A Combination of Direct Viable Counting, Fluorescence in situ Hybridization, and Green Fluorescent Protein Gene Expression for Estimating Plasmid Transfer at the Single Cell Level

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(Received December 5, 2005—Accepted February 11, 2006)

Plasmid-mediated conjugation is an important mechanism through which bacteria establish new genetic traits in the environment. Here we used a combination of direct viable counting (DVC), fluorescence in situ hybridization (FISH), and green fluorescent protein (GFP) gene expression to estimate plasmid transfer frequencies (TFs) on nutrient-limited media at the single cell level. Conjugation experiments on nutrient-rich and -limited media were carried out using *Pseudomonas fluorescens* as a donor of a broad-host-range plasmid (RK2) tagged with the GFP gene and *P. putida* as the recipient. FISH-GFP and DVC-FISH-GFP allowed for the accurate detection of donor, recipient and transconjugants by fluorescence microscopy. The TFs obtained by these culture-independent approaches were 1 to more than 3 orders of magnitude higher than those determined by selective plate-counting. Lower and variable TFs obtained by selective plate-counting emphasize the importance of culture-independent approaches in plasmid transfer studies. The application of DVC before FISH-GFP on nutrient-limited medium produced elongated and/or fattened cells and resulted in a better fluorescence signal in target bacteria cells and better accumulation, maturation and fluorescence-emission of GFP within transconjugant cells, facilitating the detection and identification of cells compared with FISH-GFP alone. The DVC-FISH-GFP could be useful for other conjugation studies using bacteria with low metabolic activity under oligotrophic/stressful conditions or studies which require a precise determination of plasmid transfer events during short-term conjugation experiments.

Key words: direct viable counting, green fluorescent protein, fluorescence in situ hybridization, plasmid transfer, *Pseudomonas*

Plasmid transfer by conjugation is an important mechanism by which bacteria establish new genetic traits that allow for rapid adaptation to changing environments^{23,47,51}. Broad-host range plasmids (incompatibility groups IncP, W, N or C) are of particular interest because they can be transferred and stably maintained in a wide range of bacteria²². The transfer of self-transmissible and mobilizable plasmids among bacteria in diverse terrestrial and aquatic habitats has been studied¹⁴. *Pseudomonas* spp. are among the most widely used microorganisms in plasmid transfer studies because they can function in a pathogenic,

plant-colonizing, or xenobiotic-degrading context and are ubiquitous in various environments including soil, fresh water, sediment, sea water, hospitals and natural biofilms^{18,32,48,49}.

Studies on plasmid transfer have shown that nutrient availability and the physiological state of the mating partner affect plasmid transfer frequencies^{11,14,52}. The influence of starvation and oligotrophic conditions on plasmid transfer has been addressed using culture-dependent methods²¹. Plasmid transfer during mating in nutrient-rich broth is undetectable when *Escherichia coli* cells (donor) are starved for 24 h beforehand in river water³⁵. In contrast, the ability of *E. coli* cells starved (recipient) before mating under the same conditions, to receive and express plasmid-encoded

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genes, does not vary⁵). In seawater, plasmid transfer between marine *Vibrio* spp. is below the detectable limit when donors and recipients are starved for more than 14 days before mating²⁰. Using a culture-independent approach, Dahlberg *et al.*¹⁰ transferred GFP-tagged plasmids from starved *P. putida* to different phylogenetically recipient cells (including bacteria indigenous to seawater) in artificial and natural seawater without adding nutrients.

Methods used to investigate plasmid-mediated conjugation can be classified into two major types depending upon whether or not they are based on bacterial cultivation. The major limitation of culture-dependent methods is that in natural habitats only a minority of the bacteria present can be cultured by conventional methods². In addition, whether all transconjugants can grow on selective media is unclear, rendering identification difficult and erroneous. Molecular techniques have recently been added to conventional selective plate-counting methods for studies of conjugal plasmid transfer⁵². Green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria*, is a valuable tool for examining biological phenomena because cells can be studied in the absence of exogenous substrate or energy¹⁷. A GFP-based method can detect single cells, thus allowing in situ monitoring of plasmid transfer without transconjugant cultivation. However, transconjugant cells showed a high degree of variation in the level of GFP they expressed, implying that cells with high levels of GFP fluorescence harbor the plasmid for a longer period of time than those showing low levels of fluorescence (and consequently received the plasmid more recently)¹⁹. Moreover, studies using a GFP marker for environmental applications have showed extensive variability in the expression of GFP in different bacterial species and that the intensity of GFP fluorescence increases over time, but the influence of environmental conditions on GFP expression is unknown¹⁷.

Fluorescence in situ hybridization (FISH) is used for the simultaneous visualization, detection, identification, enumeration and localization of phylogenetically targeted bacteria at the species, genus and family levels³⁴. FISH has been combined with GFP gene expression to specifically detect transconjugants during conjugation in biofilms^{23,43}, flow chamber⁸ and activated sludge¹⁹. Direct viable counting (DVC), commonly used when counting viable but non-culturable (VBNC) bacterial cells, has also been combined with FISH to improve the detection and enumeration of VBNC bacteria in aquatic environments^{24,25,37}. The DVC method is based on the incubation of samples in the presence of nutrients and a single antimicrobial inhibitor of DNA-gyrase. The inhibitor prevents cell division without

affecting other metabolic activities allowing cells to continue to metabolize nutrients and become elongated and/or fattened after incubation^{27,53}.

In this study, DVC was combined with FISH and GFP gene expression to improve the sensitivity of the FISH method by increasing intracellular rRNA levels within target cells and provide more time for the accumulation, maturation, and expression of GFP within transconjugant cells in conjugation studies on nutrient-limited media. *P. fluorescens* was used as the donor of a derivative broad-host-range plasmid RK2 (IncP-1 α plasmid), tagged with a GFP gene by transposition, and the *P. putida* recipient was visualized by FISH with a specific Cy3-labeled probe. Plasmid transfer frequencies were estimated by fluorescence microscopy and compared with results of plate-counting on selective media.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Table 1 lists the bacterial strains and plasmids used in the present study. All *E. coli* strains were cultured at 37°C on Luria Bertani (LB) agar (10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar per liter of distilled water) and LB broth, containing the appropriate antibiotic (if required). *Pseudomonas* strains were cultured in LB broth and on LB agar at 30°C. The GFP gene was inserted into the RK2 plasmid using the transposon delivery vector pBSL299¹), comprising a mini-Tn5 transposon and streptomycin resistance gene. This vector was chosen because in mini-Tn5 transposon family vectors, the DNA fragment is transferred without transposition functions. This feature prevents secondary transposition within bacterial cells and stable DNA insertion^{12,13}. The strain used for maintenance of the *pir*-dependent recombinant plasmids (pBSL299 and its derivatives) was *E. coli* MA9. Rifampicin-resistant *E. coli* was isolated as spontaneous mutants on LB plates supplemented with 150 µg/ml of this antibiotic.

Construction of a broad-host-range plasmid tagged with the GFP gene

The GFP-tagged derivative of RK2 was constructed essentially as described by Christensen *et al.*⁸ for tagging the plasmid TOL with a GFP gene. A synthetic GFP gene (pGFPuv), which emits signal at 18-fold the fluorescence intensity of the wildtype⁹, was obtained from the vector pGFPuv and cloned downstream of the promoter/operator element (P_{T5/lac}; comprising the phage T5 promoter and two *lac* operators) from the cloning vector pQE70, and upstream

Table 1. Characteristics of bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description	Source or reference
<i>Bacteria</i>		
<i>E. coli</i> MA9	λ pir lysogen, <i>deluidA</i> :: λ +, <i>hsdR</i> 17, <i>endA</i> 1, <i>sr</i> C300, <i>creB</i> 510, <i>zbf</i> 5, <i>thi</i> , <i>lacI</i> ::Cm, <i>lacI</i> :: λ 116, <i>lacZ</i> ΔM15Δ, λ -, RP4-2 Tc::Mu-1 Km::Tn7 Cm ^r , Km ^s	NCCB 2877
<i>E. coli</i> C600	F-, <i>supE</i> 44, <i>lacY</i> 1, <i>thr</i> -1, <i>leuB</i> 6, <i>mcrA</i> , <i>thi</i> -1, <i>rfbD</i> 1, <i>fhuA</i> 21, λ -	ATCC 23724
<i>E. coli</i> W3110	F-, IN(<i>rrnD</i> - <i>rrnE</i>)1, λ -	ATCC 27325
<i>P. putida</i>	Biotype A bacteriophage host, degrades aromatic acids, Ap ^r , Cm ^r , Sm ^s , Km ^s	ATCC 12633
<i>P. fluorescens</i>	Ap ^r , Sm ^s , Km ^s	RIMD 1615005
<i>P. fluorescens</i>	Ap ^r , Sm ^s , Km ^s	ATCC 13525
<i>Plasmids</i>		
pGFPuv	GFPuv gene vector, Ap ^r	Clontech, Inc.
pQE70	Expression vector, Ap ^r	Qiagen, Inc.
pBSL299	Mini-Tn5 transposon delivery vector Ap ^r , Sm ^r	NCCB 3383
RK2	Self-transmissible 60-Kbp broad-host-range plasmid Ap ^r , Km ^r , Tc ^r	ATCC 37125
pQE70:: <i>gfp</i>	<i>Sph</i> I- <i>Hind</i> III GFPuv gene fragment cloned downstream of P _{T5/lac} and upstream of T _{10/T1} Ap ^r	This study
pBSL299:: <i>gfp</i>	<i>Not</i> I fragment P _{T5/lac} ::GFPuv::T _{10/T1} from pQE70:: <i>gfp</i> cloned into pBSL299 vector Ap ^r , Sm ^r	This study
RK2:: <i>gfp</i>	Self-transmissible broad-host-range plasmid, P _{T5/lac} ::GFPuv::T _{10/T1} from pBSL299:: <i>gfp</i> inserted at random into RK2 plasmid Ap ^r , Km ^r , Tc ^r , Sm ^r	This study

NCCB: The Netherlands Culture Collection of Bacteria; ATCC: American Type Culture Collection; RIMD: Research Institute for Microbial Diseases, Osaka University, Japan.

of a region with two strong transcriptional terminators (T_{10/T1}; t_o from phage lambda and T1 from the *rrnB* operon of *E. coli*). pQE70 harboring the GFP fragment (designated pQE70::*gfp*) was prepared by alkaline lysis using *E. coli* JM109 as the host. The fragment containing P_{T5/lac}, a synthetic ribosomal binding site (RBSII), the GFPuv gene, the translational stop codons, and the transcriptional terminator was amplified by PCR using forward (5'-GGC GTA TCA CGA GGC CGC GGC CGC TTC ACC TCG AGA-3') and reverse (5'-TTC ACC GTC ATC AGC GGC CGC CGC GAG GCA GCT C-3') primers. These primers were designed to produce an amplified fragment containing a *Not*I site (underlined) that allowed efficient insertion of the fragment into the transposon delivery vector pBSL299 after digestion with *Not*I. The PCR conditions comprised incubation at 95°C for 9 min followed by 30 cycles of denaturation at 94°C for 1 min and annealing-extension at 72°C for 3 min, with a final extension at 72°C for 7 min. The PCR products containing the GFP gene were digested with *Not*I (all restriction endonucleases were purchased from Takara Bio Inc., Shiga, Japan), purified using a MinElute PCR purification kit (Qiagen K.K., Tokyo, Japan), and inserted into the *Not*I site of the pBSL299 vector resulting in pBSL299::*gfp*. This vector was introduced into *E. coli* MA9

and transformants were isolated on LB agar containing ampicillin (50 µg/ml) and streptomycin (10 µg/ml). Transformants carrying the GFPuv gene were detected by colony-PCR using the *uvr*1f forward (5'-ATG AGT AAA GGA GAA GAA CTT TTC-3') and *uvr*17rl reverse (5'-TTA TTT GTA GAG CTC ATC CAT GC-3') primer set. The PCR conditions comprised incubation at 95°C for 9 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. In addition, we confirmed that the GFP cassette was inserted into pBSL299 by digestion with *Not*I and *Sma*I.

The GFP cassette was inserted into the plasmid RK2 in two steps. First, *E. coli* C600 and *E. coli* MA9 underwent triparental mating as donors of RK2 and the vector pBSL299::*gfp*, respectively, with *P. fluorescens* RIMD 1615005 as the recipient. Selection on M9 minimal medium plates⁴⁴, containing 10 mM glucose as the sole carbon source, 30 µg/ml of kanamycin (RK2 marker) and 10 µg/ml of streptomycin (GFP cassette marker) resulted in the isolation of *P. fluorescens* derivatives carrying a GFP cassette inserted into either the chromosome or RK2. Around 1,000 clones were directly visualized by microscopy and those that emitted intense green fluorescence under blue excita-

tion (450–490 nm) were transferred to fresh M9 minimal medium supplemented with glucose, kanamycin and streptomycin. Clones with the cassette integrated into RK2 were selected from a second mating. Fifteen clones from the first mating were suspended in 1 ml of 0.9% NaCl and mated with *P. putida* ATCC 12633. Selection on M9 minimal medium plates supplemented with 10 mM of benzoate as the sole carbon source (which cannot be metabolized by *P. fluorescens* RIMD 1615005), kanamycin (30 µg/ml) and streptomycin (10 µg/ml) resulted in *P. putida* derivatives carrying RK2 with randomly inserted GFP cassettes (GFP-tagged RK2 plasmid). Thirty four clones were isolated based on green fluorescence emission under direct microscopic visualization. *P. putida* cells carrying a single plasmid of a size similar to that of untagged RK2 were selected.

Analysis of GFP cassette in RK2 by Southern blotting

Four *P. putida* isolates carrying GFP-tagged RK2 plasmids were suspended in 1 ml of 0.9% NaCl and mated with spontaneous rifampicin-resistant *E. coli* W3110. Transconjugants were selected on LB plates supplemented with rifampicin (150 µg/ml), kanamycin (30 µg/ml) and streptomycin (10 µg/ml). *E. coli* W3110 cells were confirmed to carry the GFP-tagged RK2 based on emissions of green fluorescence under blue excitation. GFP-tagged RK2 plasmids were prepared using a HiSpeed™ Plasmid Maxi Kit (Qiagen K.K., Tokyo, Japan) and digested with *KpnI* and *SmaI* endonucleases. Double digestion with *KpnI* and *SmaI* cut the GFP cassette at one site and the plasmid at nine sites, according to a digestion-based analysis of the Birmingham IncP- α plasmid sequence⁴⁰⁾ (GenBank accession number L27758) performed using freeware pDRAW32 (AnaClone Software, <http://www.acaclone.com>). Digested plasmids were resolved by electrophoresis in agarose gels and the plasmid DNA was capillary blotted overnight onto hybridization membranes (GeneScreen Plus, NEN™ Life Science Products, Boston, MA, USA). Fluorescein-labeled probes for detection of the GFP gene on membranes were prepared using ECF Random Prime Labelling (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Briefly, the GFP gene was amplified by PCR using the primers uv1f and uv717rl, and the products were denatured by heating for 5 min. The DNA was labeled at 37°C for 1 h, and nucleotides, primers (random nanomers), denatured DNA, and enzyme (Klenow fragment) were mixed according to the volumes recommended by the manufacturer. The membrane containing plasmid DNA was hybridized with the fluorescein-labeled GFP probe for 16 h at 60°C. Thereafter, the membrane was washed twice for 15 min at 60°C. The fluorescent

signal was amplified using an anti-fluorescein alkaline phosphatase (AP) conjugate and the ECF Signal Amplification System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) to increase the sensitivity of detecting the GFP gene. In this system, probe-bound AP catalyzes the conversion of the detection reagent into a highly fluorescent product. The GFP gene was finally detected on the membrane by scanning using a Molecular Dynamics FluorImager™ (PMT value 550; 570 nm filter) (Sunnyvale, CA, USA).

Comparison of transfer frequencies (TFs) of RK2 and RK2::gfp determined by plate-counting

We used *E. coli* W3110 carrying the GFP-tagged RK2 with one insertion, designated RK2::gfp, as donor cells in a preliminary comparison of the TFs against the untagged RK2. The recipient strain was *E. coli* MA9 which is resistant to chloramphenicol and sensitive to kanamycin. Donors and recipients in the stationary phase from the culture broth were diluted separately in 0.9% NaCl at a final concentration of 1×10^8 cells/ml (culturability of 10^7 CFU/ml on LB agar for both strains). Equal portions taken from these dilutions were mixed at a donor:recipient ratio of 1:1 and 30 µl aliquots spotted onto cellulose acetate membranes (pore size 0.45 µm, Advantec Co., Tokyo, Japan) were placed on LB agar plates and incubated for 16 h at 37°C. Membranes carrying mated cells were placed into microtubes containing 1 ml of 0.9% NaCl and mixed by vortex to suspend the bacteria. Serial dilutions were spread on LB agar containing chloramphenicol for the enumeration of recipient cells and on LB containing kanamycin and chloramphenicol for that of transconjugants. The plates were incubated for 2 days at 37°C. All matings proceeded in triplicate and the TFs were compared with those of *E. coli* W3110 carrying untagged RK2. The TFs were calculated using the equation T/R , where T is the number of transconjugants and R is the number of recipients.

The second comparison used *Pseudomonas* species as both donor and recipient. The plasmid RK2::gfp was transferred by biparental mating from *E. coli* W3110 to *P. fluorescens* ATCC 13525. Derivatives of *P. fluorescens* carrying RK2::gfp were isolated from M9 minimal medium supplemented with 10 mM of acetate as the sole carbon source, 30 µg/ml of kanamycin and 10 µg/ml of streptomycin. Diluted suspensions of *P. fluorescens* carrying RK2::gfp were mated on membranes with a diluted suspension of *P. putida* at a final concentration 1×10^8 cells/ml (donor:recipient=1:1) (culturability of $0.5\text{--}1 \times 10^7$ CFU/ml on M9 minimal medium supplemented with 10 mM glucose

for both strains) and incubated for 16 h at 30°C. To enumerate recipients and transconjugants, serial dilutions of bacterial suspensions were spread on M9 minimal medium supplemented with benzoate and kanamycin when required. The plates were incubated for 4 days at 30°C and the TFs were compared with those of *P. fluorescens* ATCC 13525 carrying untagged RK2. Selective media supplemented with kanamycin and/or tetracycline were also assayed during the selection of transconjugants.

TFs of RK2::gfp determined by FISH-GFP on nutrient-rich medium

The donor used for quantifying the ratio of TF to *P. putida* ATCC 12633 by FISH-GFP was *P. fluorescens* ATCC 13525 carrying RK2::gfp. Conjugation proceeded essentially as described above. The mating mixture of donor and recipient (30 µl; donor:recipient, 1:1) was spotted in triplicate onto cellulose acetate membranes that were placed on nutrient-rich medium (LB agar) and incubated for 16 h at 30°C. Thereafter, the membranes were placed in microtubes containing 1 ml of 0.9% NaCl and mixed by vortex. Suspended bacterial cells were fixed in PBS containing 8% paraformaldehyde for 4 h. Appropriately diluted fixed cells (20 µl) were spotted into the holes of polyester seals attached to gelatin-coated (0.1% gelatin [Becton, Dickinson and Company, Sparks, MD, USA]; 0.01% KCr[SO₄]₄) glass slides and vacuum-dried as described by Maruyama *et al.*³³⁾ for the direct enumeration of bacteria. In situ hybridization proceeded as described by Kitaguchi *et al.*²⁶⁾ with some modifications. Fifty, 70 and finally 100% ethanol was dropped onto the sample for 3 min each, and then the glass slides were vacuum-dried and incubated in a humid chamber for 10 min at 48°C before hybridization. Warm hybridization buffer (30 µl; 1×SSC buffer [0.15 M NaCl and 0.015 M sodium citrate], 0.01% sodium dodecyl sulfate [SDS] and 0.02 M Tris-HCl [pH 7.5])²⁹⁾ containing 1 ng/µl of Cy3-labeled *P. putida*-specific probe (5'-TTG CCA GTT TTG GAT GCA GT-3')¹⁵⁾ was dropped onto the sample. After hybridization for 2 h at 48°C in the humid chamber, the samples were washed three times with warm washing solution (0.9 M NaCl, 0.01% SDS and 0.02 M Tris-HCl [pH 7.5]) for 15 min at 48°C.

Recipients and transconjugants were also enumerated by counting on plates containing selective medium (M9 minimal medium supplemented with benzoate). When transconjugants were enumerated on the plates, the selective medium was supplemented with kanamycin and/or streptomycin as markers on the plasmid RK2::gfp. Epifluorescence microscopy confirmed that colonies were positive for GFP and

TFs were expressed as the number of transconjugants per recipient cell (T/R).

TFs of RK2::gfp determined by FISH-GFP and DVC-FISH-GFP on nutrient-limited medium

Conjugation experiments on nutrient-limited medium (M9 minimal medium supplemented with glucose [10 mM] as the sole carbon source) proceeded essentially as described above. Donors and recipients were mixed at a ratio of 1:1 and spotted onto cellulose acetate membranes that were placed on nutrient-limited medium and incubated for 24 h at 30°C. Mated bacterial suspensions were fixed with 8% paraformaldehyde and labeled with FISH as described above (FISH-GFP). To increase the sensitivity for detection and identification of mated cells, DVC was applied to increase the rRNA content of target cells before FISH and provide more time for the accumulation, maturation and expression of GFP within transconjugant cells. Nalidixic acid (40 µg/ml) and LB broth (diluted two-fold) were added to bacterial suspensions⁵³⁾ and incubated at 30°C for 16 h in the dark. The bacterial suspensions were then fixed with 8% paraformaldehyde and attained using the FISH method. Before and after the DVC method, plasmid TFs were quantified by selective plate-counting and fluorescence microscopy.

Microscopy and image analysis

Samples were directly observed using a Nikon model E-400 epifluorescence microscope at a magnification of ×1000, equipped with a mercury lamp (100 W) as well as B-2A and G-2A filters for blue (GFP) and green (Cy3) excitation, respectively. Microscopic images were captured using a CoolSnap color CCD camera (Roper Scientific, Inc., Tucson, AZ, USA). Ten images were captured per sample for counting. Images of the donor, recipient and transconjugant were analyzed using Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA, USA). Measurements of the relative fluorescence of GFP gene expression before and after application of the DVC method were analyzed using IPLab Scientific Imaging software (Scanalytics, Inc., Rockville, MD, USA).

Results and Discussion

Construction and analysis of broad-host-range plasmid tagged with the GFP gene

Use of the vector pBSL299::gfp (mini-Tn5 transposon) and the mating procedure allowed for the isolation of *P. putida* ATCC 12633 carrying derivatives of RK2 with ran-

Fig. 1. Analysis of GFP-tagged RK2 plasmids by digestion with endonucleases and Southern blotting. (A) Electrophoretic agarose gel profile after double digestion of GFP-tagged derivatives of RK2 with *Kpn*I and *Sma*I. (B) Image scanned after Southern blotting using a fluorescent probe to detect the GFPuv gene. 1, DNA marker (λ -*Hind*III digest); 2, untagged RK2 plasmid (control negative); 3, 4, 5 and 6, GFP-tagged RK2 plasmids; 7, pBSL299::gfp vector (control positive).

domly inserted GFP cassettes. Figure 1A shows double digestion with endonucleases (*Kpn*I and *Sma*I) of GFP-tagged RK2 plasmid DNA. Electrophoresis on agarose gels revealed different band profiles of GFP-tagged RK2 (Fig. 1A, number 3, 4, 5 and 6) compared with untagged RK2 (Fig. 1A, number 2). Comparing the profile of untagged RK2 with the profiles of GFP-tagged plasmids, the upper band (21,412 bp) was digested into two smaller bands in all the tagged plasmids. This result suggests that one GFP cassette is inserted into the region between bp 38,682 and 903 of RK2 according to Pansegrau *et al.*⁴⁰). However, identifying the exact position of the cassette will require further analysis. By using Southern blotting with the GFPuv gene probe, it was possible to determine the number of GFP cassettes inserted into GFP-tagged RK2. The results revealed one insertion in two plasmids (Fig. 1B, number 5 and 6) and two insertions in another two plasmids (Fig. 1B, number 3 and 4). We selected plasmid number 6 with one GFPuv insertion for plasmid transfer experiments and designated it as

RK2::gfp.

Comparison of TFs of RK2 and RK2::gfp determined by plate-counting

Table 2 compares the TFs of RK2 and RK2::gfp using *E. coli* strains. The TFs of RK2::gfp obtained using selective medium supplemented with kanamycin did not significantly differ from ratios observed with untagged RK2, indicating that insertion of the GFP cassette into the plasmid did not affect its transfer mechanism. This observation was confirmed using *Pseudomonas* strains as donors and recipients of RK2 and RK2::gfp, respectively. Similar TFs were obtained using kanamycin and/or tetracycline as markers on plasmids (data not shown).

Use of the constructed vectors proved to be a rapid and simple way of tagging plasmids with the GFP gene using *Pseudomonas* and *E. coli* strains as donors. This type of delivery system has been widely applied to investigation of microbial ecology and the genetic tagging of bacteria^{4,13,36}). All the GFP-tagged plasmids (one or two GFP inserts) achieved a stable and rapid accumulation of GFP protein in donor bacterial strains allowing clear visualization under blue and UV excitation.

Visualization of transconjugant cells by FISH-GFP

We validated the FISH-GFP method before applying it to transfer experiments, by means of fluorescence in situ hybridization combined with GFP expression applied to an artificial mixture of fixed cells of *P. fluorescens* carrying RK2::gfp, *P. putida* and *P. putida* carrying RK2::gfp (Fig. 2). Each bacterium was specifically detected using a combination of images. Figure 2C shows that the donor is stained green, the recipient red, and the transconjugant yellow, as a consequence of both green and red fluorescence. Similarly, FISH-GFP in the transfer plasmid experiment on nutrient-rich medium allowed for the obvious discrimination of transconjugants, donors, and recipient cells (Fig. 2D). The expression of GFP in transconjugant cells was heterogeneous, but the presence of yellow indicated GFP expression

Table 2. Transfer frequencies determined by plate-counting between *E. coli* and *Pseudomonas* species carrying RK2 or RK2::gfp plasmids

Donor	Recipient	Plasmid	TFs ^a (10 ⁻²)
<i>E. coli</i> W3110	<i>E. coli</i> MA9	RK2	5.8±1.6 ^b
		RK2::gfp	4.6±1.0
<i>P. fluorescens</i> ATCC 13525	<i>P. putida</i> ATCC 12633	RK2	3.1±1.2
		RK2::gfp	2.8±1.7

^a Transfer frequency was taken as the number of transconjugants per recipient.

^b The data represent the means±SE of three independent experiments.

Fig. 2. Discrimination of donor, recipient and transconjugants by using FISH-GFP. (A, B and C) FISH-GFP was applied to an artificial mixture of fixed *P. fluorescens* carrying RK2::gfp, *P. putida* and *P. putida* carrying RK2::gfp. (A) *P. putida* cells emitted red Cy3 fluorescence under green excitation. (B) *P. fluorescens* and *P. putida* carrying RK2::gfp emitted green fluorescence under blue excitation. (C) Composite image of (A) and (B) showing *P. fluorescens* carrying RK2::gfp in green, *P. putida* in red and *P. putida* carrying RK2::gfp in yellow. (D) Composite image after conjugation on nutrient-rich medium showing donor (*P. fluorescens* carrying RK2::gfp; green), recipient (*P. putida*; red) and transconjugant (*P. putida* carrying RK2::gfp; yellow, arrow) cells. Bars, 10 μ m.

in those cells. During the conjugation period, recipient cells increased in number more rapidly than donor cells. The lower number of donor cells might be attributed to costs related to the presence of RK2::gfp. It is well documented that plasmid size, plasmid replication, expression of plasmid-encoded genes, and culture conditions confer important physiological or energy costs for a bacterial host, affecting its fitness and competitiveness^{30,31,32}.

Observation of transconjugant cells using DVC-FISH-GFP

On nutrient-limited medium, the use of FISH-GFP and DVC-FISH-GFP also allowed for the discrimination of transconjugants from donor and recipient cells on computer-generated images. However, the growth of donors and recipients was minimal on nutrient-limited as opposed to nutrient-rich medium. Furthermore, the level of GFP was low

in most transconjugant cells that appeared orange after the computer images were combined. The DVC method combined with the FISH-GFP approach facilitated the discrimination of transconjugant cells (Fig. 3). The incubation elongated and/or fattened bacterial cells and increased the intensity of the signal from FISH-targeted cells and GFP-positive cells according to the relative fluorescence analyzed using the imaging software IPLab. DVC also resulted in a better accumulation, maturation and expression of GFP within transconjugant cells. Analysis before as opposed to after DVC showed that the total number of GFP-positive cells and TFs did not differ.

Comparison of TFs determined by plate-counting, FISH-GFP and DVC-FISH-GFP

The plasmid TFs determined by FISH-GFP and DVC-FISH-GFP on nutrient-rich and -limited media were higher

Fig. 3. Detection of transconjugant cells using FISH-GFP (A) and DVC-FISH-GFP (B) after conjugation on nutrient-limited medium. In B, the incubation produced fattened and/or elongated cells and better emissions of fluorescence from GFP within transconjugant cells compared with FISH-GFP alone. Arrows: transconjugant cells. Bars, 10 μ m.

(10^{-1}) than those obtained by selective plate-counting (10^{-2} – 10^{-4}) (Table 3). The values differed significantly depending on the type of antibiotic used as the selection marker in plate counting. O'Morchoe *et al.*³⁹⁾ observed a similar discrepancy in the number of transconjugants recovered using various antibiotic resistance markers in a study of conjugation in freshwater. Selection with carbenicillin (a penicillin derivative) yielded more transconjugants than that with geneticin (G-418; an aminoglycoside). The difference in recovery might not be due to marker instability, but rather to the specific mechanism of drug resistance, which may not allow sufficient expression of resistance determinants in time for the bacteria to survive exposure to the antibiotic. Differences in the uptake of streptomycin or kanamycin plus streptomycin and/or accumulation of the drugs in recipient cells might also affect the recovery. Reduced uptake of aminoglycosides (kanamycin and streptomycin), mostly in *Pseudomonas* spp., is likely to be due to membrane permeabilization, but the underlying molecular mechanisms remain unclear^{3,7)}. The reduced permeability of the *P. aeruginosa* membrane to streptomycin during bacterial cell growth and the nature of the growth medium obviously af-

fected both the incorporation of streptomycin and the minimal inhibitory concentration, resulting in a low level of resistance to streptomycin⁵⁰⁾.

Although mating on nutrient-limited medium decreased bacterial growth and GFP expression in transconjugants, the transfer rates determined by plate-counting, FISH-GFP and DVC-FISH-GFP were of similar orders of magnitude compared with those on nutrient-rich medium. These observations are consistent with the findings that nutrient limitation does not seriously impede conjugation in natural systems even among starved bacterial cells, and that although a minimum level of activity appeared necessary for plasmid transfer to proceed, transfer rates do not correlate with levels of metabolic activity^{28,38,45)}. Interestingly, Hauster and Wuertz²³⁾ observed the highest TFs for plasmids under poor nutrient conditions and postulated that the formation of clusters at low concentrations of nutrients during the formation of a biofilm contributes to the high transfer rates under these conditions. The study of Pearce *et al.*⁴²⁾ showed that TFs were reduced at high nutrient concentrations, suggesting that plasmid transfer is either stimulated when growth-limiting nutrients are scarce or inhibited when they are abundant.

To summarize, the TFs obtained by the FISH-GFP and DVC-FISH-GFP culture-independent approach were 1 to >3 orders of magnitude higher than those obtained by determining the number of transconjugants based on CFU. This could be due to the VBNC status of the transconjugants or a loss of plasmids upon cell division even under selective conditions²³⁾.

Despite that TFs did not differ significantly between FISH-GFP and DVC-FISH-GFP on nutrient-limited medium, DVC allowed for storage GFP expression within transconjugant cells. The counting of cells became easier; therefore, more transconjugant cells were recorded resulting in higher TFs compared with FISH-GFP alone. Previous reports have showed that the expression from a single copy of the wild-type GFP gene in a chromosome is not strong

Table 3. Transfer frequencies of RK2::gfp plasmids between *Pseudomonas* species on nutrient-rich and nutrient-limited media determined by plate-counting, FISH-GFP and DVC-FISH-GFP

Medium	Plate-counting ^a			FISH-GFP	DVC-FISH-GFP
	Km	Sm	Km+Sm		
Rich	$3.8 \pm 2.0 \times 10^{-2}$ ^b	$8.4 \pm 2.8 \times 10^{-4}$	$2.0 \pm 0.9 \times 10^{-3}$	$1.9 \pm 0.2 \times 10^{-1}$	Not done
Limited	$1.7 \pm 0.9 \times 10^{-2}$	$1.9 \pm 1.1 \times 10^{-4}$	$1.2 \pm 0.5 \times 10^{-3}$	$1.1 \pm 0.3 \times 10^{-1}$	$1.5 \pm 0.6 \times 10^{-1}$

^a antibiotics used as a selection marker on the plasmid for the transconjugant on selective media. Km: kanamycin, Sm: streptomycin.

^b The data represent the means $\pm$ SE of three independent experiments.

enough to be detected by fluorescence microscopy and it has been suggested that approximately 10,000 GFP molecules in the cytoplasm of a cell are necessary for accurate microscopic detection^{16,41}). A new series of GFP mutants have been developed allowing detection of single cells with one copy of the GFP gene in the chromosomes¹⁷). However, GFP-tagged plasmids show limitations related to i) stability under natural conditions, ii) low sensitivity for single cell detection when there is only a single copy of the GFP gene encoded on the plasmid and a low copy number of plasmids (e.g. large plasmids) within cells, and iii) insufficient time for maturation of GFP within cells under growth-limiting and/or stressful conditions. DVC would provide enough time for GFP molecules to accumulate within bacterial cells and consequently permit a better maturation and expression of GFP resulting in a higher level of detection of transconjugant cells, especially in bacteria with low metabolic activity.

Sørensen *et al.*⁴⁷) recently indicated important methodological problems with the study of plasmid transfer in situ. First, conventional methods for detecting plasmid transfer do not distinguish between increased numbers of transfer events and post-transfer selection (clonal expansion of parental cells and transconjugants during long-term conjugation or cultivation). Second, traditional methods produce population-averaged measures of plasmid transfer, which generally do not provide an insight into spatial expansion in non-homogeneous environments. Third, traditional approaches invariably rely on cultivation of donor, recipient and transconjugant populations. DVC combined with FISH-GFP could be an useful culture-independent method in studies which require short-term conjugation allowing for a more precise detection of plasmid transfer events, because this method prevents an increase in the number of bacterial cells and post-transfer during mating and DVC. It also provides more time to improve the fluorescence signal level of the transconjugant.

The development of sensitive methods for monitoring conjugative plasmid transfer is of considerable importance in environmental microbiology. Oligotrophic and stressful conditions during conjugation might induce bacterial cells to enter the VBNC state and exhibit low levels of metabolic activity (e.g. insufficient rRNA content detectable by microscopy after FISH and incomplete maturation of GFP protein within cells). Low metabolic activity makes detecting plasmid transfer difficult and the culture-dependent approach underestimates actual transfer frequencies. Investigations into plasmid transfer using culture-independent methods under oligotrophic and stressful conditions are

scarce. A recent study using a metal-stressed microcosm revealed plasmid transfer from an *E. coli* strain to a sediment-derived microbial community and long-term plasmid retention in a low-carbon flow⁴⁶). Conjugal transfer of the plasmid TOL among *Pseudomonas* under toxic stress has also been studied by Arango Pinedo⁶). Premating exposure to toxicants such as ethanol, toluene and phenol did not result in positive effects on plasmid transfer. However, exposure to sodium-dodecyl-sulfate before mating increased the amount of conjugal TFs, supporting the notion that if plasmid transfer is indeed part of an adaptive response to stress, then stress should increase the ability to release and receive plasmids.

In conclusion, the DVC-FISH-GFP method could be an adequate option for in situ monitoring of conjugative plasmid transfer in laboratory-based experiments and model microcosm, under oligotrophic or stressful conditions where conventional culture methods are difficult to apply, and studies which require short-term conjugation for a precise determination of plasmid transfer rates preventing post-transfer or bacterial growth during mating.

Acknowledgements

This study was supported in part by a JSPS Grant-in-aid for Scientific Research (A) (no. 15201006). M. Jorquera acknowledges the Mombukagakusho scholarship funded by the Ministry of Education, Science, Sport and Culture, Japan.

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