Journal of Visualized Experiments

High-resolution comparison of bacterial conjugation frequencies

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57812R2
Full Title:	High-resolution comparison of bacterial conjugation frequencies
Keywords:	plasmid; Conjugation; conjugation frequency; most probable number; initiation of transfer; FACS
Corresponding Author:	Masaki Shintani Shizuoka University Hamamatsu, JAPAN
Corresponding Author's Institution:	Shizuoka University
Corresponding Author E-Mail:	shintani.masaki@shizuoka.ac.jp
First Author:	Masaki Shintani
Other Authors:	Moriya Ohkuma
	Kazuhide Kimbara
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

TITLE:

High-Resolution Comparison of Bacterial Conjugation Frequencies

AUTHORS & AFFILIATIONS:

Masaki Shintani[1,2,3], Moriya Ohkuma[3], Kazuhide Kimbara[1]

[1]Applied Chemistry and Biochemical Engineering Course, Department of Engineering, Graduate School of Integrated Science and Technology, Shizuoka University, Hamamatsu, Shizuoka, Japan
[2]Department of Bioscience, Graduated School of Science and Technology, Shizuoka University, Hamamatsu, Shizuoka, Japan
[3]Japan Collection of Microorganisms, RIKEN BioResource Research Center, Tsukuba, Ibaraki, Japan

Moriya Ohkuma (mohkuma@riken.jp)
Kazuhide Kimbara (kimbara.kazuhide@shizuoka.ac.jp)

Corresponding Author:

Masaki Shintani
shintani.masaki@shizuoka.ac.jp

KEYWORDS:

plasmid; conjugation; conjugation frequency; most probable number; initiation of transfer; FACS

SHORT ABSTRACT:

With an aim to understand the behaviors of various bacterial conjugative DNA elements under different conditions, we describe a protocol for detecting differences in conjugation frequency, with high resolution, to estimate how efficiently the donor bacterium initiates conjugation.

LONG ABSTRACT:

Bacterial conjugation is an important step in the horizontal transfer of antibiotic resistance genes via a conjugative DNA element. In-depth comparisons of conjugation frequency under different conditions are required to understand how the conjugative element spreads in nature. However, conventional methods for comparing conjugation frequency are not appropriate for in-depth comparisons because of the high background caused by the occurrence of additional conjugation events on the selective plate. We successfully reduced the background by introducing a most probable number (MPN) method and a higher concentration of antibiotics to prevent further conjugation in selective liquid medium. In addition, we developed a protocol for estimating the probability of how often donor cells initiate conjugation by sorting single donor cells into recipient pools by fluorescence-activated cell sorting (FACS). Using two plasmids, pBP136 and pCAR1, the differences in conjugation frequency in *Pseudomonas putida* cells could be detected in liquid medium at different stirring rates. The frequencies of conjugation initiation were higher for pBP136 than for pCAR1. Using these results, we can better understand the conjugation features in these two plasmids.

INTRODUCTION:

Bacterial conjugation of mobile genetic elements, conjugative plasmids, and integrative and conjugative elements (ICEs) is important for the horizontal spread of genetic information. It can promote rapid bacterial evolution and adaptation and transmit multidrug resistance genes[1,2]. The conjugation frequency can be affected by proteins encoded on the conjugative elements for mobilization of DNA (MOB) and mating pair formation (MPF), including sex pili, which are classified according to MOB and MPF type[3-5]. It can also be affected by the donor and recipient pair[6] and the growth conditions of the cells[7-12] (growth rate, cell density, solid surface or liquid medium, temperature, nutrient availability, and the presence of cations). To understand how the conjugative elements spread among bacteria, it is necessary to compare conjugation frequency in detail.

The conjugation frequency between donor and recipient pairs after mating are usually estimated by conventional methods as follows. (i) First, the numbers of donor and recipient colonies are counted; (ii) then, the recipient colonies, which received the conjugative elements (= transconjugants) are counted; (iii) and finally, the conjugation frequency is calculated by dividing the colony forming units (CFU) of the transconjugants by those of the donor and/or recipient[13]. However, when using this method, the background is high due to additional conjugation events that can also occur on the selective plates used to obtain transconjugants when the cell density is high[10]. Therefore, it is difficult to detect small differences in frequency (below a 10-fold difference). We recently introduced a most probable number (MPN) method using liquid medium containing a higher concentration of antibiotics. This method reduced the background by inhibiting further conjugation in selective medium; thus, the conjugation frequency could be estimated with higher resolution.

Conjugation can be divided into three steps: (1) attachment of the donor-recipient pair (2) initiation of conjugative transfer, and (3) dissociation of the pair[14]. During steps (1) and (3), there is physical interaction between the donor and recipient cells; thus, cell density and the environmental conditions can influence these steps, although the features of the sex pili are also important. Step (2) is likely regulated by the expression of several genes involved in conjugation in response to external changes, which could be affected by various features of the plasmid, donor, and recipient. Although the physical attachment or detachment of donor-recipient pairs can be mathematically simulated using an estimation of cells as particles, the frequency of step (2) should be experimentally measured. There have been a few reports on direct observations of how often donors can initiate conjugation [step (2)] using fluorescence microscopy[15,16]; however, these methods are not high-throughput because a large number of cells must be monitored. Therefore, we developed a new method to estimate the probability of the occurrence of step (2) by using fluorescence activated cell sorting (FACS). Our method can be applied to any plasmid, without identification of the essential genes for conjugation.

PROTOCOL:

1. Preparation of a Donor with Green Fluorescent Protein (*GFP*)- and Kanamycin Resistance Gene-Tagged Plasmids

1.1. Introduction of marker genes into the target plasmid pBP136

Note: The goal of this protocol is to generate pBP136::*gfp*. The bacterial strains and plasmids used in this study are listed in **Table 1**.

1.1.1. Grow cultures of *Escherichia coli* DH10B harboring pBP136[17] in 5 mL of sterile Luria broth (LB) and *E. coli* S17-1λ*pir*[18] harboring pJBA28[19] [containing a kanamycin (Km)-resistance gene and *gfp*mut3* gene with its promoter and terminator in a mini-Tn*5*] in 5 mL of sterile LB containing 50 µg/mL Km at 37 °C overnight (O/N, 16–24 h) with shaking at 200 revolutions per minute (rpm).

1.1.2. Harvesting and washing

1.1.2.1. Harvest 1 mL of each culture, place it into a 2 mL microtube, and centrifuge (10,000 × *g*, room temperature, 2 min). Then, discard the supernatant and resuspend the cell pellet in 2 mL of sterile phosphate buffered saline (PBS).

1.1.2.2. Centrifuge again (10,000 × *g*, room temperature, 2 min), and resuspend in 500 µL of sterile PBS.

1.1.3. Filter mating

1.1.3.1. Prepare sterile LB plates (with 1.6% agar), and place a sterile 0.22 µm pore size membrane filter on it. Mix 500 µL of *E. coli* S17-1λ*pir* harboring pJBA28 with *E. coli* DH10B harboring pBP136 and spot the mixture on the filter on the LB plate. Incubate the plate O/N at 30 °C. Remove the filter from the LB plate, place it into a sterile 50 mL plastic tube, and add 1 mL of sterile PBS.

Note: pJBA28 can replicate in the presence of Π protein, encoded by the *pir* gene[18], and can be transferred from S17-1λ*pir* to DH10B. pBP136 carries no marker gene[17] and can be transferred from DH10B to S17-1λ*pir*. Therefore, we could not distinguish S17-1λ*pir* harboring pBP136 and pJBA28 from DH10B harboring pBP136 and the mini-Tn*5* (transposed into the chromosome or pBP136) at this stage. Then, we used mixtures of them as donors in subsequent steps (1.1.4.–1.1.5.).

1.1.4. Grow an O/N culture of the above mating mixture in sterile LB containing 50 µg/mL Km at 37 °C with shaking at 200 rpm and a culture of *Pseudomonas putida* KT2440 [Km-sensitive (Km^s), rifampicin-sensitive (Rif^s), gentamicin-sensitive (Gm^s), and tetracycline resistant (Tc^r)] in medium containing 12.5 µg/mL Tc at 30 °C with shaking at 200 rpm.

1.1.5. After harvesting and washing the cells as in step 1.1.2, use them (the mating mixture and KT2440) for filter mating (O/N, 30 °C) as in step 1.1.3.

1.1.6. Prepare sterile LB plates containing 50 µg/mL Km and 12.5 µg/mL Tc (LB + Km + Tc plates).

1.1.7. Dilute the resuspended mixture on the membrane filter with sterile PBS (10^1–10^5-fold), and then spread each dilution onto LB + Km + Tc plates and incubate the plates at 30 °C for 2–3 d.

1.1.8. Pick colonies from the plates, grow an O/N culture in sterile LB containing Km and Tc as well as *P. resinovorans* CA10dm4RG (Rif^r and Gm^r)[6] in sterile LB containing Rif (25 µg/mL) and Gm (30 µg/mL) at 30 °C and 200 rpm.

Note: As described in the previous note, the colonies on the LB + Km + Tc plates (from 1.1.7.) may be KT2440 harboring pBP136 carrying a mini-Tn*5* and KT2440 with a mini-Tn*5*, because pJBA28 could be directly transferred from S17-1λ*pir* harboring pBP136 and pJBA28 to KT2440. This is why another mating with *P. resinovorans* CA10RG is required to obtain the target pBP136 with a mini-Tn*5* in the following steps.

1.1.9. After harvesting and washing the cells as in step 1.1.2, use them for filter mating (O/N, 30 °C) as in step 1.1.3.

1.1.10. Prepare sterile LB plates containing Rif, Gm, and Km (LB + Rif + Km + Gm plates).

1.1.11. Resuspend the mixture on the filter and then dilute it 10^1–10^5-fold, spread it onto LB + Rif + Km + Gm plates, and incubate the plates for 2–3 d at 30 °C.

1.1.12. Pick the colonies and check if they harbor pBP136 by PCR using specific primers for the plasmid.

1.2. Introduction of a selective marker gene into the target plasmid pCAR1

Note: The goal of this protocol is to generate pCAR1::*gfp*

1.2.1. Grow an O/N culture of *P. putida* KT2440 harboring pCAR1 (Km^s, Gm^s, Rif^s, Tc^r)[20] at 200 rpm and 30 °C and *E. coli* S17-1λ*pir*[18] harboring pJBA28 in 5 mL of sterile LB containing 50 µg/mL Km at 200 rpm and 37 °C.

1.2.2. After harvesting and washing the cells as in step 1.1.2, use them for filter mating (O/N, 30 °C) as in step 1.1.3.

1.2.3. Remove the filter from the LB plate, place it into a sterile 50 mL plastic tube, and add 1 mL of sterile PBS.

1.2.4. Dilute the resuspended mixture with sterile PBS (10^1–10^5-fold), and spread the diluted mixture onto sterile selective LB + Tc + Km plates.

Note: pCAR1 does not replicate in *E. coli*; thus, *P. putida* KT2440 harboring pCAR1 with a mini-Tn*5* can be selected on LB + Tc + Km plates.

1.2.5. Pick a colony from the plate, and grow an O/N culture in sterile LB containing Km and Tc and a culture of *P. resinovorans* CA10dm4RG in sterile LB containing Rif and Gm (200 rpm, 30 °C).

1.2.6. After harvesting and washing as in step 1.1.2, use the cells for filter mating (O/N, 30 °C) as in step 1.1.3.

1.2.7. Resuspend the mixture on the filter and then dilute it, spread onto LB + Rif + Km + Gm plates, and incubate the plates for 2–3 d at 30 °C.

1.2.8. Pick the colonies and check if they harbor pCAR1 by PCR with specific primers for the plasmid.

1.3. Confirm the transferability of the tagged-plasmids and prepare the donors for the next steps

Note: The goal of this protocol is to confirm the transferability of the above constructed plasmids and prepare the donors for the next steps.

1.3.1. Grow an O/N culture of *P. resinovorans* CA10dm4RG harboring pBP136::*gfp* or pCAR1::*gfp* in sterile LB containing Km and a culture of *P. putida* SMDBS [Km^s, Gm^s, Rif^r, Tc^r, $lacI^q$, in which $P_{A1/O4/O3}$-*gfpmut3** is not expressed because of its chromosomal $lacI^q$ gene][21] in 3 mL of LB containing Tc (200 rpm, 30 °C).

1.3.2. After harvesting and washing the cells as in step 1.1.2, use them for filter mating (O/N, 30 °C) as in step 1.1.3.

1.3.3. Place the filter into a sterile 50 mL plastic tube, and resuspend with 1 mL of sterile PBS. Dilute the resuspended mixture with sterile PBS (10^1–10^5-fold), spread the diluted mixture onto sterile selective LB + Tc + Km plates.

1.3.4. Pick the colonies and check if they harbor each of the plasmids by PCR with specific primers.

Note: Confirmation of the insertion position of the mini-Tn*5* by direct sequencing after plasmid extraction is optional, to confirm that the insertion does not affect the transfer function of the plasmids.

2. Calculation of Conjugation Frequency by the MPN Method

2.1. Prepare sterile LB + Km and LB + Gm plates.

2.2. Grow an O/N culture of *P. putida* SMDBS harboring pBP136::*gfp* or pCAR1::*gfp* in 3 mL of sterile LB containing Km and a culture of *P. putida* KT2440RGD (Gm^r, Rif^r) in 3 mL of LB containing Gm (140 rpm, 30 °C).

2.3. After harvesting and washing the cells as in step 1.1.2, use them for filter mating at 30 °C for 45 min as in step 1.1.3.

2.4. Serially dilute the above donor and recipient culture (10^1–10^7) and spread it onto LB + Km (donor) or LB + Gm (recipient) plates (each in triplicate) to count the colony forming units (CFU). Incubate the plates at 30 °C for 2 d.

2.5. Resuspend the mixture on the filter in sterile LB containing Km and Gm, and serially dilute (from 2^1 to 2^{24}–$10^{7.2}$) using a 96-well cell culture plate (in quadruplicate).

2.6. Incubate the 96-well plate for the appropriate time (2 d at 30 °C).

2.7. Count the CFU of the donor and recipient on the plates (step 2.4.) and count the number of wells in which the transconjugants grow.

2.8. Calculate the MPN and its deviation by using the MPN calculation program developed by Jarvis *et al*.[22], which is available at http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/MPN_ver5.xls.

2.8.1. Enter the name of the experiment (ex., 'test'), the date of the experiment (ex., 2018/4/9), the number of test series, and the max. no. of dilutions (enter '24') in row #7 of the 'Program' sheet of the Excel file ('MPN_ver5.xls').

2.8.2. Enter '2^{-1} (= 0.5) to 2^{-24} (= 5.96×10^{-8})' in the 'dilution factor *d*' column, '0.01' in 'volume in ml or g *w*' column, and '4' in 'No. of tubes n' in the automatically produced tables of 'input data'.

2.8.3. Enter the number of wells in which the transconjugants grow at each sample dilution (0–4).

2.8.4. Push the upper right 'Calculate Results' button, and then obtain the results (in MPN/µL) and their 95% confidence limits (lower and upper).

2.9. Calculate the conjugation frequency of the plasmids by dividing the number of transconjugants (MPN/mL) by the numbers of donor and recipient cells (CFU/mL).

3. Preparation for Estimation of the Probability of Donor-Initiated Conjugation

3.1. Grow an O/N culture of *P. putida* SMDBS harboring pBP136::*gfp* or pCAR1::*gfp* in 3 mL of sterile LB containing Km and a culture of *P. putida* KT2440RGD (Gm^r, Rif^r) in 3 mL of sterile LB containing Gm using 300 mL flasks (140 rpm, 30 °C) as precultures.

3.2. Transfer 200 µL of the preculture into 200 mL of fresh sterile LB containing Km or Gm in 500 mL flasks and incubate at 30 °C with shaking at 140 rpm.

3.3. Measure the turbidity at 600 nm (OD_{600}) of the culture using a UV-VIS spectrophotometer and spot the culture, diluted in LB (10^1–10^8-fold dilutions), onto an LB plate containing Km or Gm. Incubate these LB plates at 30 °C for 1–2 d, and determine the CFU.

3.4. Plot the OD_{600} values and the CFU with growth time to generate growth curves of the donor and recipient.

3.5. Grow cultures of the donor and recipient strain to mid-log phase, based on the growth curve.

3.6. After harvesting and washing the cells, prepare 10^1–10^3 CFU of the donor in 10 µL of LB and 10^5–10^7 CFU of the recipient in 100 µL of LB.

3.7. Mix 10 µL of the donor and 100 µL of the recipient cultures at different densities in 96-well plates (in triplicate). For example, mix 10^1 CFU of the donor and 10^5 CFU of the recipient and add it to each of the 96 wells, and mix 10^1 CFU of the donor and 10^6 CFU of the recipient in another 96-well plate, and so on.

3.8. Incubate the mixture at 30 °C for 45 min, and then add high concentrations of antibiotics (100 µg/mL Km and 60 µg/mL Gm) to each well to inhibit further conjugation.

3.9. Incubate the plate at 30 °C for 2 d.

3.10. Count the number of wells in which transconjugants grow.

3.11. Choose the recipient density that is appropriate for estimation of the probability of donor-initiated conjugation based on the above data (transconjugants should be found in at least 1 well of a 96-well plate).

Note: Transconjugants will grow in all wells when the densities of the donor and recipient cells are high and more than one conjugation occurs in a well. In contrast, no transconjugants will be found in any wells when the cell density is too low. In the following section, a single donor cell is sorted into a well. Therefore, the recipient density should be at maximum.

4. Estimation of the Probability of Donor-Initiated Conjugation

4.1. Prepare 200 mL of a mid-log phase culture of donor *P. putida* SMDBS harboring

pBP136::*gfp* or pCAR1::*gfp* and that of recipient *P. putida* KT2440RGD, as described in 3.6–3.7.

4.2. Place 10^6 CFU of the recipient in 100 µL of LB in each well of a 96-well plate.

4.3. Set up the FACS system (flow cytometry and cell sorter with a robotic arm, a 488 nm argon laser, and a 70 µm nozzle orifice). Set to forward scatter (FSC), with a 1% threshold as the acquisition trigger. Tune the H gain and A gain of the FSC and side scatter (SSC) at maximum sensitivity, which can exclude false positive signals, using PBS as a negative control. Set the sort gate based on FSC and SSC and 0.5 drop sort mode for maximal sort purity.

4.4. Sort a single donor cell by FACS on an LB plate (384 different spots), incubate the plate at 30 °C for 2 d, and then count how many colonies appear on the plate from the sorted cells.

Note: This procedure is for validation of the set gate. If there are 384 colonies on the plate, it means that 100% of the sorted cells could form colonies. The average validity of the sorting is always 90–95%.

4.5. Sort a single donor cell by FACS into each well of a 96-well plate with the recipient (4.2).

4.6. Incubate the plate for 45 min at 30 °C, and then add high concentrations of antibiotics (100 µg/mL Km and 60 µg/mL Gm) to each well to prevent further conjugation.

4.7. Incubate the plate at 30 °C for 2 d.

4.8. Count the number of wells in which transconjugants grew as determined by visual inspection with the naked eye.

4.9. Calculate the probability of donor-initiated conjugation by dividing the number of wells with transconjugants by the total number of wells in which the donor was sorted.

REPRESENTATIVE RESULTS:

Comparison of conjugation frequency by the MPN method

In our previous report, we compared the conjugation frequencies of pBP136::*gfp* and pCAR1::*gfp* in three-fold diluted LB (1/3 LB) liquid medium with different stirring rates after a 45 min mating using 125 mL spinner flasks[10]. We compared the conjugation frequencies of pBP136::*gfp* and pCAR1::*gfp* with 10^6 CFU/mL of donor and recipient strains under different stirring conditions (0–600 rpm). The conjugation frequency of both plasmids increased at higher stirring rates, and the maximum difference in the conjugation frequency was <10-fold for pBP136::*gfp* (between 0 and 400 rpm), while that of pCAR1::*gfp* was ~25-fold (between 0 and 200 rpm; Fig. 1).

Estimation of the probability of donor-initiated conjugation

The previously estimated probability of donor-initiated conjugation is shown in **Table 2**. To determine the density of recipient cells required to compare the probability of conjugation, mating assays were performed with different densities of donor and recipient. As shown in **Table**

2, pBP136::*gfp* transconjugants were detected in 100% (96/96) of wells containing 10^3 CFU of donor and 10^5–10^7 CFU of recipient, and those with 10^2 CFU of donor and 10^6–10^7 CFU of recipient, indicating that the cell density was too high. Mating assays with 10^1 CFU of donor and 10^6 or 10^5 CFU of recipient resulted in a decreased number of transconjugant-positive wells (66% and 2.1%, respectively, **Table 2**). Thus, >10^5 CFU of recipient was predicted to be required for mating with a single donor cell. Similarly, we performed the mating assays with pCAR1::*gfp* at different densities of donor and recipient strains. The percentages of transconjugant-positive wells were much lower than those of pBP136::*gfp* (**Table 2**). Assuming that the donor and recipient cells can attach to each other similarly, the probability of conjugation initiation for the pCAR1 donor was lower than that for the pBP136 donor. Based on these results, we determined that 10^7 CFU of recipient was required for a single donor cell sorted by FACS.

Then, the numbers of transconjugant-positive wells were counted. The percentage of transconjugant-positive wells for pBP136::*gfp* was larger (1.9%) than that for pCAR1::*gfp* (<0.052%; **Table 2**). Thus, there was more than a 36-fold difference in the probability of donor-initiated conjugation between these two plasmids.

FIGURE AND TABLE LEGENDS:

Figure 1. Comparison of the conjugation frequencies of pBP136::*gfp* and pCAR1::*gfp* with 10^6 colony forming units (CFU) mL^{-1} of donor (*Pseudomonas putida* SMDBS) and recipient (*P. putida* KT2440RGD) at different stirring rates (0–600 rpm). The error bars were calculated based on 95% confidence limits by the MPN method and the standard deviation of CFU of donor and recipient.

Table 1. Bacterial strains and plasmids.

Table 2. The number of wells, with different cell densities, containing transconjugants to compare the probability of donor-initiated conjugation between pBP136::*gfp* and pCAR1::*gfp*.

DISCUSSION:

Here, we present a high-resolution protocol for detecting differences in conjugation frequency under different conditions, using a MPN method to estimate the number of transconjugants. One important step in the protocol is diluting the mixture of donor and recipient after mating until no transconjugants grow. Another step is adding high concentrations of antibiotics to the selective liquid medium to prevent further conjugation. These procedures can reduce the background caused by further conjugation in the selective medium. We could successfully detect differences, even after a short mating duration between the donor and recipient. The conjugative frequency calculated by this protocol could be altered by small differences in the growth conditions of the donor and recipient strains. Thus, these conditions should be carefully designed.

In addition, we present a protocol for estimating the second step of conjugation by using FACS for single donor cell sorting. The most important step in this protocol is determining the appropriate density of recipient cells for a sorted single donor cell. When the number of recipient cells surrounding a single donor cell is large enough, physical contact between the donor and

recipient is certain. Then, the conjugation frequency can be influenced, not by the probability of how often the donor and recipient cells contact each other, but by the probability of donor-initiated conjugation. Sorting a single donor cell by FACS is not difficult; however, 96 wells are not always sufficient to estimate the probability. Therefore, 10–100 plates should be prepared. One of the limits of the protocol is that it is not appropriate for measuring the probability of donor-initiated conjugation of a plasmid with low-frequency transmissibility.

Based on these methods and their results, we recently reported that two plasmids showed different conjugation frequencies in liquid media by changing the stirring rates, which can affect the first and third steps of conjugation, attachment and detachment of donor-recipient pairs. In addition, we also found differences in the probability of the second step[10]. These results demonstrate how the conjugation frequency changes under different conditions. These protocols are useful for comparing the conjugation features of plasmids under various conditions, including aerobic or anaerobic conditions, different donor-recipient pairs, different temperature or pH, and in the presence or absence of specific chemicals, such as cations, nutrients, and antibiotics.

ACKNOWLEDGMENTS:

We thank Dr. K. Kamachi of the National Institute of Infectious Diseases (Japan) for providing pBP136 and Prof. Dr. H. Nojiri of the University of Tokyo (Japan) for providing pCAR1. We are also grateful to Professor Dr. Molin Sølen of the Technical University of Denmark for providing pJBA28. This work was supported by JSPS KAKENHI (Grant Numbers 15H05618 and 15KK0278) to MS (https://kaken.nii.ac.jp/en/grant/KAKENHI-PROJECT-15H05618/, https://kaken.nii.ac.jp/en/grant/KAKENHI-PROJECT-15KK0278/).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Cabezon, E., Ripoll-Rozada, J., Pena, A., de la Cruz, F. & Arechaga, I. Towards an integrated model of bacterial conjugation. *FEMS Microbiology Reviews* **39** (1), 81-95 (2015).

2 Johnson, C. M. & Grossman, A. D. Integrative and conjugative elements (ICEs): what they do and how they work. *Annual Review of Genetics* **49**, 577-601 (2015).

3 Garcillán-Barcia, M. P., Alvarado, A. & de la Cruz, F. Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiology Reviews* **35** (5), 936-956 (2011).

4 Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. & de la Cruz, F. Mobility of plasmids. *Microbiology and Molecular Biology Reviews* **74** (3), 434-452 (2010).

5 Garcillán-Barcia, M. P., Francia, M. V. & de la Cruz, F. The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiology Reviews* **33** (3), 657-687 (2009).

6 Shintani, M. *et al.* Recipient range of IncP-7 conjugative plasmid pCAR2 from *Pseudomonas putida* HS01 is broader than from other *Pseudomonas* strains. *Biotechnology Letters* **27** (23-24), 1847-18 53 (2005).

7 Yanagida, K. *et al.* Comparisons of the transferability of plasmids pCAR1, pB10, R388, and

441 NAH7 among *Pseudomonas putida* at different cell densities. *Bioscience, Biotechnology, and*
442 *Biochemistry* **80** (5), 1020-1023 (2016).
443 8 Schuurmans, J. M. *et al.* Effect of growth rate and selection pressure on rates of transfer
444 of an antibiotic resistance plasmid between *E. coli* strains. *Plasmid* **72,** 1-8 (2014).
445 9 Bradley, D. E., Taylor, D. E. & Cohen, D. R. Specification of surface mating systems among
446 conjugative drug resistance plasmids in *Escherichia coli* K-12. *Journal of Bacteriology* **143** (3),
447 1466-1470 (1980).
448 10 Nakazawa, S. *et al.* Different transferability of incompatibility (Inc) P-7 plasmid pCAR1 and
449 IncP-1 plasmid pBP136 in stirring liquid conditions. *PLoS One* **12** (10)**,** e0186248 (2017). Erratum
450 in: PLoS One. 2018 Jan 11;13(1):e0191393.
451 11 Verma, T., Ramteke, P. W. & Garg, S. K. Effect of ecological factors on conjugal transfer of
452 chromium-resistant plasmid in *Escherichia coli* isolated from tannery effluent. *Biotechnology and*
453 *Applied Biochemistry* **102-103** (1-6), 5-20 (2002).
454 12 Sakuda, A. *et al.* Divalent cations increase the conjugation efficiency of the incompatibility
455 P-7 group plasmid pCAR1 among different *Pseudomonas* hosts. *Microbiology* **164** (1), 20-27
456 (2018).
457 13 Corliss, T. L., Cohen, P. S. & Cabelli, V. J. R-plasmid transfer to and from *Escherichia coli*
458 strains Isolated from human fecal samples. *Applied and Environmental Microbiology* **41** (4), 959-
459 966 (1981).
460 14 Zhong, X., Krol, J. E., Top, E. M. & Krone, S. M. Accounting for mating pair formation in
461 plasmid population dynamics. *Journal of Theoretical Biology* **262** (4), 711-719 (2010).
462 15 Gilmour, M. W., Lawley, T. D. & Taylor, D. E. The cytology of bacterial conjugation. *EcoSal*
463 *Plus* **2004 (**2004).
464 16 Minoia, M. *et al.* Stochasticity and bistability in horizontal transfer control of a genomic
465 island in *Pseudomonas*. Proceedings of the National Academy of Sciences of the United States of
466 America **105** (52), 20792-20797 (2008).
467 17 Kamachi, K. *et al.* Plasmid pBP136 from *Bordetella pertuss*is represents an ancestral form
468 of IncP-1beta plasmids without accessory mobile elements. *Microbiology* **152** (12), 3477-3484
469 (2006).
470 18 Simon, R., Priefer, U. & Pühler, A. A broad host range mobilization system for *in vivo*
471 genetic engineering: transposon mutagenesis in gram negative bacteria. *Nature Biotechnology* **1**
472 (9), 784-791 (1983).
473 19 Andersen, J. B. e*t al.* New unstable variants of green fluorescent protein for studies of
474 transient gene expression in bacteria. *Applied and Environmental Microbiology* **64** (6), 2240-2246
475 (1998).
476 20 Shintani, M. e*t al.* Characterization of the replication, maintenance, and transfer features
477 of the IncP-7 plasmid pCAR1, which carries genes involved in carbazole and dioxin degradation.
478 *Applied and Environmental Microbiology* **72** (5), 3206-3216 (2006).
479 21 Shintani, M. e*t al.* Single-cell analyses revealed transfer ranges of IncP-1, IncP-7, and IncP-
480 9 plasmids in a soil bacterial community. *Applied and Environmental Microbiology* **80** (1), 138-
481 145 (2014).
482 22 Jarvis, B., Wilrich, C. & Wilrich, P. T. Reconsideration of the derivation of most probable
483 numbers, their standard deviations, confidence bounds and rarity values. *Journal of Applied*
484 *Microbiology* **109** (5), 1660-1667 (2010).

23 Haagensen, J. A., Hansen, S. K., Johansen, T. & Molin, S. *In situ* detection of horizontal transfer of mobile genetic elements. *FEMS Microbiology Ecology* **42** (2), 261-268 (2002).
24 Herrero, M., de Lorenzo, V. & Timmis, K. N. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *Journal of Bacteriology* **172** (11), 6557-6567 (1990).
25 Bagdasarian, M. *et al*. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16** (1-3), 237–247 (1981).
26 Maeda, K. *et al*. Complete nucleotide sequence of carbazole/dioxin-degrading plasmid pCAR1 in *Pseudomonas resinovorans* strain CA10 indicates its mosaicity and the presence of large catabolic transposon Tn*4676*. *Journal of Molecular Biology* **326** (1), 21-33 (2003).
27 Takahashi, Y., Shintani, M., Yamane, H., Nojiri, H. The complete nucleotide sequence of pCAR2: pCAR2 and pCAR1 were structurally identical IncP-7 carbazole degradative plasmids. *Bioscience, Biotechnology, and Biochemistry*, **73** (3), 744-746 (2009).

Figure

Click here to download Figure Figure_1.eps

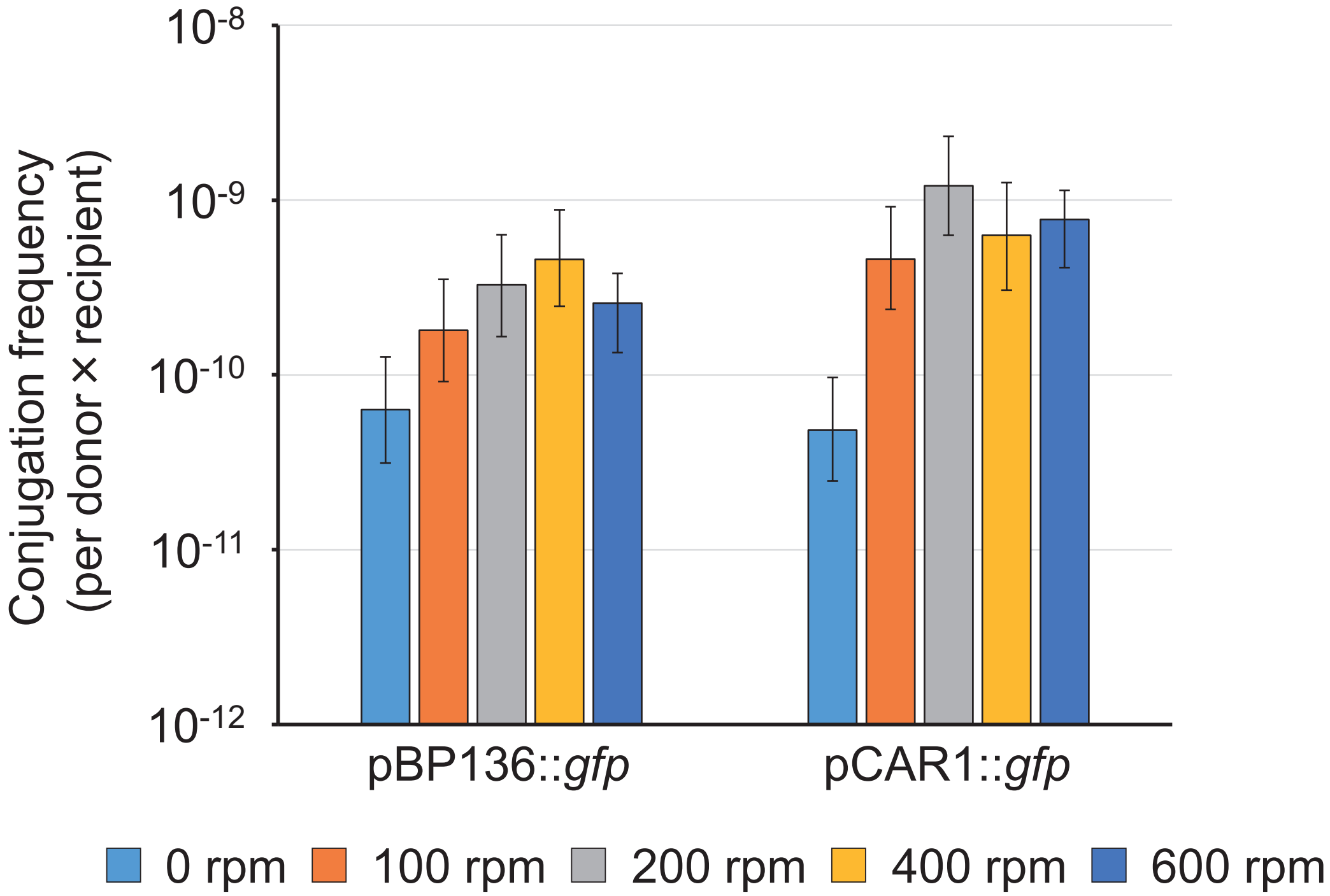

Table 1. Bacterial strains and plasmids.

Bacterial strains	Genotype and relevent phenotype	Reference or source
Escherichia coli DH10B	F^-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC)*, Φ80d*lacZ* ΔM15, Δ*lacX74*, *deoR*, *recA1*, *araD139*, Δ(*ara leu*)7697, *galU*, *galK*, λ^-, *rpsL*, *endA1*, *nupG*	Thermo
E. coli S17-1(λ*pir*)	Tm^r, Sm^r, *recA*, *thi*, *pro*, $hsdR^- M^+$, RP4: 2-Tc:Mu: Km Tn*7* *λpir*	18
Pseudomoans putida KT2440	Km^s, Rif^s, Gm^s, Tc^r	25
Pseudomoans putida KT2440(pCAR1)	KT2440 harboring pCAR1	20
Pseudomoans putida KT2440RGD	Km^s, Rif^r, Gm^r, Tc^r, miniTn*7* (Gm) $P_{A1/O4/O3}$ *DsRedExpress-a* is inseted in chromosome	10
Pseudomoans putida SMDBS	Derivative strain of *P. putida* KT2440, *dapB*-deleted, Km^s, Gm^s, Rif^r, Tc^r, $lacI^q$ is inserted in chromosome	21
P. resinovorans CA10RG	Km^s, Rif^r, Gm^r, Tc^s	6
Plasmids		
pBP136	IncP-1, MOB_P, MPF_T plasmid	17
pBP136::*gfp*	pBP136 carrying Km^r and $P_{A1/04/03}$-*gfp* cassette in *parA* (26,137 nt)	21
pCAR1	IncP-7, MOB_H, MPF_F, carbazole degradative plasmid	26, 27
pCAR1::*gfp*	pCAR1 carrying Km^r and $P_{A1/04/03}$-*gfp* cassette in ORF171 (182,625 nt)	21
pJBA28	Ap^r, Km^r, delivery plasmid for mini-Tn*5*-Km-$P_{A1/04/03}$-RBSII-*gfp* mut3*-T_0-T_1	18

Table 2. The number of wells with transconjugants with different cell density to compare the probability of donor-initiated conjugation between pBP136::*gfp* and pCAR1::*gfp*

Plasmid	[a]Donor [CFUs or cell]	[a]Recipient [CFUs]	The numbers of wells with transconjugants per 96 wells	Percentage [%]
pBP136::*gfp*	10^3	10^7	96/96	100
		10^6	96/96	100
		10^5	96/96	100
	10^2	10^7	96/96	100
		10^6	96/96	100
		10^5	54/96	56
	10^1	10^7	71/96	74
		10^6	63/96	66
		10^5	2/96	2.1
	1	10^7	23/1212	**1.9**
pCAR1::*gfp*	10^3	10^7	6/96	6.3
		10^6	6/96	6.3
		10^5	0/96	0
	10^2	10^7	1/96	1
		10^6	1/96	1
		10^5	0/96	0
	10^1	10^7	0/96	0
		10^6	0/96	0
		10^5	0/96	0
	1	10^7	1/1920	**< 0.052**

Materials Table

Click here to download Materials Table JoVE_Materials_Shintani.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
MoFlo XDP	Beckman-Coulter	ML99030	FACS
IsoFlow	Beckman-Coulter	8599600	Sheath solution
Fluorospheres (10 µm)	Beckman-Coulter	6605359	beads to set up the FACS
Incubator	Yamato Scientific Co. Ltd	211197-IC802	
UV-VIS Spectrophotometer UV-1800	SIMADZU Corporation	UV-1800	
96-well plates	NIPPON Genetics Co, Ltd	TR5003	
microplate type Petri dish	AXEL	1-9668-01	for validation of sorting
membrane filter	ADVANTEC	C045A025A	for filter mating
pippettes	Nichiryo CO. Ltd	00-NPX2-20, 00-NPX2-200, 00-NPX2-1000	0.5-10 µL, 20-200 µL, 100-1000 µL
multi-channel pippetes	Nichiryo CO. Ltd	00-NPM-8VP, 00-NPM-8LP	0.5-10 µL, 20-200 µL
Tryptone	BD Difco	211705	
Yeast extract	BD Difco	212750	
NaCl	Sigma	S-5886	
Agar	Nakarai tesque	01162-15	
rifampicin	Wako	185-01003	
gentamicin	Wako	077-02974	
kanamycin	Wako	115-00342	
Petri dish	AXEL	3-1491-51	JPND90-15

microtubes	Fukaekasei	131-815C
500 mL disposable spinner flask	Corning	CLS3578

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: High-resolution comparison of bacterial conjugation frequencies

Author(s): Masaki Shintani, Moriya Ohkuma, Kazuhide Kimbara

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Masaki Shintani
Department:	Department of Engineering
Institution:	Graduate School of Integrated Science and Technology, Shizuoka University
Article Title:	High-resolution comparison of bacterial conjugation frequencies
Signature:	新谷 政己
Date:	31, Dec. 2017

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

RE: Editorial comments:
NOTE: The manuscript has been modified to include minor grammar and formatting changes. The updated manuscript 57812_R1.docx is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink to download the .docx file. Please download the .docx file and use this updated version for future revisions. The file is also attached.
Thank you very much for your handling for our manuscript. We carefully rewrote the manuscript based on the comments from the editor and reviewers. We added Table 1 to show bacterial strain and plasmids for this study, and former Table 1 was renamed as Table 2 in the revised manuscript.

• The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors.
The revised manuscript was edited by professional English editing services (editage.com).

• Protocol Detail:
1) 4.3: Please describe all button clicks and menu selections.
Because we replaced the commercial name of FACS into generic one (L303-307), the procedures with Summit software were also removed in the revised manuscript.
2) 3.8: By naked eye?
This might be for 4.8, and yes, by naked eye (L324).

• Protocol Highlight:
1) Please ensure that the manuscript title best reflects the filmable content (i.e. the portions you highlight).
We slightly change the title in the revised manuscript.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Notes cannot be filmed and should be excluded from highlighting.
4) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.
5) We cannot film in 2 different locations, so I suggest excluding the FACS steps (which would need to be filmed in Riken from highlighting), while ensuring that the above conditions are met.
We excluded notes from highlighting and the FACS steps in the revised manuscript.

• References: Please edit your references to comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).]
We edited our reference lists according to the JoVE instructions.

• Commercial Language: Please replace the commercial names [MoFlo XDP IntelliSort

II instrument (Beckman Coulter), CyClone, Summit software (Beckman Coulter), with generic alternatives.
We replaced the instrument name into generic one (L303-307).

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
The figure in the present manuscript was originally made.

RE:Reviewer #1:

Manuscript Summary:

The revised manuscript now breaks down the protocol into a number of very clear sections that describe the tagging of plasmids, checking that the plasmids still transfer and then estimating the transfer rate parameters.

Thank you very much for your reviewing and comments.

Major Concerns:

The language of the manuscript still needs improvement.

The revised manuscript was edited by professional English editing services (editage.com).

Minor Concerns:

The title for sections 3 and 4 are identical but the methods are different. The titles needs to be revised.

The title of section 3 was changed into “Preparation for estimation of the probability of donor-initiated conjugation”.

RE:Reviewer #2:

The clarity of the paper has significantly improved compare with the first version. Few grammatical errors and typos need to be fixed.

Thank you very much for your reviewing and comments. The revised manuscript was edited by professional English editing services (editage.com).

RE:Reviewer #3:
I appreciate the authors' effort to include additional step-by-step details of the protocol in the revised manuscript. The added details improved the clarity of the core protocols (Protocol 2, 3, and 4) in the manuscript.
Thank you very much for your reviewing and comments.

However, the description of protocol 1 was poorly written and difficult to follow. I had to go back to the original submission to figure out the objectives of these steps. I recommend re-write this section combining the big picture/goal-orientated description writing style from the original submission and the step-by-step style in this revised version. A table listing all the strains used and their purpose would be greatly helpful. So is adding a summary statement to clearly describe the purpose of each experiment. For example, the goal for protocol 1.1 could be something like 'generating a donor strain P. putita SMDBS carrying the pPB136::gfp plasmid'.

Currently it reads the goal is to generate a plasmid, which indicates the end product of this protocol is a plasmid DNA. This is clearly not the case.
Thank you for your important comments. Steps 1.1 and 1.2 were written for construction of tagged plasmids, and 1.3 was for preparing donors in next steps. We added brief sentences to show goal of each step (L92-93, 158, 189-190) and Table 1 to show lists of strains and plasmids used in the present study.

The revised manuscript still needs significant editing to improve its English. Professional editing is highly recommended.
The revised manuscript was edited by professional English editing services (editage.com).