High-Frequency Phage-Mediated Gene Transfer among *Escherichia coli* Cells, Determined at the Single-Cell Level

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Recent whole-genome analysis suggests that lateral gene transfer by bacteriophages has contributed significantly to the genetic diversity of bacteria. To accurately determine the frequency of phage-mediated gene transfer, we employed cycling primed in situ amplification-fluorescent in situ hybridization (CPRINS-FISH) and investigated the movement of the ampicillin resistance gene among *Escherichia coli* cells mediated by phage at the single-cell level. Phages P1 and T4 and the newly isolated *E. coli* phage EC10 were used as vectors. The transduction frequencies determined by conventional plating were 3 × 10⁻⁸ to 2 × 10⁻⁶, 1 × 10⁻⁸ to 4 × 10⁻⁸, and ≤4 × 10⁻⁸ per PFU for phages P1, T4, and EC10, respectively. The frequencies of DNA transfer determined by CPRINS-FISH were 7 × 10⁻⁴ to 1 × 10⁻³, 9 × 10⁻¹ to 3 × 10⁻³, and 5 × 10⁻⁴ to 4 × 10⁻³ for phages P1, T4, and EC10, respectively. Direct viable counting combined with CPRINS-FISH revealed that more than 20% of the cells carrying the transferred gene retained their viabilities. These results revealed that the difference in the number of viable cells carrying the transferred gene and the number of cells capable of growth on the selective medium was 3 to 4 orders of magnitude, indicating that phage-mediated exchange of DNA sequences among bacteria occurs with unexpectedly high frequency.

Recent nucleotide and whole-genome analyses have revealed that most bacterial genomes contain large amounts of bacteriophage DNA (18). This finding suggests that lateral gene transfer by bacteriophages has contributed significantly to the acquisition of new genetic traits, the ability of bacteria to exploit new environments, and the genetic diversity of bacteria (27). Since bacteriophage-mediated gene transfer was first recognized (32), transduction has been found to occur in many phage-host systems, and various aspects of transduction, including molecular mechanisms, physiologic and genetic characterization of transductants, and ideal environments for transduction, have been investigated (29).

For nearly a half-century, culture methods using selective agar media have played a leading role in the study of gene transfer (5). Genetic characteristics such as amino acid deficiency repair and antibiotic resistance have been used for selection of transductants (9). Transduction frequencies were shown to differ over orders of magnitude from 10⁻¹¹ to 10⁻⁵ per bacteriophage, which are lower than those for conjugation and transformation (14, 27). However, current knowledge of horizontal gene transfer via bacteriophages in the environment is rather limited because of methodological constraints.

Conventional methods for the detection of gene transfer depend on high levels of gene expression and culturability on selective media. Although these methods have led to an understanding of the genetic and physiologic characteristics of transductants and the molecular mechanism of transduction, they have limited abilities to quantify the genetic material introduced into individual cells and provide little information about gene flow among bacteria at the DNA level. The expression level of the transferred gene and the culturability on media may differ for each recipient cell. In addition, many prokaryotic genomes contain a large fraction of foreign genes; for example, more than 15% of the genomes of *Escherichia coli* have been acquired by lateral transfer (13). This suggests that lateral gene transfer contributes to the genetic diversity of bacterial genomes (2), and we hypothesized that DNA fragments are transferred among bacteria at higher rates than those shown by culture-based methods using selective media. In order to accurately quantify DNA movement, gene-targeting approaches without the requirement for cultivation or gene expression are necessary.

In this study, we employed an in situ DNA amplification technique (cycling primed in situ amplification-fluorescent in situ hybridization [CPRINS-FISH]) in which the target sequence is amplified inside the cell (11). With CPRINS-FISH, gene movement among *Escherichia coli* cells mediated by bacteriophage was examined at the single-cell level. To explore the viabilities of cells that acquired the gene from phage, direct viable counting (DVC) was carried out (12). The DVC method is based on the incubation of samples with antimicrobial agents and nutrients. The antibiotic cocktail acts as a specific inhibitor of DNA synthesis and prevents cell division without affecting other metabolic activities. The resulting cells can continue to metabolize nutrients and elongate and/or become fattened after incubation. Simultaneous DVC-positive and CPRINS-FISH-positive cells represent viable cells carrying the transferred gene.

In this study, three *E. coli* phages were used as vectors. Phage P1 is the most commonly used generalized transducing phage for *E. coli* (22). P1 packages DNA by the “headful packaging” mechanism (20, 23). The DNA packaging of concatemeric DNA consisting of repeating units of the viral ge-
tone, 0.5% yeast extract, 0.5% NaCl). E. coli Bacteria were cultured at 37°C in aerobic Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Enrichment cultures were incubated overnight, whereupon the samples were treated with chloroform and centrifuged to eliminate all viable bacterial cells. DNA from bacteriophages was isolated by an enrichment procedure in E. coli containing a 1:100 (vol/vol) dilution of an overnight culture of strains which carry the bla gene in plasmid RK2 is a part of the chromosome; E. coli NBRC 12713 KEN201, which carries Tn1 of the chromosome; E. coli NBRC 12713 RK2, which carries plasmid RK2; Hafnia alvei 1; Klebsiella oxytoca ATCC 1382T; Salmonella enterica serovar Enteritidis I1 640; Shigella sonnei I1 969; and Yersinia enterocolitica I1 981. Plasmid RK2 carried the bla gene, a tetracycline resistance gene (tetA), and a kanamycin resistance gene (aph3). The bla gene in plasmid RK2 is a part of the chromosome. A PCR product was purified using the CEQ8000 genetic analysis system (Beckman Coulter) with M13 primers. The dye terminator cycle-sequencing reactions were performed according to the manufacturer’s procedures.

Viabilities of recipient cells determined by DVC. In order to explore the viabilities of the recipient cells carrying the transferred gene, DVC was carried out. After the recipient cell culture was mixed with each transducing phage (P1E, T4GT7, and EC10) under the above-described conditions. A portion was filtered through a gelatin [0.1% gelatin, 0.001% CuSO4, 0.001% CeCl3·7H2O]-coated polycarbonate white filter (0.2-μm pore size, 25-mm diameter; ADVANTEC) and rinsed twice with filtered deionized water. Then, samples were stored at 20°C. Before transduction and DNA transfer experiments, we extracted DNA from each phage (P1, T4, and EC10) which infected E. coli without the bla gene by using the Wizard Lambda Prep DNA purification system (Promega) as described above and confirmed that the bla gene was not a part of the phage genome by using PCR as follows. A PCR mixture containing 1× PCR buffer II (Applied Biosystems), 2.0 mM MgCl2, 0.2 mM concentrations of each deoxynucleoside triphosphate, 0.4 μM concentrations of the AmpR20f [5′-GTG TCGGCCTTATTCCCTTTS-3′] and AmpR480r [5′-GGCACCTATCTCAGCGAAC-3′] primer sets (11), and 2.5 U of AmpliTaq Gold (Applied Biosystems) was made up with DNA-free water. PCR 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 extension at 72°C for 1.5 min. Amplification was repeated for 30 cycles with a thermal cycler (PTC-200; MJ Research Inc.).

Viabilities of recipient cells determined by DVC. In order to explore the viabilities of the recipient cells carrying the transferred gene, DVC was carried out. After the recipient cell culture was mixed with each transducing phage (P1E, T4GT7, EC10) at 37°C for 20 min as described above, 20 μl of the mixture was transferred to 180 μl of LB broth containing an antibiotic cocktail (final concentration: 20 μg of nalidixic acid, 10 μg of pipromic acid, 10 μg of pipemidic acid, 10 μg of cephalaxin, 0.1 μg of ciproflaxin per ml) (10) and incubated at 37°C for 3 h. Cells that exceeded at least twice the mean length of cells determined before DVC under epifluorescence microscopy as described below were scored as elongated. After incubation for DVC, samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 16 h after the recipient cell culture was mixed with each transducing phage (P1E, T4GT7, and EC10) under the above-described conditions. A portion was filtered through a gelatin [0.1% gelatin, 0.01% CuSO4, 0.01% CeCl3·7H2O]-coated polycarbonate white filter (0.2-μm pore size, 25-mm diameter; ADVANTEC) and rinsed twice with filtered deionized water. Then, samples were stored at 20°C. Before transduction and DNA transfer experiments, we extracted DNA from each phage (P1, T4, and EC10) which infected E. coli without the bla gene by using the Wizard Lambda Prep DNA purification system (Promega) as described above and confirmed that the bla gene was not a part of the phage genome by using PCR as follows. A PCR mixture containing 1× PCR buffer II (Applied Biosystems), 2.0 mM MgCl2, 0.2 mM concentrations of each deoxynucleoside triphosphate, 0.4 μM concentrations of the AmpR20f [5′-GTGTT CGGCCTTATTCCCTTTS-3′] and AmpR480r [5′-GGCACCTATCTCAGCGAAC-3′] primer sets (11), and 2.5 U of AmpliTaq Gold (Applied Biosystems) was made up with DNA-free water. PCR 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 extension at 72°C for 1.5 min. Amplification was repeated for 30 cycles with a thermal cycler (PTC-200; MJ Research Inc.).
through a gelatin (0.1% gelatin, 0.01% Ca(SO)4)-coated polycarbonate white filter (0.2-μm pore size, 25-mm diameter; ADVANCE) and rinsed twice with filtered deionized water. Then, samples were stored at −20°C.

**CPRINS-FISH.** In order to detect cells carrying the **bla** gene transferred by bacteriophage, CPRINS-FISH was performed. Permeabilization for CPRINS-FISH was carried out as described by Kenzaka et al. (11). The filters with bacterial cells were coated with gelatin to avoid cell loss during extensive cell wall permeabilization. After bozoyme treatment, each filter was cut into 16 sections and subjected to CPRINS-FISH. A 1/16 section of the filter was transferred to a microscope (volume, 0.2 ml) and immersed in 100 μl of the CPRINS buffer, containing 1× PCR buffer II (Applied Biosystems), 2.0 mM MgCl2, 0.2 mM of each deoxynucleoside triphosphate, 0.4 μM AmpliTag Gold ( Applied Biosystems), CPRINS 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 extension at 72°C for 1.5 min. Amplification was repeated for 30 cycles with a thermal cycler (PTC-200; MJ Research Inc.). After the amplification, filters were rinsed with 0.1% Nonidet P-40 and sterile deionized water, dehydrated in 99% ethanol, and vacuum dried. Multiply labeled fluorescent probes were used to hybridize amplicons and improve specificity and sensitivity. Filters were soaked in 100 μl of hybridization buffer (1 M Betain, 20 mM Tris-HCl [pH 8.8], 10 mM KCl, 10 mM Na2SO4, 0.1% Triton X-100) containing 5 to 20 ng of an Alexa Fluor 546-labeled probe set (AmpliTag Gold; Applied Biosystems), 0.4 mM of each deoxynucleoside triphosphate, 0.4 μM AmpliTag Gold (Applied Biosystems), and 0.4 μM of each deoxynucleoside triphosphate, 0.4 μM primers, and 2.5 U of TaqTag LA Taq (Takara Bio Inc.), was made up with DNA-free water. The PCR cycles consisted of denaturation at 98°C for 20 s and annealing and extension at 68°C for 15 min. Amplification was repeated for 30 cycles. Sequence analysis was performed on a CEQ8000 genetic analysis system (Beckman Coulter) with the AmpliTag Gold, AmpliPloyLA, and Altt6698R primers (see below).

After a sequence downstream of the **bla** gene on the transductant chromosome was revealed to be a part of the **tau** gene next to the terminal inserted-repeat sequence of transposon Tn1, the **usu** gene-specific primers (AmpliTag Gold; Applied Biosystems), 0.4 mM of each deoxynucleoside triphosphate, 0.4 μM primers, and 2.5 U of TaqTag LA Taq (Takara Bio Inc.), was made up with DNA-free water. The PCR cycles consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 5 min. Amplification was repeated for 30 cycles. If Tn1 was inserted, the size of the PCR product was expected to be 4150 bp when the combination of the Altro6460F and Amp154LAr primers was used. Amplification of the **bla** gene was then carried out. PCR cycles were 0.1% Nonidet P-40 in PBS) at 70°C for 30 min and then washed with hybridization buffer (0.1% Nonidet P-40 in PBS) at 70°C for 10 min. Finally, filters were counterstained with 1 μg ml−1 4′,6-diamidino-2-phenylindole (DAPI) for 10 min.

Filters were mounted in VECTASHELL (Vector Laboratories Inc., Burlingame, CA) for observation by epifluorescence microscopy. In order to exclude the possibility of nonspecific probe binding to cell structures other than target DNA in the target cells, the following were performed: (i) FISH using laboratory strains without amplification of target DNA; (ii) CPRINS-FISH targeting of the **BLA** gene sequence; and (iii) CPRINS-FISH targeting of the chloroamphenicol acetyltransferase gene, using laboratory strains that did not carry the chloroamphenicol acetyltransferase gene (11).

**Epifluorescence microscopy.** The filters were observed under an epifluorescence microscope (E-400; Nikon, Tokyo, Japan) with the Nikon filter sets (FT565, and BP610/75) for Alexa Fluor 546. Images were acquired by a cooled charge-coupled device camera (CoolSNAP; Roper Photometrics) and stored as high-resolution images for analysis.

**DNA extraction.** After a sequence downstream of the **bla** gene on the transductant chromosome was revealed to be a part of the **tau** gene next to the terminal inserted-repeat sequence of transposon Tn1, the **usu** gene-specific primers (AmpliTag Gold; Applied Biosystems), 0.4 mM of each deoxynucleoside triphosphate, 0.4 μM primers, and 2.5 U of TaqTag LA Taq (Takara Bio Inc.), was made up with DNA-free water. The PCR cycles consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 5 min. Amplification was repeated for 30 cycles. If Tn1 was inserted, the size of the PCR product was expected to be 4150 bp when the combination of the Altro6460F and Amp154LAr primers was used. Amplification of the **bla** gene was then carried out. PCR cycles were 0.1% Nonidet P-40 in PBS) at 70°C for 30 min and then washed with hybridization buffer (0.1% Nonidet P-40 in PBS) at 70°C for 10 min. Finally, filters were counterstained with 1 μg ml−1 4′,6-diamidino-2-phenylindole (DAPI) for 10 min.

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**Nucleotide sequence accession numbers.** The GenBank accession numbers of the sequences which we determined in this study are AY819041 to AY819044.

**RESULTS**

**Gene transfer via phage P1.** Phage P1 is the most commonly used generalized transducing phage for *E. coli*. Transfer of the **bla** gene, which is a part of transposon Tn1 on the chromosome of *E. coli* NBRC 12713 KEN1 via phage P1kc, was first examined by both a culture-based method using selective medium containing ampicillin and a culture-independent method with CPRINS-FISH targeting of the **bla** gene. *E. coli* NBRC 12713 was used as the recipient. Transduction was observed in this phage-host system, and the transduction frequencies for the **bla** gene on selective medium were 3 × 10−7 to 5 × 10−7 transductants per CFU and 0.2 × 10−6 to 2 × 10−6 per PFU at MOIs of 0.2 to 2 (Table 1).

The frequencies of DNA transfer determined by CPRINS-FISH were 0.1 × 10−3 to 1 × 10−3 per TDC. Colony-forming bacteria in the same samples constituted about 90% of the total cells, and the frequencies of DNA transfer were represented as numbers of CPRINS-FISH-positive cells per number of total DAPI-stained cells. Representative photographs of *E. coli* NBRC 12713, to which the **bla** gene was transferred by phage P1kc, are shown in Fig. 1A and D. V. D. C. analysis re-
revealed that almost all cells carrying the \textit{bla} gene became elongated and/or fattened independent of the MOI (Table 1 and Fig. 1C and D); that is, they still possessed protein synthesis activity. The quantitative differences between the viable cells that carry the transferred \textit{bla} gene (5 × 10^{-7} to 7 × 10^{-4} per PFU) and those that can grow on the selective medium containing ampicillin (0.2 × 10^{-6} to 2 × 10^{-6} per PFU) were on average 3 orders of magnitude.

Next, we examined the transfer of the \textit{bla} gene, which was carried on Tn1 on the low-copy-number plasmid RK2 in \textit{E. coli} NBRC 12713 via phage \textit{P1kc}. \textit{E. coli} NBRC 12713 was used as the recipient. Although the phage P1 terminase recognizes a specific pac sequence present in the DNA substrate, packaging of plasmid DNA that lacks the pac sequence by P1 is possible at lower frequencies (3). As shown in Table 1, transductants that acquired the \textit{bla} gene on Tn1 from RK2 were obtained at lower frequencies than those with the chromosomal gene (\(P < 0.05\)). We found that there were two groups of \textit{P1kc} transductants. Fifty-three percent of the transductants (group A) could grow on the selective medium containing three antibiotics (ampicillin, tetracycline, and kanamycin) and carried the \textit{bla}, \textit{tetA}, and \textit{aphA} genes derived from RK2. Other transductants (group B) were unable to grow on the selective medium and lacked the \textit{tetA} and \textit{aphA} genes. The size of RK2 is 60 kbp, which is smaller than the DNA-packaging capacity of \textit{P1kc} (about 100 kbp). Thus, group A transductants were thought to have acquired the entire RK2 plasmid. Analysis of sequences up- and downstream of the \textit{bla} gene on the chromosomes of all group B transductants revealed that a DNA fragment of Tn1 (4,951 bp) was inserted into the \textit{uutB} gene for altronate oxidoreductase on the recipient chromosome (Fig. 2A). A report by Coren et al. (3) showed that phage P1 packages multiple DNA molecules within a single phage head both in vitro and in vivo by the discontinuous headful packaging mechanism. Phage \textit{P1kc}, which infected group B transductants, might package both the partial plasmid DNA including Tn1 and the partial chromosomal DNA of the donor.

Although the transduction frequencies for the plasmid gene on selective media were lower than those for the chromosomal gene, the DNA transfer frequencies determined by CPRINS-FISH were similar for both gene sources (Table 1). RK2 replicated at about two copies per host chromosome in our preliminary experiments, and host \textit{E. coli} cells carried a single copy of the \textit{bla} gene on the chromosome. Thus, the transducing phages packaged the \textit{bla} gene at similar rates independent of the location of the gene (low-copy-number plasmid or chromosome).

CPRINS-FISH revealed that DNA was transferred from phage \textit{P1kc} to recipient cells at a high rate, and DVC analysis revealed that more than 20% of the remaining cells carrying

<table>
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<th>Location</th>
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<th>Transductant frequency per indicated unit*</th>
<th>DNA transfer frequency per indicated unit as determined by indicated method</th>
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* Frequencies are shown per CFU, PFU, or TDC. Values indicate means ± standard deviations for triplicate samples.
* Transductants were incubated on LB agar medium containing ampicillin for 2 days.
* Recipient cells carrying the \textit{bla} gene were detected by CPRINS-FISH.
* Viable cells carrying the \textit{bla} gene were detected by combined DVC and CPRINS-FISH.

FIG. 1. Visualization of \textit{E. coli} cells carrying the ampicillin resistance (\textit{bla}) gene transferred by bacteriophage \textit{P1kc}. (A and B) \textit{E. coli} NBRC12713 cells were mixed with phages for 20 min and subjected to CPRINS-FISH targeting of the \textit{bla} gene. (C and D) Viable \textit{E. coli} NBRC12713 cells carrying the \textit{bla} gene transferred by bacteriophage \textit{P1kc} were detected by a combination of CPRINS-FISH and DVC. (E and D) All DAPI-stained bacterial cells were visualized under UV excitation (exposure time, 0.1 s). (A and C) Only cells having \textit{bla} gene-amplified products emitted the fluorescence of the Alexa Fluor 546-labeled probe under green excitation (exposure, 0.5 s).
FIG. 2. Partial sequences down- and upstream of the bla gene around the terminal inverted-repeat sequence of transposon Tn1 in transductant (A) and inside the phage head (B) were represented. The underlined, bold, and italic portions are the plasmid RK2 sequences, the terminal inverted-repeat sequence of transposon Tn1, and the uxaB sequences on the E. coli chromosome, respectively.

the bla gene became elongated and/or fattened. The frequencies determined by DVC combined with CPRINS-FISH (3 × 10^{-4} to 10 × 10^{-4} per PFU) were 4 orders of magnitude higher than those for colony-forming bacteria on selective medium (3 × 10^{-8} to 10 × 10^{-8} per PFU). Based on phage P1kc-mediated gene transfer experiments with the bla gene on the chromosome and plasmid, the number of viable cells carrying the transferred bla gene and the number that were able to grow on the selective medium containing ampicillin differed by 3 to 4 orders of magnitude.

**Gene transfer via phage T4.** Transfer of the bla gene on the chromosome of E. coli NBRC 12713 KEN1 via phage T4GT7 was also examined by both selective agar plating and CPRINS-FISH targeting of the bla gene (Table 2). The transduction frequencies for the bla gene on selective medium were 0.7 × 10^{-8} to 3 × 10^{-8} per CFU and 1 × 10^{-8} to 4 × 10^{-8} per PFU. The frequencies of DNA transfer determined by CPRINS-FISH were 0.7 × 10^{-3} to 2 × 10^{-3} per TDC and 0.9 × 10^{-3} to 3 × 10^{-3} per PFU. DVC analysis revealed that more than 40% of the remaining cells carrying the bla gene possessed protein synthesis activity independent of the MOI. These results revealed that DNA was transferred from phage to viable recipient cells at a rate 10^{4} times higher than that estimated by conventional plating.

**Gene transfer via isolated phage EC10.** In order to further clarify the quantitative differences between the viable E. coli cells that carry the transferred gene and those that can grow on the selective medium following phage-mediated gene transfer, the transducing phage EC10 was newly isolated from an aquatic environment and used in the transduction experiment. The host range of phage EC10 was examined by a plaque assay. Plaque formation was observed in only some E. coli strains (C600 RK2, HB101, NBRC 12713, and W3110) but not in ATCC 43888 O157:H7. No plaque formation was observed

<table>
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<th>Location</th>
<th>MOI</th>
<th>CFU</th>
<th>PFU</th>
<th>DNA transfer frequency per indicated unit as determined by indicated method</th>
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* Frequencies are shown per CFU, PFU, or TDC. Values indicate means ± standard deviations for triplicate samples.

* Transductants were incubated on LB agar medium containing ampicillin for 2 days.

* Recipient cells carrying the bla gene were detected by CPRINS-FISH.

* Viable cells carrying the bla gene were detected by combined DVC and CPRINS-FISH.
with other Enterobacteriaceae (Citrobacter freundii IFO 12681, Enterobacter aerogenes BM 2688, Enterobacter gergoviae JCM 1234, Hafnia alvei 1, Klebsiella oxytoca ATCC 13827, Shigella sonnei IID 969, Salmonella enterica serovar Enteritidis IID 640, or Yersinia enterocolitica IID 981).

Partial nucleotide sequences of EC10 (AY819041 [120 bp], AY819042 [120 bp], AY819043 [351 bp], and AY819044 [775 bp]) had similarity to an unknown sequence of enterobacterial phage T1 (79%), a second unknown sequence of enterobacterial phage T1 (77%), a sequence encoding the Vs.8 conserved hypothesis of protein of enterobacterial phage T4 (92%), and the terminase sequence of enterobacterial phage T4 (92%), respectively.

DNA packaging is currently thought to proceed by similar mechanisms for most double-stranded DNA phages, although there are several mechanisms of concatemeric DNA cleavage for generating the mature form of phage DNA present in virions (6). In most double-stranded DNA phages, a noncapsid protein called terminase is responsible for recognition of its own DNA, prohead binding, DNA translocation, and DNA cleavage during packaging of DNA from the concatemer. Phage EC10 had a higher similarity to the terminase sequence of phage T4 than to other T4-like phages, and thus, the DNA packaging machinery of EC10 was thought to resemble that of T4. The genome size of EC10 was about 34 kbp, which is much smaller than that of T4; thus, the packaging capacity of host DNA was thought to be lower than those of T4 (170 kbp) and P1 (100 kbp). However, it should be noted that some phages can carry DNA larger than their normal genome sizes (6), and further experiments are required to determine how DNA is packaged in EC10.

Transfer of the bla gene present on the chromosome and on plasmid via EC10 was also examined by both selective agar plating and CPRINS-FISH targeting of the bla gene. The transduction frequencies for the chromosomal bla gene were \(<4 \times 10^{-9}\) to \(2 \times 10^{-9}\) per PFU (Table 3). The frequencies of DNA transfer determined by CPRINS-FISH were \(0.7 \times 10^{-3}\) to \(2 \times 10^{-3}\) per PFU. DVC analysis revealed that more than 20% of the remaining cells carrying the bla gene possessed protein synthesis activity independent of the MOI.

Transduction of the bla gene on plasmid RK2 was also observed in this phage-host system, and the transduction frequencies determined by selective agar plating were \(<1 \times 10^{-9}\) to \(4 \times 10^{-8}\) per PFU (Table 3). The transductants that were unable to grow on the selective medium containing tetracycline and kanamycin lacked the tetA and aphA genes. Analysis of sequences up- and downstream of the bla gene on the chromosome in the transductants revealed that an approximately 5-kbp DNA fragment of Tn1 was inserted into the usab gene on the recipient chromosome in the same sequences as those shown in Fig. 2A. The size of RK2 (60 kbp) is larger than that of the EC10 genome (34 kbp), and thus, EC10, which had packaged the partial plasmid DNA including Tn1, was thought to transfer the bla gene to the recipients.

The frequencies of DNA transfer determined by CPRINS-FISH were \(0.5 \times 10^{-7}\) to \(4 \times 10^{-3}\) per PFU. These results revealed that DNA was transferred from phage to recipient cells at a rate \(10^3\) to \(10^5\) times higher than that estimated by conventional plating. DVC analysis revealed that more than 20% of the remaining cells carrying the bla gene possessed protein synthesis activity. Based on the phage EC10-mediated gene transfer experiments with the bla gene on the chromosome and plasmid, the number of viable cells carrying the transferred gene was found to be about 4 orders of magnitude higher than the number able to grow on selective medium containing ampicillin.

In all DNA transfer experiments with the three E. coli phages (P1lc, T4G7T, and EC10), the difference in the number of viable cells carrying the transferred gene and those able to grow on the selective medium was about 4 orders of magnitude. In order to examine whether the high frequencies were due to integration of the bla gene into the phage genome, we analyzed sequences upstream and downstream of the bla gene inside the phage head. If the bla gene was integrated into the phage genome, heterogeneous DNA consisting of sequences of Tn1 and phage would be expected to be present inside the phage head, and phage sequences would be expected to be located near Tn1. However, sequence analysis showed that there was no phage sequence near Tn1 in each phage (Fig. 2B). Thus, DNA fragments including the bla gene were thought to be present inside the phage head separate from the phage genome.

**DNA transfer frequency in non-plaque-forming strain.** The plaque assay has played an important role in the study of the infection range of phage. The phage life cycle consists of adsorption to the host, injection of nucleic acid, commandeering...
of host machinery, production of phage proteins and nucleic acid, assembly, and release by either lysis or extrusion. If lytic genes carried on phage DNA were not expressed in the host cell after injection of nucleic acid, no plaque formation would result. The plaque-negative result may cause underestimation of the infection range of the phage. In the present study, DNA transfer via the three phages was further investigated with other \textit{E. coli} strains which were plaque positive (W3110) and negative (ATCC 43888). The frequencies of the transfer of the \textit{bla} gene on the chromosome via phages Plkc, T4GT7, and EC10 were determined with \textit{E. coli} W3110 at an MOI of 1 (Table 4). The quantitative differences between the viable cells that carry the transferred \textit{bla} gene and those that can grow on the selective medium containing ampicillin were 2 to 5 orders of magnitude.

When \textit{E. coli} ATCC 43888, which was plaque negative, was used as a recipient, the number of viable recipient cells decreased at a rate similar to that for the plaque-forming strain (W3110) after infection by phage for 20 min (Table 4). Transfer of the \textit{bla} gene on the chromosome via phages Plkc, T4GT7, and EC10 was also observed in \textit{E. coli} ATCC 43888 (Table 4). The frequencies of \textit{bla} gene transfer in ATCC 43888 as determined by CPRINS-FISH and DVC-CPRINS-FISH were similar to those in plaque-forming strains. When \textit{E. coli} ATCC 43888 cells were plated at high cell densities (>10⁷ CFU/ml) on LB agar medium containing ampicillin, they formed colonies, perhaps due to overproduction of a multidrug-resistant efflux pump (30).

**DISCUSSION**

The present study attempted to accurately determine the rate of DNA transfer mediated by phages among \textit{E. coli} cells by 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 (2), transduction frequencies in laboratory experiments using selective media in most studies were generally reported to be low. For a more accurate estimation, we employed a culture-independent method with CPRINS-FISH targeting of the \textit{bla} gene. CPRINS-FISH allows visualization of specific DNA sequences inside bacterial cells (11). The advantages of CPRINS-FISH with respect to other in situ DNA amplification methods (16, 24) include (i) the generation by CPRINS of long single-stranded DNA, preventing amplicons from leaking outside the cell; (ii) the use of multiply labeled fluorescent probes, which improves specificity and sensitivity; (iii) in situ DNA amplification on a polycarbonate filter, making it possible to concentrate target cells through filtration; and (iv) applicability to diverse bacteria. In addition, in order to analyze the viabilities of cells that acquired the \textit{bla} gene from phage, DVC was carried out prior to CPRINS-FISH. Simultaneous DVC-positive and CPRINS-FISH-positive cells represent viable cells carrying the \textit{bla} gene.

CPRINS-FISH clearly demonstrated that the \textit{bla} gene was transferred from phages into recipient cells at a significantly higher rate than previously thought (Tables 1, 2, and 3). Several possibilities for the fate of the transferred gene in recipient cells were envisioned: (i) since bacteria possess both DNA restriction systems that destroy foreign DNA and DNA repair systems that severely inhibit recombination of nonhomologous DNA, the transferred DNA may be broken down in living cells; (ii) cells infected by phage might die, but the transferred gene would remain in dead cells; (iii) the transferred gene would remain in living cells but would not be integrated into the bacterial chromosome, and since it could not replicate effectively, it would be diluted out by further growth (abortive transduction); or (iv) the transferred gene would recombine into the bacterial chromosome or would be maintained as a separate replicon. CPRINS-FISH following DVC supports protein synthesis activity for cells carrying the transferred gene. Thus, the method could exclude the dead cells in which the transferred gene remains.

During the 3-h incubation for DVC, the transferred gene inside recipient cells might be destroyed because the antibiotics used for DVC do not inhibit enzymatic activity for DNA modification or restriction. Only viable cells carrying the transferred gene were detected by CPRINS-FISH following DVC.
The transferred gene inside a viable recipient cell has a higher chance of integrating into the recipient chromosome and being maintained. Potential future work should examine the frequency and mechanism of maintenance for transferred genes and understand the mechanism of maintenance based on DNA sequence.

In recent years, the use of reporter-gene technology, such as green fluorescent protein (GFP), has provided increasingly popular tools for studying plasmid transfer without the need for culturing (4, 8, 17). This method allows estimation of bacterial cells in which the reporter gene is expressed. Studies have estimated the frequencies for transfer of a GFP-marked conjugal plasmid among laboratory strains or from an E. coli donor to indigenous freshwater bacteria. The transfer frequencies determined by GFP expression were reported to be 100- to 1,000-fold higher than those determined by conventional plate-counting methods (7, 21). However, not all recipient cells carrying the reporter gene can be detected by methods based on gene expression. Some bacteria might not transcribe the reporter gene efficiently, and their codon usage might also hinder translation of reporter gene mRNA in certain bacteria. Indeed, our preliminary experiments showed that the fluorescence intensities of cells expressing GFP differed even among E. coli strains (data not shown). Because methodological limitations have hampered quantification of recipient cells carrying the transferred gene at the DNA level, the difference between the number of cells receiving the transferred gene and the number of cells that express the gene remains unclear. Furthermore, the difference in the frequencies of gene transfer determined by the gene-targeting approach and the culture-dependent method is questionable, too.

The gene-targeting approach described here enabled us to target a specific DNA sequence at the single-cell level and clarified the quantitative difference in phage-mediated gene transfer.

The transfer of a foreign DNA molecule (or injection of DNA) into a viable recipient cell is the important first step of lateral gene transfer. CPRINS-FISH following DVC allows the detection of viable cells which received the foreign DNA molecule. In contrast, the culture-dependent method allows the detection of viable cells that maintain the transferred gene and grow on the given selective medium. The use of GFP allows estimation of cells in which the reporter gene is expressed. The combination of these techniques will lead to a further understanding of the dynamics of gene transfer at the DNA level.

With the non-plaque-forming strain, CPRINS-FISH revealed that the bla gene was transferred to strains which had not been considered previously to be host and that the transferred DNA remained in the viable cells (Table 4). Plaque formation requires the complete phage life cycle, that is, adsorption of phage to the host, injection of nucleic acid, commandeering of host machinery, assembly, and release, etc. (29). Our results show that, at least, adsorption and injection of nucleic acid happened in the non-plaque-forming strain used in this study and imply that the transfer of a foreign DNA molecule into a viable recipient cell, which is the first step of gene transfer, might happen in a wider range of strains than that estimated by the conventional plaque assay.

Many prokaryotic genomes contain a large percentage of foreign genes, and therefore, gene transfer events have driven the diversification of the bacterial genome (2, 13). The gene-targeting approach described here has great potential to provide more-useful information about the extent of gene transfer in the natural environment.

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