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Determination of conjugation rates on solid surfaces

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ABSTRACT

A cytometric method for the estimation of end-point conjugation rates is developed and adapted to surface conjugation. This method improves the through-put of conjugation assays based on replica-plating and results in less noisy experimental data. Although conjugation on solid surfaces deviates from ideal conditions in which cells are continuously mixed, results show that, within the limits of high initial population densities and short mating times, end-point estimates of the conjugation rates are robust measurements. They are independent of the donor/recipient ratios and, to some extent, of the sampling time. Remixing the mating population in the course of a conjugation experiment results in a boost in the frequency of transconjugants.

1. Introduction

Conjugation is a mechanism of DNA transfer among bacteria (de la Cruz et al., 2010; Smillie et al., 2010). It is one of the main forces contributing to shape bacterial genomes (de la Cruz and Davies, 2000). Perhaps more importantly, it is responsible for the rapid spread of antibiotic resistance (AbR) among human bacterial pathogens. In fact, the mortality rate of patients afflicted with infectious diseases has shown a steady increase during the last years (Boucher et al., 2009). Analyses of the mobile platforms (plasmids, bacteriophages, transposons, etc.) that operate in bacterial populations, and the fluxes of AbR genes within them, requires precise quantitative determination of conjugation rates (Levin et al., 1997). These quantitative data will provide essential parameters for the development of mathematical models to explain AbR dissemination among bacterial populations and the effect of conjugation on the spread of AbR (Garcillan-Barcia et al., 2011). This knowledge, in turn, could suggest strategies to control the spread of AbR to human pathogens by using specifically designed drugs (Baquero et al., 2011).

Different approaches have been used to determine the efficiency of plasmid transfer. The most widely used indicator is the transfer frequency, that is, the ratio of the number of transconjugants (T) to either the number of donors (D) or of recipients (R). In an aim to establish a universal parameter of transfer rate, independent of the experimental procedure involved, Levin et al. (1979) described a method to estimate the rate constant of conjugative transfer. That work demonstrated that, in an ideal setting, conjugation dynamics fits a simple mass action model. In its simplest realization, a bacterial chemostat, a conjugation rate (γ) could be defined, if some assumptions were made: matings are random events between cells in suspension; there is no loss of plasmids by segregation; newly formed transconjugants act like donors immediately; and every cell has the same growth rate. Simonsen et al. (1990) adapted this model to describe plasmid transfer in batch culture. Importantly, the end-point method for estimating transfer rates is ideal because it is insensitive to initial cell density, initial D/R ratio and mating time (see Simonsen, 1990 for a more detailed analysis). The end-point estimate of the conjugation rate is given by the equation:

$$\gamma_{ep} = \psi_{max}(N - N_0)^{-1} \ln\left(1 + \frac{T \cdot N}{D \cdot R}\right) \quad (1)$$

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Table 1
Plasmids.

Plasmid	Replicon	Marker	Description	Reference
pAP711Δ*oriT*::tet	R388	Gm, Tc	Non-mobilizable R388 derivative in which *oriT* was deleted and substituted by *tetA* (coding for TcR)	Demarre et al. (2005)
pGP12 [a]	pSC101	Km	Derivative of plasmid pUA66 that contains R388_*oriT* with its promoter P*stbA* controlling *gfp-mut2* expression	This work
pROD17	ColE1	Ap Km	Plasmid containing *mCherry* gene (Shaner et al., 2004) under P*lac* control	Gift of D. Sherratt
pAC1 [b]	ColE1	Ap	Km-sensitive pROD17 derivative. Contains *mCherry* gene under P*lac* control	This work
pAC2 [b]	ColE1	Ap	Km-sensitive pROD17 derivative. Contains *DsRed2* gene under P*lac* control	This work
pAC4 [b]	ColE1	Ap	Km-sensitive pROD17 derivative. Contains *mKate2* gene under P*lac* control	This work

[a] Plasmid pGP12 was constructed as follows: plasmid pUA66 (Zaslaver et al., 2006) DNA was digested with endonucleases *XhoI* and *BamHI*. This fragment was ligated to a PCR amplicon of R388_*oriT* (Llosa et al., 1991) obtained using oligonucleotides *stbAdir* (5′-GTCGTCCTCGAGTACTTGGATGGGGTCGCCTA) and *stbArev* (5′-GTCGTCGGATCCCGTCCGTTTCATTCACTTGT).

[b] Plasmids containing the different RFP genes were constructed as pROD17 derivatives. pROD17 KmR gene was eliminated by digestion with endonucleases *BglII* and *BamHI* to obtain a ApR Km-sensitive derivative (called pAC1). pAC2 and pAC4 were obtained by *EcoRI* + *SalI* endonuclease digestion of pROD17 and ligation of a PCR amplicon of genes *dsred2* (Bevis and Glick, 2002) or *mkate2* (Shcherbo et al., 2009), respectively. Amplicons were obtained by PCR amplification using the same oligonucleotide primers *rfp_EcoRI* (5′-GCTATGAATTCAAAAGTGCCACCTGACGTCTGAAGG) and *rfp_SalI* (5′-GCTATGTCGACTTCATATGGACCATGGCTAATTCCC).

where γ_{ep} is the transfer rate (mL cell^{-1} h^{-1}), ψ_{max} is the bacterial growth rate (h^{-1}), and D, R, T and N are the densities (cells mL^{-1}) of donors, recipients, transconjugants and total culture cells, respectively, at any given time. Although the end point estimate was originally developed for liquid cultures, due to its independence from changes in some experimental conditions and to its simple implementation, the method has been used to measure transfer rates even in experimental set-ups involving surface-associated conjugation (Licht et al., 1999; Lilley and Bailey, 2002; Normander et al., 1998). More recently, Zhong et al. (2011) analyzed the theoretical feasibility of application of the method to surface conjugation, using simulated data from an interactive particle system model. They concluded that transfer rates (γ) were more robust measurements than transfer frequencies (T/D or $T/(R+T)$) both in liquid conjugation and in surface-associated conjugation but only if the donor and recipient populations are forming a well-mixed confluent layer. It was shown that, unlike the T/D, T/R or T/N ratios, the end-point estimate of γ is insensitive to the D/R ratio and, to some extent, to the sampling time.

In this work we develop a simplified method of measuring conjugation rates under laboratory conditions (on the surface of agar plates) by analyzing conjugation of simple *oriT*s. In this way, transconjugants are not converted to new donors and we measure the transfer rates of the donor population *sensu stricto*. By using a cytometric analysis of the mating populations, we can estimate γ directly from the cytometer readings. We provide experimental evidence that supports the statement of Zhong et al. (2011) that γ is a robust parameter, resistant to changes in the initial experimental conditions. We further demonstrate that conjugation frequencies can be boosted by remixing the mating populations.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All experiments were carried out using the *Escherichia coli* K12 derivative BW27783 (*lacI*^{q} *rrnB3* Δ*lacZ4787* *hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568* DE(*araFGH*) φ(Δ*araEp* P_{CP8}-*araE*) (Khlebnikov et al., 2001). Two BW27783 spontaneous mutants, one resistant to nalidixic acid (BW-NxR) and another one resistant to rifampicin (BW-RfR) were obtained for this work by plating out BW27783 in LB-agar containing the relevant antibiotic. Spontaneous mutants arose at a frequency of roughly 10^{-8}. The mutants were not further characterized. Bacteria were grown in Luria–Bertani broth (LB) or LB-agar at 37 °C supplemented with the appropriate antibiotics. P*lac* expression was induced by adding 0.5 mM IPTG to culture media. Antibiotics were used at the following concentrations: tetracycline (Tc) = 10 mg/L; ampicillin (Ap) = 100 mg/L; kanamycin (Km) = 25 mg/L; nalidixic acid (Nx) = 20 mg/L and rifampicin (Rf) = 50 mg/L.

2.2. Plasmid construction

Plasmids used and details of their construction are shown in Table 1. They were constructed by using standard recombinant DNA technology (Sambrook and Russell, 2001). DNA inserts were obtained by endonuclease digestion or PCR amplification with specific oligonucleotides and subsequent ligation to the appropriate plasmid vector. The integrity of all constructions was confirmed by DNA sequencing.

2.3. Conjugation assays

Plate-mating experiments were carried out in 24-well plates containing 1.0 mL LB-agar per well. Donor and recipient strains were grown o/n from single colonies in LB medium at 37 °C with the appropriate antibiotics. After washing, fresh suspensions from stationary phase cultures of donor and recipient strains were mixed in appropriate volumes according to their OD_{600} to get the desired proportion of donor and recipient. The mixture was centrifuged for 5 min at 4000*g* and resuspended in 1/10 volume of LB medium. Fifteeen microliters of this cell mixture (corresponding to a total OD_{600} of 0.6 units (roughly 6×10^8 cells)) were placed directly onto an agar surface within a 24 well plate (previously filled with 1.0 mL