Video Article

High-Resolution Comparison of Bacterial Conjugation Frequencies

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URL: https://www.jove.com/video/57812
DOI: doi:10.3791/57812

Keywords: plasmid, conjugation, conjugation frequency, most probable number, initiation of transfer, FACS

Date Published: 12/3/2018

Citation: Shintani, M., Ohkuma, M., Kimbara, K. High-Resolution Comparison of Bacterial Conjugation Frequencies. *J. Vis. Exp.* (), e57812, doi:10.3791/57812 (2018).

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.

Video Link

The video component of this article can be found at https://www.jove.com/video/57812/

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[3,4,5]. It can also be affected by the donor and recipient pair[6] and the growth conditions of the cells[7,8,9,10,11,12] (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. **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. 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).
   2. Harvesting and washing
      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).
      2. Centrifuge again (10,000 × *g*, room temperature, 2 min), and resuspend in 500 µL of sterile PBS.
   3. Filter mating
      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.).
   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.
   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.
   6. Prepare sterile LB plates containing 50 µg/mL Km and 12.5 µg/mL Tc (LB + Km + Tc plates).
   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.
   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.
   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.
   10. Prepare sterile LB plates containing Rif, Gm, and Km (LB + Rif + Km + Gm plates).
   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.
   12. Pick the colonies and check if they harbor pBP136 by PCR using specific primers for the plasmid.

2. **Introduction of a selective marker gene into the target plasmid pCAR1**
   Note: The goal of this protocol is to generate pCAR1::*gfp*
   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.
   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.
   3. Remove the filter from the LB plate, place it into a sterile 50 mL plastic tube, and add 1 mL of sterile PBS.
   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.
   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).
   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.
   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.
   8. Pick the colonies and check if they harbor pCAR1 by PCR with specific primers for the plasmid.

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. 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).
   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.

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.
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

1. Prepare sterile LB + Km and LB + Gm plates.
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).
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.
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.
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).
6. Incubate the 96-well plate for the appropriate time (2 d at 30 °C).
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.
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.
    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. 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'.
    3. Enter the number of wells in which the transconjugants grow at each sample dilution (0–4).
    4. Push the upper right 'Calculate Results' button, and then obtain the results (in MPN/µL) and their 95% confidence limits (lower and upper).

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

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.
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. 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.
4. Plot the OD_{600} values and the CFU with growth time to generate growth curves of the donor and recipient.
5. Grow cultures of the donor and recipient strain to mid-log phase, based on the growth curve.
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.
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.
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.
9. Incubate the plate at 30 °C for 2 d.
10. Count the number of wells in which transconjugants grow.
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

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

5. Sort a single donor cell by FACS into each well of a 96-well plate with the recipient (4.2).
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.
7. Incubate the plate at 30 °C for 2 d.
8. Count the number of wells in which transconjugants grew as determined by visual inspection with the naked eye.
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.

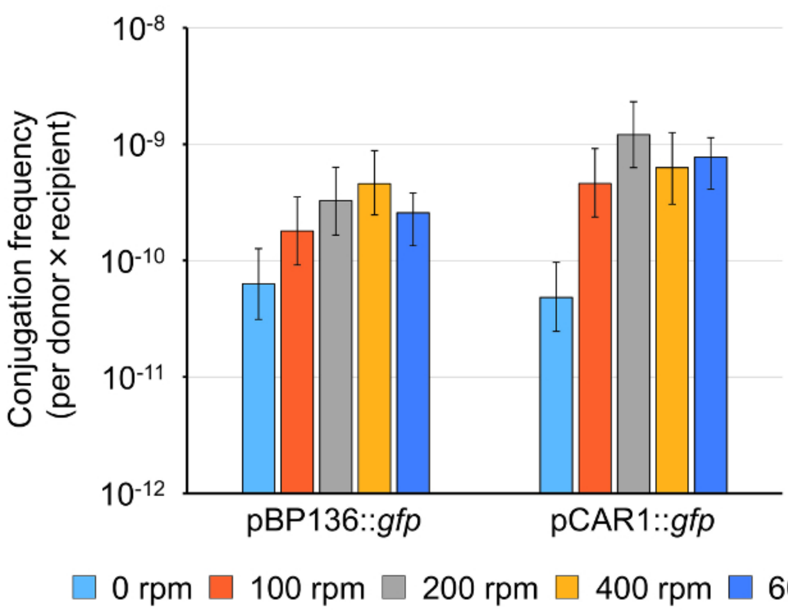

Figure 1. Comparison of the conjugation frequencies of pBP136::*gfp* and pCAR1::*gfp* with 10^6 colony forming units (CFU) mL^{-1} of donor (*Pseudomonas putida* SMDBS) and recipient (*P. putida* KT2440RGD) at different stirring rates (0-600 rpm). The error bars were calculated based on 95% confidence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

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 1. Bacterial strains and plasmids.

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**

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.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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/).

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