. The transfer frequencies determined by DVC combined with CPRINS-FISH were at least three (P1*kc*) or five (T4GT7) orders of magnitude higher than for colony-forming bacteria on selective medium in plaque-forming strains. Our results show that DNA transfer mediated by phage P1*kc* and T4GT7 happened in more divergent strains than those estimated by conventional methods.

Gene transfer via isolated phage EC10

To examine the potential for gene transfer by phages in a freshwater environment, we prepared isolated transducing phage EC10 packed with the *gfp* gene, and DNA transfer experiments were performed using the *gfp* gene as an indicator of gene transfer. Phage EC10 that was propagated with *E. coli* NBRC 12713 RK2::*gfp* was mixed with *Enterobacteriaceae* strains.

Transfer of the *bla* and *gfp* gene on the plasmid RK2::*gfp* via phage EC10 was examined by both selective agar plating and CPRINS-FISH targeting the *bla* and *gfp* genes (Table 3). Frequencies on selective media containing ampicillin were undetectable in this study using *E. coli* NBRC 12713 RK2::*gfp* as a donor, although phage EC10 has the ability to transfer *E. coli* genes to both plasmids and chromosomes (Kenzaka *et al.*, 2007). Frequencies of DNA transfer determined by CPRINS-FISH targeting the *bla* gene were $7–10 \times 10^{-3}$ per PFU. Frequencies of DNA transfer of the *gfp* gene were slightly lower than those of the *bla* gene ($P<0.05$). Most gene-positive cells determined by CPRINS-FISH retained protein synthesis activity.

Next, the possible range of transfer of the *gfp* gene via EC10 was investigated using other *Enterobacteriaceae* strains as recipients (Table 4). Frequencies of DNA transfer determined by CPRINS-FISH targeting the *gfp* gene in the strains ranged from 3 to 4×10^{-3} per PFU. Viable cells carrying the transferred *gfp* gene determined by DVC combined with CPRINS-FISH constituted more than 30% of the *gfp* gene-positive cells determined by CPRINS-FISH. These frequencies were similar to plaque-forming *E. coli* (Table 3). The results demonstrated that isolated phage EC10 also has the ability to transfer the gene from *E. coli* to other *Enterobacteriaceae* strains, and conventional methods underestimate the range of phage-mediated gene transfer.

Frequency of DNA transfer at low bacterial concentrations

In natural aquatic environments, bacterial concentration is significantly lower than in laboratory conditions. Thus, DNA-transfer frequencies at low bacterial concentration were examined by CPRINS-FISH (Table 5). Although it was expected that phage absorption by recipients was reduced by the low bacterial concentration, DNA-transfer frequencies were similar at a concentration of $10^5–10^8$ *E. coli* cells per ml. Usually bacterial communities in aquatic environments consist of a small number of hosts and a large number of non-hosts. As phage EC10 did not transfer the *gfp* gene into *P. putida*, *E. coli* was added to *P. putida* and DNA transfer experiments were performed to examine the effect of a small population size on DNA-transfer frequency. The CPRINS-FISH analysis on a polycarbonate filter

Figure 2 Visualization of viable bacterial cells carrying the specific gene transferred by phage or stained by SYBR Gold-labeled phage. (**a** and **b**) *Citrobacter freundii* cells were mixed with phage P1 *kc* for 10 min. Viable *C. freundii* cells carrying the *bla* gene transferred by phage P1*kc* were detected by a combination of cycling primed *in situ* amplification-fluorescent *in situ* hybridization (CPRINS-FISH) and direct viable counting (DVC). (**a**) 4′,6-diamidino-2-phenylindole (DAPI) image. (**b**) Alexa Fluor 546 image. (**c** and **d**) Indigenous bacteria in river water taken at Juhachijo in the Kanzakigawa River were mixed with SYBR Gold-labeled phage EC10 for 20 min. The cells to which phage EC10 injected their DNA were detected by fluorescent bacteriophage assay. (**c**) DAPI image. (**d**) SYBR Gold image. (**e** and **f**) Indigenous bacteria in river water taken at Juhachijo in the Kanzakigawa River were mixed with phage EC10 for 20 min. Viable indigenous bacterial cells carrying the *gfp* gene transferred by phage EC10 were detected by a combination of CPRINS-FISH and DVC. (**e**) DAPI image. (**f**) Alexa Fluor 546 image. A full-color version of this figure is available at the ISME Journal online.

made it possible to concentrate target cells through filtration, and has advantages in estimating the frequency of gene transfer in samples with low bacterial concentrations, such as natural aquatic environments.

Gene transfer with indigenous bacterial communities as recipients

Potential gene transfer by phages in freshwater environments was examined with bacterial communities from oligotrophic and eutrophic rivers as

Table 3 DNA-transfer frequencies of *bla* gene and *gfp* gene via phage EC10 with *E. coli* NBRC12713 as recipient[a]

MOI	*Transductant frequency per indicated unit*[b]		*DNA transfer frequency of bla gene per indicated unit as determined by indicated method*				*DNA transfer frequency of gfp gene per indicated unit as determined by indicated method*			
			CPRINS[c]		*DVC-CPRINS*[d]		*CPRINS*[c]		*DVC-CPRINS*[d]	
	Per CFU	*Per PFU*	*Per TDC*	*Per PFU*	*Per TDC*	*Per PFU*	*Per TDC*	*Per PFU*	*Per TDC*	*Per PFU*
2	$<4\times10^{-9}$	$<1\times10^{-10}$	$(1\pm0.2)\times10^{-2}$	$(7\pm1)\times10^{-3}$	$(1\pm0.2)\times10^{-2}$	$(6\pm1)\times10^{-3}$	$(8\pm3)\times10^{-4}$	$(4\pm2)\times10^{-4}$	$(1\pm0.4)\times10^{-3}$	$(5\pm2)\times10^{-4}$
1	$<4\times10^{-9}$	$<2\times10^{-10}$	$(8\pm2)\times10^{-3}$	$(8\pm2)\times10^{-3}$	$(9\pm0.7)\times10^{-3}$	$(9\pm0.7)\times10^{-3}$	$(6\pm2)\times10^{-4}$	$(6\pm2)\times10^{-4}$	$(1\pm0.6)\times10^{-3}$	$(1\pm0.6)\times10^{-3}$
0.2	$<4\times10^{-9}$	$<1\times10^{-9}$	$(3\pm0.8)\times10^{-3}$	$(1\pm0.4)\times10^{-2}$	$(4\pm0.9)\times10^{-3}$	$(2\pm0.5)\times10^{-2}$	$(3\pm2)\times10^{-4}$	$(2\pm0.7)\times10^{-3}$	$(6\pm2)\times10^{-4}$	$(3\pm0.7)\times10^{-3}$

Abbreviations: CFU, colony-forming unit; CPRINS-FISH, cycling primed *in situ* amplification-fluorescent *in situ* hybridization; DVC, direct viable count; LB, Luria–Bertain medium; MOI, multiplicity of infection; ND, not detectable; PFU, plaque-forming unit; TDC, total direct count.
[a]Frequencies are shown per CFU, PFU or TDC. Values indicate means ± s.d. values for triplicate samples.
[b]Transductants were incubated on LB agar medium containing ampicillin for 2 days. ND, not detectable.
[c]Frequencies were determined by CPRINS-FISH.
[d]Viable cells carrying *gfp* gene were detected by combined DVC and CPRINS-FISH.

Table 4 Infection range of phage EC10 and DNA transfer frequencies of *gfp* gene with *Enterobacteriaceae* strains as recipient[a]

Recipient	*Plaque*[b]	*DNA transfer frequency per indicated unit as determined by indicated method*			
		CPRINS[c]		*DVC-CPRINS*[d]	
		Per TDC	*Per PFU*	*Per TDC*	*Per PFU*
C. freundii	−	$(6 \pm 0.7) \times 10^{-3}$	$(3 \pm 0.3) \times 10^{-3}$	$(3 \pm 0.9) \times 10^{-3}$	$(1 \pm 0.5) \times 10^{-3}$
E. aerogenes	−	$(8 \pm 2) \times 10^{-3}$	$(4 \pm 0.7) \times 10^{-3}$	$(3 \pm 0.6) \times 10^{-3}$	$(1 \pm 0.3) \times 10^{-3}$
P. mirabilis	−	$(5 \pm 1) \times 10^{-3}$	$(3 \pm 0.6) \times 10^{-3}$	$(1 \pm 1) \times 10^{-4}$	$(5 \pm 5) \times 10^{-4}$
S. enteritidis	−	$(7 \pm 0.8) \times 10^{-3}$	$(3 \pm 4) \times 10^{-3}$	$(5 \pm 2) \times 10^{-3}$	$(2 \pm 1) \times 10^{-3}$
S. marcescens	−	$(8 \pm 2) \times 10^{-3}$	$(4 \pm 1) \times 10^{-3}$	$(6 \pm 2) \times 10^{-3}$	$(3 \pm 1) \times 10^{-3}$

Abbreviations: CFU, colony-forming unit; CPRINS-FISH, cycling primed *in situ* amplification-fluorescent *in situ* hybridization; DVC, direct viable count; MOI, multiplicity of infection; ND, not detectable; PFU, plaque-forming unit; TDC, total direct count.
[a]Frequencies are shown per TDC or PFU. Values indicate means ± s.d. values for triplicate samples.
[b]Infection range was determined by plaque assay. +, positive; −, negative.
[c]Frequencies were determined by CPRINS-FISH at MOI of 2.
[d]Viable cells carrying *gfp* gene were detected by combined DVC and CPRINS-FISH.

Table 5 DNA transfer frequencies of *gfp* gene via phage EC10 with different concentration of *Escherichia coli* NBRC12713 as recipients[a]

Sample	*TDC (cells ml^{-1})*	*DNA transfer frequency per indicated unit as determined by CPRINS*[b]	
		Per TDC	*Per PFU*
E. coli	1.3×10^{8}	$(8 \pm 4) \times 10^{-3}$	$(4 \pm 2) \times 10^{-3}$
E. coli	1.0×10^{7}	$(3 \pm 2) \times 10^{-3}$	$(2 \pm 0.9) \times 10^{-3}$
E. coli	1.6×10^{6}	$(7 \pm 6) \times 10^{-3}$	$(4 \pm 3) \times 10^{-3}$
E. coli	1.6×10^{5}	$(3 \pm 2) \times 10^{-3}$	$(2 \pm 0.8) \times 10^{-3}$
P. putida+*E. coli*[c]	3.7×10^{7}	$(1 \pm 0.5) \times 10^{-3}$	$(7 \pm 2) \times 10^{-2}$
P. putida+*E. coli*	3.3×10^{6}	$(8 \pm 0.7) \times 10^{-4}$	$(4 \pm 0.3) \times 10^{-2}$
P. putida+*E. coli*	4.3×10^{5}	$(2 \pm 0.8) \times 10^{-3}$	$(7 \pm 4) \times 10^{-2}$

Abbreviations: CFU, colony-forming unit; CPRINS-FISH, cycling primed *in situ* amplification-fluorescent *in situ* hybridization; DVC, direct viable count; MOI, multiplicity of infection; ND, not detectable; PFU, plaque-forming unit; TDC, total direct count.
[a]Frequencies are shown per TDC or PFU. Values indicate means ± s.d. values for triplicate samples.
[b]Frequencies were determined by CPRINS-FISH at MOI of 2.
[c]1% of *E. coli* were added to *P. putida*.

recipients. Four samples were collected from different rivers including the Kanzakigawa River from which phage EC10 was isolated. Plasmid RK2::*gfp* encodes ampicillin, kanamycin and tetracycline resistance genes (Thomas, 1981), but a significant part of indigenous bacteria from these sites (10^{-5}–10^{-4} per TDC) were resistant to these antibiotics. The presence of antibiotic-resistant bacteria in the river samples made it difficult to detect transductants by culture-dependent methods using selective medium. Both *E. coli* W3110 RK2::*gfp* and NBRC 12713 RK2::*gfp* strains were used as donors. There was no difference in the DNA-transfer frequencies between these donor strains when *E. coli* NBRC 12713 was used as a recipient (data not shown).

To assess the possible range of DNA injection by phage EC10, fluorescent bacteriophage assay was carried out first (Hennes *et al.*, 1995; Noble and Fuhrman, 2000). Although plaque formation was observed only in *E. coli*, phage DNA was injected by phage EC10 into *C. freundii*, *E. aerogenes*, *P. mirabilis*, *S. enteritidis* and *S. marcescens*. Fluorescent bacteriophage assay showed that phage EC10 could infect indigenous bacterial cells in river water samples (Figures 2c and d). Only cells stained by fluorescently labeled-phage EC10 emitted the green fluorescence of SYBR Gold under blue excitation (Figure 2d). The frequency of cells stained by SYBR gold-labeled phage EC10 constituted $0.4–2 \times 10^{-2}$ per TDC in the river water samples (Table 6). The abundance of *E. coli* in these rivers determined by FISH with a ribosomal RNA-targeted probe for *E. coli* (Kenzaka *et al.*, 2001) was about 10^{-4} of TDC, which was lower than the numbers determined by fluorescent bacteriophage assay. Thus, phage EC10 was thought to inject DNA into non-*E. coli* cells in the samples.

The 'mixing ratio' was calculated on the basis of the number of cells stained by SYBR gold-labeled phage EC10 in the river water samples. Then the frequencies of the *gfp* gene transfers to indigenous bacterial communities were investigated by DVC combined with CPRINS-FISH (Table 6). The DVC-positive cells in river water samples accounted for 15–30% of the total bacteria at Juhachijo, 9–34% at Kitahashi, 8–28% at Kuwazu and 17–24% at Takiue through the sampling periods. Independent of the mixing ratio (2, 20 and 200), the DNA-transfer frequencies per TDC determined by CPRINS-FISH or DVC combined with CPRINS-FISH at Juhachijou were similar. Thus, the indigenous bacterial populations to which phage EC10 could inject the *gfp* gene in river water samples were confined to limited cells.

Gene transfer by phage EC10 was also observed in other river water samples from oligotrophic (Takiue) and eutrophic (Juhachijo, Kitahashi and Kuwazu) sites (Table 6). Indigenous bacteria from these four locations did not show positive signals with CPRINS-FISH before transducing phages were

Table 6 DNA transfer frequencies of *gfp* gene via phage EC10 with indigenous bacteria in rivers as recipients[a]

Sampling site	*Date (mo/day/yr)*	*TDC (cells ml^{-1})*	*DVC (cells ml^{-1})*	*DNA injection frequency per TDC as determined by FBA*[b]	*Donor E. coli strain with RK2::gfp*	*Mixing ratio*	*DNA transfer frequency per indicated unit as determined by indicated method*			
							CPRINS[c]		*DVC-CPRINS*[d]	
							Per TDC	*Per PFU*	*Per TDC*	*Per PFU*
Juhachijou	02/22/05	2.2×10^6	5.9×10^5	$(2 \pm 0.7) \times 10^{-2}$	W3110	200	$(2 \pm 0.1) \times 10^{-3}$	$(4 \pm 0.3) \times 10^{-4}$	$(6 \pm 2) \times 10^{-4}$	$(2 \pm 0.5) \times 10^{-4}$
					W3110	20	$(1 \pm 0.2) \times 10^{-3}$	$(3 \pm 0.4) \times 10^{-3}$	$(6 \pm 1) \times 10^{-4}$	$(2 \pm 0.3) \times 10^{-3}$
					W3110	2	$(2 \pm 0.1) \times 10^{-3}$	$(5 \pm 0.3) \times 10^{-2}$	$(1 \pm 0.1) \times 10^{-3}$	$(3 \pm 0.3) \times 10^{-3}$
	03/07/05	1.6×10^6	3.2×10^5	$(2 \pm 1) \times 10^{-2}$	W3110	2	$(4 \pm 0.7) \times 10^{-4}$	$(1 \pm 0.2) \times 10^{-2}$	$(2 \pm 0.1) \times 10^{-4}$	$(4 \pm 0.2) \times 10^{-3}$
					NBRC12713	2	$(3 \pm 2) \times 10^{-4}$	$(6 \pm 4) \times 10^{-3}$	$(8 \pm 0.4) \times 10^{-4}$	$(2 \pm 0.1) \times 10^{-2}$
	10/18/09	3.1×10^6	7.0×10^5	$(8 \pm 5) \times 10^{-3}$	W3110	2	$(1 \pm 0.3) \times 10^{-3}$	$(6 \pm 2) \times 10^{-2}$	$(6 \pm 0.9) \times 10^{-4}$	$(4 \pm 0.5) \times 10^{-2}$
	10/26/09	3.1×10^6	9.2×10^5	$(6 \pm 3) \times 10^{-3}$	W3110	2	$(1 \pm 0.5) \times 10^{-3}$	$(9 \pm 6) \times 10^{-2}$	$(1 \pm 0.4) \times 10^{-3}$	$(9 \pm 5) \times 10^{-2}$
	11/09/09	2.3×10^6	3.4×10^5	$(7 \pm 1) \times 10^{-3}$	W3110	2	$(8 \pm 1) \times 10^{-4}$	$(8 \pm 1) \times 10^{-2}$	$(2 \pm 1) \times 10^{-4}$	$(2 \pm 1) \times 10^{-2}$
Kitahashi	03/07/05	1.1×10^6	5.6×10^4	$(1 \pm 0.4) \times 10^{-2}$	W3110	2	$< 5 \times 10^{-5}$	$< 1 \times 10^{-3}$	$(3 \pm 0.7) \times 10^{-4}$	$(6 \pm 2) \times 10^{-3}$
					NBRC12713	2	$(1 \pm 0.2) \times 10^{-4}$	$(3 \pm 0.5) \times 10^{-3}$	$< 5 \times 10^{-5}$	$< 1 \times 10^{-3}$
	10/18/09	1.7×10^6	5.8×10^5	$(2 \pm 0.3) \times 10^{-2}$	W3110	2	$(1 \pm 0.1) \times 10^{-3}$	$(3 \pm 0.2) \times 10^{-2}$	$(8 \pm 3) \times 10^{-4}$	$(3 \pm 0.1) \times 10^{-2}$
	10/26/09	1.8×10^6	6.4×10^5	$(7 \pm 5) \times 10^{-3}$	W3110	2	$(2 \pm 0.6) \times 10^{-3}$	$(9 \pm 4) \times 10^{-2}$	$(7 \pm 5) \times 10^{-4}$	$(5 \pm 4) \times 10^{-2}$
	11/09/09	1.5×10^6	3.9×10^5	$(7 \pm 1) \times 10^{-3}$	W3110	2	$(9 \pm 1) \times 10^{-4}$	$(6 \pm 0.9) \times 10^{-2}$	$(6 \pm 1) \times 10^{-4}$	$(4 \pm 0.8) \times 10^{-2}$
Kuwazu	03/07/05	1.0×10^6	8.2×10^4	$(1 \pm 0.6) \times 10^{-2}$	W3110	2	$(2 \pm 0.1) \times 10^{-3}$	$(7 \pm 0.3) \times 10^{-2}$	$(4 \pm 0.9) \times 10^{-4}$	$(2 \pm 0.4) \times 10^{-2}$
					NBRC12713	2	$(1 \pm 0.1) \times 10^{-3}$	$(5 \pm 0.6) \times 10^{-2}$	$(6 \pm 0.5) \times 10^{-4}$	$(2 \pm 0.2) \times 10^{-2}$
	10/18/09	1.2×10^6	3.3×10^5	$(2 \pm 1) \times 10^{-2}$	W3110	2	$(6 \pm 4) \times 10^{-4}$	$(1 \pm 1) \times 10^{-2}$	$(2 \pm 2) \times 10^{-4}$	$(6 \pm 4) \times 10^{-3}$
	10/26/09	7.2×10^5	1.7×10^5	$(2 \pm 0.3) \times 10^{-2}$	W3110	2	$(1 \pm 0.8) \times 10^{-3}$	$(4 \pm 3) \times 10^{-2}$	$(8 \pm 6) \times 10^{-4}$	$(3 \pm 2) \times 10^{-2}$
	11/09/09	7.7×10^5	1.1×10^5	$(6 \pm 1) \times 10^{-3}$	W3110	2	$(1 \pm 0.4) \times 10^{-3}$	$(9 \pm 3) \times 10^{-2}$	$(1 \pm 0.3) \times 10^{-3}$	$(8 \pm 2) \times 10^{-2}$
Takiue	03/07/05	2.3×10^5	4.5×10^4	$(4 \pm 2) \times 10^{-3}$	W3110	2	$(3 \pm 0.6) \times 10^{-4}$	$(3 \pm 0.8) \times 10^{-2}$	$(1 \pm 0.1) \times 10^{-4}$	$(1 \pm 0.1) \times 10^{-2}$
					NBRC12713	2	$(3 \pm 0.3) \times 10^{-4}$	$(4 \pm 0.3) \times 10^{-2}$	$(3 \pm 0.7) \times 10^{-4}$	$(4 \pm 0.8) \times 10^{-2}$
	10/18/09	2.8×10^5	4.8×10^4	$(1 \pm 0.3) \times 10^{-2}$	W3110	2	$(4 \pm 3) \times 10^{-4}$	$(2 \pm 2) \times 10^{-2}$	$(4 \pm 4) \times 10^{-4}$	$(2 \pm 2) \times 10^{-2}$
	10/26/09	4.8×10^5	1.1×10^5	$(4 \pm 2) \times 10^{-3}$	W3110	2	$(9 \pm 4) \times 10^{-4}$	$(9 \pm 5) \times 10^{-2}$	$(5 \pm 4) \times 10^{-4}$	$(6 \pm 4) \times 10^{-2}$
	11/09/09	7.5×10^5	6.3×10^4	$(8 \pm 2) \times 10^{-3}$	W3110	2	$(6 \pm 3) \times 10^{-4}$	$(4 \pm 2) \times 10^{-2}$	$(1 \pm 0.9) \times 10^{-4}$	$(8 \pm 6) \times 10^{-3}$

Abbreviations: CFU, colony-forming unit; CPRINS-FISH, cycling primed *in situ* amplification-fluorescent *in situ* hybridization; DVC, direct viable count; FBA, fluorescent bacteriophage assay; MOI, multiplicity of infection; ND, not detectable; PFU, plaque-forming unit; TDC, total direct count.
[a]Frequencies are shown per TDC or PFU. Values indicate means ± s.d. values for triplicate samples.
[b]DNA injection was determined by fluorescent bacteriophage assay with phage EC10.
[c]Frequencies were determined by CPRINS-FISH.
[d]Viable cells carrying *gfp* gene were detected by combined DVC and CPRINS-FISH.

added, but they were CPRINS-FISH positive after gene transfer experiments were performed with phage EC10. The frequencies of *gfp* gene transfer in these bacterial communities ranged from undetectable to 2×10^{-3} cells per TDC, which correspond to undetectable to 9×10^{-2} cells per PFU. Viable cells carrying the transferred *gfp* gene were detected by DVC combined with CPRINS-FISH (Figures 2e and f), and they constituted more than 20% of the *gfp* gene-positive cells in most cases. The frequencies of *gfp* gene-positive viable cells ranged from undetectable to 9×10^{-2} cells per PFU (undetectable to 1×10^{-3} cells per TDC). Although it was hard to detect transductants using the culture-dependent method, DVC combined with CPRINS-FISH clarified the DNA-transfer frequencies through the *E. coli* phage in the river water samples at the single-cell level. The frequencies of *gfp* gene-positive viable cells per PFU at each site were compared between early spring and autumn, and among three sampling dates in autumn. Overall, the obtained frequencies at each site were not significantly different among sampling dates ($P>0.05$) except those at Juhachijo between 7 March 2005 and 18 October 2009 ($P<0.05$).

Discussion

This study attempted to accurately determine the frequency and possible range of DNA transfer mediated by *E. coli* phages using a gene-targeting approach. Although advances in genomics and nucleotide sequence analysis have highlighted the significance of phages in lateral gene transfer as possible contributors to bacterial evolution (Bordenstein and Reznikoff, 2005), transduction frequencies in laboratory experiments using selective media in most studies were generally reported to be low and the range of phage-mediated gene transfer was considered narrow, although broad host-range phages were occasionally found in aquatic environments (Beumer and Robinson, 2005). To understand gene flow among bacteria, sensitive and reliable methods of detecting transferred DNA are necessary, which should have the following properties: (i) recognition of transferred gene in individual cells without the requirement of cultivation or gene expression, (ii) effective concentration of target bacteria that acquire the indicator gene, for example, by filtration, and (iii) identical conditions that can be applied to diverse bacteria because the gene is expected to be transferred to bacteria physiology of which is unclear. In this study, we used a gene-targeting approach based on *in situ* DNA amplification, which enabled us to determine the frequency and possible range of gene transfer at the single-cell level.

Previous studies show transduction frequencies determined by culture-dependent methods using selective agar media varied over orders of magnitude from 10^{-11} to 10^{-5} per PFU in freshwater and marine systems (Weinbauer and Rassoulzadegan, 2004). In contrast, DNA-transfer frequencies determined by DVC combined with CPRINS-FISH for *gfp* gene were up to 3×10^{-3} per PFU in *Enterobacteriaceae* strains, and up to 9×10^{-2} per PFU in natural bacterial communities; a few orders of magnitude greater than those previously measured throughout the studied periods.

Conventional methods confirmed that the range of gene transfer by phage P1*kc*, T4GT7 and EC10 was limited to plaque-forming strains (*E. coli* and *E. aerogenes*), but CPRINS-FISH revealed that the three *E. coli* phages could mediate transfer of the gene from *E. coli* to non-plaque-forming *Enterobacteriaceae* strains. Specifically, some bacteria, which were not thought to be a 'host' or 'recipient' when conventional methods were used, were able to receive the bacterial gene via the *E. coli* phages. The phage life cycle consists of adsorption into a cell, injection of nucleic acid, commandeering of host machinery, production of phage proteins and nucleic acid, assembly and release by either lysis or extrusion (Wommack and Colwell, 2000). For visible plaques, it is necessary to complete these processes and disperse effectively within soft agar. Certain phages in natural environments do not form plaques on any known host because they have the mechanism to suppress host lysis (lysogeny), have a mutation in the genes responsible for lysis or lack genetic material themselves (defective phage; Garro and Marmur, 1970). The presence of such phages masks actual host range. The gene-targeting approach described here makes it possible to detect target DNA sequences in bacterial cells without cultivation or gene expression, and is less likely to be biased by physiological or genetic (that is, marker expression) limitations. Consequently, our results suggest that DNA exchange among bacteria via phage may occur in a more divergent range of bacteria than previously thought using conventional methods.

The entry of DNA into a recipient bacterium is an important first step in genetic diversification through lateral gene transfer. On DNA entry into a cell, binding between the phage receptor on the cell surface and the tail fiber on the phage is necessary. Our results show that three *E. coli* phages (P1*kc*, T4GT7 and EC10) attached on more divergent strains than those estimated by conventional methods, and transferred the *E. coli* gene to the strains (Table 1, 2, and 4). These results indicate that the binding between the phage receptor and the tail fiber might be more plastic than previously thought. In addition, the structure of the phage receptor on the cell surface and the tail fiber on the phage could be changed by mutation (Yu and Mizushima, 1982; Tetart *et al.*, 1996). This would allow the movement of DNA molecules among more divergent genera mediated by phages more frequently.

The fate of the transferred gene in recipient cells is of interest for genomic diversity studies. During the incubation for DVC, a proportion of transferred genes inside recipient cells may be destroyed,

but DVC combined with CPRINS-FISH showed that a significant proportion of the transferred gene remained in viable recipient cells. Potential future study should examine the frequency and mechanism of the maintenance of transferred genes and aim to elucidate the mechanism of maintenance based on DNA sequence.

Previous studies have shown that a high degree of genomic diversity may occur among closely related genomes (Ohnishi *et al.*, 2002). Genomes of *Vibrio splendidus* in natural coastal areas showed extensive allelic diversity, and the group consists of at least 1000 distinct genotypes in 1 ml of sea water (Thompson *et al.*, 2005). Such variation might arise because of DNA exchange among microorganisms at the single-cell level. Several prokaryotic genomes contain large fractions of foreign genes, for example, more than 15% of the genes of *E. coli* have been acquired by lateral gene transfer (Lawrence and Ochman, 1998). The events of gene transfer have significantly driven diversification in the bacterial genome.

This study offers a way to address the movement of a specific gene among bacterial cells *in situ*, regardless of culturability or gene expression. Our results suggest that DNA exchange among bacteria via phages in natural aquatic environments may occur in a more divergent range of bacteria and more frequently than thought previously using conventional methods. 'Lateral gene transfer', in the general meaning, consists of several steps: entry of the foreign gene into viable recipient cells, gene replication, maintenance during cell growth, gene expression and so forth. Reporter gene technology, such as green fluorescent protein, allows the estimation of bacterial cells in which the reporter gene is expressed (Dahlberg *et al.*, 1998; Hendrickx *et al.*, 2003), although potential limitations and advantages have been discussed (Sorensen *et al.*, 2005; Maruyama *et al.*, 2006). Our approach has a great potential to provide more quantitative information on gene transfer steps in the natural environment. It will be important in the future to clarify whether such extensive gene transfer events via phages observed at the single-cell level are a general feature of interactions between natural bacteria and phages using other phage-host systems.

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