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Estimating plasmid conjugation rates: A new computational tool and a critical comparison of methods

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ABSTRACT

Plasmids are important vectors for the spread of genes among diverse populations of bacteria. However, there is no standard method to determine the rate at which they spread horizontally via conjugation. Here, we compare commonly used methods on simulated and experimental data, and show that the resulting conjugation rate estimates often depend strongly on the time of measurement, the initial population densities, or the initial ratio of donor to recipient populations. Differences in growth rate, e.g. induced by sub-lethal antibiotic concentrations or temperature, can also significantly bias conjugation rate estimates. We derive a new 'end-point' measure to estimate conjugation rates, which extends the well-known Simonsen method to include the effects of differences in population growth and conjugation rates from donors and transconjugants. We further derive analytical expressions for the parameter range in which these approximations remain valid. We present an easy to use R package and web interface which implement both new and previously existing methods to estimate conjugation rates. The result is a set of tools and guidelines for accurate and comparable measurement of plasmid conjugation rates.

1. Introduction

Plasmids are extra-chromosomal, self-replicating genetic elements that can spread between bacteria via conjugation. They spread genes within and between bacterial species and are a primary source of genetic innovation in the prokaryotic realm (Ochman et al., 2000; Hall et al., 2017). Genes disseminated by plasmids include virulence factors, heavy metal and antibiotic resistance, metabolic genes, as well as genes involved in cooperation and spite (Hall et al., 2017; Von Wintersdorff et al., 2016; Lopatkin et al., 2016; Rankin et al., 2011). To understand how these traits shape the ecology and evolution of bacteria (Martínez, 2018), it is of fundamental importance to understand and quantify how plasmids spread.

The maintenance and spread of a plasmid in a population is determined by two factors: (i) the horizontal transmission of plasmids between neighbouring bacteria (conjugation) and (ii) the vertical transmission of a plasmid with its host upon cell division (clonal expansion). Plasmid conjugation requires physical contact between donor cells (D), carrying the plasmid, and recipient cells (R), to create transconjugant cells (T), i.e. recipients carrying the plasmid (Ochman et al., 2000). The transconjugants then further contribute to the transfer of the plasmid to recipients. The conjugation rates from transconjugants can be substantially higher due to transitory derepression of the conjugative pilus synthesis (Lundquist and Levin, 1986; Laura, 2010), and because transconjugant and recipient cells are the same strain with the same restriction modification systems (Benz et al., 2021; Dimitriu et al., 2019). In addition, the rates of clonal expansion of D, R, T populations can differ strongly, especially when the plasmid is transferred across species boundaries (Benz et al., 2021).

Quantifying the horizontal and vertical modes of plasmid transmission separately is important for the fundamental understanding of plasmid biology and plasmid-host interactions, as well as the prediction of plasmid spread and selection in diverse environments. The burden of a plasmid on its host cell, the selection of plasmid-borne traits, and the

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regulation of plasmid conjugation machinery may all be affected by mutations and (a)biotic factors in the environment. Their interdependence can be assessed only when growth and conjugation are quantified independently. This is of particular importance for the epidemiology of antibiotic resistance plasmids, where plasmids with intrinsically high conjugation rates necessitate different interventions than clonally spreading plasmid-strain associations (Mathers et al., 2015; León-Sampedro et al., 2021).

Given the importance of plasmid spread, it is surprising that there is no generally accepted method to quantify the amount of conjugation that occurs between bacterial populations. Differences between conjugation assays are dictated by the variety of biological systems in which conjugation occurs: different species require different growth medium for instance, and some plasmids require solid matrices for conjugation. All conjugation assays have in common that the donor and recipient cells are cultured together in or on a specific growth medium for a certain amount of time t. After this time, the resulting population densities are measured. However, assays differ in which populations are measured - D, R, and T, or only a subset thereof (Benz et al., 2021; Lone et al., 1990; Dahlberg et al., 1998); in the experimental system used - e.g. well-mixed liquid cultures, filters, plates, the gut of vertebrate hosts (Benz et al., 2021; Sheppard et al., 2020; Bakkeren et al., 2019); the duration of the assay t - from 1 h to multiple days (Flett et al., 1997; Xue et al., 2012); and the way population densities are measured - e.g. through selective plating, or flow cytometry (Sheppard et al., 2020; Sørensen et al., 2005; Kneis et al., 2019). Differences in the output of such conjugation assays are then further exacerbated when the measured population densities are related to the amount of conjugation that occurred. Indeed, there is no consensus on what to call this quantity: commonly used phrases include conjugation frequency (Rozwandowicz et al., 2019; Liu et al., 2019), plasmid transfer rate constant (Lone et al., 1990; Levin et al., 1979), or transfer efficiency (Sørensen et al., 2005; Kneis et al., 2019). More than 10 different methods to quantify conjugation are currently found in the literature (see Table 1).

Many methods are based on the ratio between population densities, such as T/D or T/R, to quantify the fraction of transconjugants at the end of the conjugation assay (these will collectively be referred to as 'population density based methods') (Laura, 2010; Flett et al., 1997). However, these measures vary as a function of the initial population densities, the initial donor to recipient ratio, and the length of the conjugation assay (Lone et al., 1990; Xue et al., 2012). In addition, they are affected both by a plasmid's transfer rate, as well as its clonal expansion (Sørensen et al., 2005). Thus, experimental results reported with such measures are not comparable between studies without detailed information on the experimental conditions and growth rates of the strains involved (which are often lacking) (Sheppard et al., 2020; Xue et al., 2012). The resulting measurements are also not a priori comparable across experimental conditions that could affect the growth rate, including differing nutrient conditions (Saliu et al., 2019), recipient species (Benz et al., 2021; Flett et al., 1997; Trieu-Cuot et al., 1987), temperatures (Rozwandowicz et al., 2019), and (sub-lethal) antibiotic exposure (Liu et al., 2019). This limits the predictive power of conjugation proficiency when expressed as a ratio of population densities (Sørensen et al., 2005).

Table 1
Measures of conjugation proficiency reported in the literature. Here D, R, T stands for the population density of donors, recipients, and transconjugants at the time point of measurement, N is the total population density ($N = D + R + T$), N_0 is the initial total population density, R_0 is the initial population density of recipients, ψ_{max} is the maximum growth rate of the mating culture, and $p_0(t)$ is the probability that zero transconjugants are observed at time t.

Measure	Units	Mating culture	Name of resulting quantity
Population density based methods			
$\frac{T}{R_0}$	Dimensionless	Plate (Flett et al., 1997)	Exconjugant frequency (Flett et al., 1997)
$\frac{T}{R}$	Dimensionless	Filter (Dahlberg et al., 1998)	Gene transfer frequency (Dahlberg et al., 1998)
$\frac{T}{D}$	Dimensionless	Liquid (Rozwandowicz et al., 2019; Curtiss et al., 1969); Filter (Dahlberg et al., 1998; Liu et al., 2019; Trieu-Cuot et al., 1987)	(Plasmid) transfer frequency (Trieu-Cuot et al., 1987); Gene transfer frequency (Dahlberg et al., 1998); Conjugation frequency (Rozwandowicz et al., 2019; Liu et al., 2019), Recombinant yield (Curtiss et al., 1969), Plasmid transfer efficiency (Sørensen et al., 2005)
$\frac{T}{N}$	Dimensionless	Liquid (Saliu et al., 2019)	Conjugation frequency based on total bacterial count (Saliu et al., 2019)
$\frac{T}{R+T}$	Dimensionless	Liquid (Benz et al., 2021; Stevenson et al., 2018), Mouse gut (Benz et al., 2021; Bakkeren et al., 2019)	Proportion of transconjugants (Bakkeren et al., 2019; Stevenson et al., 2018), Fraction of transconjugants (in recipient population) (Benz et al., 2021)
Heuristic population dynamics based methods			
$\frac{T}{DR}$	$\frac{\text{mL}}{\text{CFU}}$	Filter (Pinedo and Smets, 2005)	Transconjugant frequency (Pinedo and Smets, 2005)
$\frac{T}{\sqrt{DR}}$	Dimensionless	Filter (Dionisio et al., 2002)	Conjugation frequency (Dionisio et al., 2002)
$log\left(\frac{T}{\sqrt{DR}}\right)$	Dimensionless	Liquid (Gama et al., 2017)	(Logarithm of) Conjugation rate (Gama et al., 2017)
Population dynamics based methods			
$\frac{\psi_{max}}{N(b)-N(a)} ln\left(\frac{D/N+T/R\vert_b}{D/N+T/R\vert_a}\right)$	$\frac{\text{mL}}{\text{CFU}\cdot\text{hour}}$	Liquid (batch and chemostat) (Levin et al., 1979)	Transfer rate constant (Levin et al., 1979)
$\frac{T}{DR\Delta t}$	$\frac{\text{mL}}{\text{CFU}\cdot\text{hour}}$	Liquid (chemostat) (Lopatkin et al., 2016; Levin et al., 1979), Plate (Lopatkin et al., 2016)	Transfer rate constant (Levin et al., 1979), Conjugation efficiency (Lopatkin et al., 2016)
$\psi_{max} ln\left(1+\frac{TN}{RD}\right)\frac{1}{(N-N_0)}$	$\frac{\text{mL}}{\text{CFU}\cdot\text{hour}}$	Liquid (Lone et al., 1990), Plate (Sheppard et al., 2020; Xue et al., 2012)	(Plasmid) Transfer rate (Lone et al., 1990; Sheppard et al., 2020), Plasmid transfer efficiency (Kneis et al., 2019), Conjugation rate per mating pair (Bakkeren et al., 2019), Conjugation coefficient (Fischer et al., 2014)
Fluctuation test based methods			
$-\ln p_0(t)\cdot\left(\frac{\psi_D+\psi_R}{D_0R_0(e^{(\psi_D+\psi_R)t}-1)}\right)$	$\frac{\text{mL}}{\text{CFU}\cdot\text{hour}}$	Liquid (Kosterlitz et al., 2021)	Donor conjugation rate (Kosterlitz et al., 2021)

Population dynamic models were developed specifically to disentangle the influence of horizontal and vertical plasmid transmission on final population density. In 1979, Levin et al. showed that conjugation in well-mixed liquid cultures can be accurately described with the mass action kinetics also used to describe chemical reactions (Levin et al., 1979). They described a method to estimate the conjugation rate from bacterial population densities using linear regression in the exponential or stationary growth phase (Levin et al., 1979). This method was developed further by Simonsen et al. (Lone et al., 1990), who derived a closed formula for the conjugation rate. They call this an 'end-point' method since it requires a single measurement of D, R and T population densities at the end of the conjugation assay, as opposed to time-course data. The method further requires knowledge of the mating population growth rate and initial population density. Recently a new method was introduced which explicitly takes into account the stochasticity of conjugation (Kosterlitz et al., 2021). This so-called 'Luria-Delbrück method' still assumes mass action kinetics of plasmid transfer, as well as deterministic and exponential growth of the donor and recipient populations. However, the stochastic treatment has the advantage of allowing shorter incubation times and shows low variance of the estimate across experimental replicates.

Although the Simonsen method is widely regarded as the most robust method available to estimate conjugation rates (Xue et al., 2012), more than thirty years after its publication an astounding variety of methods is still in common use (see Table 1). One can speculate whether this slow adoption of the Simonsen method has been because of a sense of unease with the model-based formulation, the extra bit of work involved in measuring the population growth rate, or the power of habit in using population density based methods. In addition, the Simonsen method has the drawback that it does not account for differences in growth rates between strains, nor in differences in conjugation stemming from donors or transconjugants. Fischer et al. (Fischer et al., 2014) extended the Simonsen model along these lines, but their approach requires time course measurements and a fitting procedure which is sensitive to the initial values of the optimisation. Thus, there is a clear need to reiterate the drawbacks of population density based methods, and to lower the barrier to widespread use of better population dynamics based alternatives.

Here, we show the limitations of existing measures of conjugation proficiency on simulated and experimental data, including their dependence on measurement time point, as well as the initial population densities and ratios. To mitigate these limitations, we extend the Simonsen model to include the effects of differential population growth and conjugation rates from donors and transconjugants. For this extended model we derive a new formula for the conjugation rate, as well as the critical time within which these approximations are valid. We show how our extended model compares to the original Simonsen model as a function of differences in the growth and conjugation rates. We further developed an R package and web interface (a Shiny app) to facilitate the calculation of a variety of conjugation rate methods from experimental data and to allow testing whether these were measured within the critical time. The result is a set of guidelines for easy, accurate, and comparable measurement of conjugation rates and tools to verify these rates.

2. Materials and methods

2.1. Simulations

We simulated bacterial population dynamics under the extended Simonsen model (ESM, described in the Theory and Calculations section) and evaluated the performance of different conjugation rate estimation methods. The code to simulate the different models, as well as the specific parameter settings for each figure are available from https://github.com/JSHuisman/conjugator_paper.

2.2. Conjugation experiments

2.2.1. Strains

We used *Escherichia coli* strains for the time course and full protocol conjugation assays. To distinguish the conjugation rate from donors (γ_D) from that of transconjugants (γ_T), the full protocol (described in the Results section) consists of two experiments: (i) the DRT assay combines donors (D) and recipients (R) to form transconjugants (T), and (ii) the TRT assay combines labelled transconjugants with recipients (both of the same genetic background) to form 2nd generation transconjugants. We recently described the *E. coli* strains used in these conjugation assays but provide details of their identity here (Duxbury et al., 2021). For DRT assays, the Donor (D) was a natural chicken isolate (ESBL-375) carrying an IncI1 plasmid with the cefotaxime resistance gene $bla_{CTX-M-1}$ (gift from Michael Brouwer, Wageningen Bioveterinary Research, The Netherlands). As Recipient (R) we used strain DA28100: a MG1655 laboratory strain chromosomally labelled with chloramphenicol resistance in the *galK* locus (MG1655, *galK::sYFP2opt-cat*; GenBank accession number KM018300) (Gullberg et al., 2014). Strain DA28100 was a kind gift from the Dan Andersson lab via Peter A Lind, constructed by Erik Gullberg/Wistrand-Yuen. For TRT assays, we derived the Donor (D′) strain from strain DA28100 by removing the *cat* gene flanked by FLP recombinase target (FRT) sites, by expression of FLP recombinase (strain constructed by Andrew Farr, Arjan de Visser lab). This strain was isogenic to strain DA28200 described in Gullberg et al. (Gullberg et al., 2014), constructed via the same method. To distinguish strain D′ from strain R, a spontaneous nalidixic acid (NAL) resistant mutant of strain D′ was isolated. Strains R and D′ were otherwise isogenic. Strain D′ received the IncI1 plasmid from ESBL-375 via a prior conjugation assay.

Compared to the full protocol described in the main text, we decided to label strain D′ (rather than R) for use in the TRT assay, since this allowed use of the same recipient strain (R) in both DRT and TRT assays and the simultaneous measurement of conjugation and growth rates of all strains in the same assay. The TRT assay is thus more correctly labelled the D'RT assay.

Strains were cryopreserved by storing in 20% v/v glycerol in LB medium (10 g L^{-1} (bacto) tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) at −80°C (Duxbury et al., 2021). Strains were revived prior to growth or conjugation assays by streaking to single colonies on agar plates (LB or VL medium as described in assays below). Due to cryopreservation and revival, any transient de-repression state in strain D′ has likely been lost.

2.2.2. Time course conjugation assay

To compare the sensitivity of different conjugation rate estimation methods to the measurement time point, we performed a DRT conjugation assay with sampling at multiple time points. Overnight cultures were incubated at 37°C in LB medium with shaking at 250 rpm. Cultures were then diluted in LB medium and grown into early stationary phase. Strains D and R were mixed in a 50:50 ratio and 100 μL of culture was diluted into 10 mL LB medium in a 50 mL falcon tube, resulting in an approximate initial density of $1.5 \cdot 10^7$ CFU/mL per strain. The culture was vortex-mixed and sampled for plating on selective agar at $t = 0$. The culture was then incubated under static conditions at 37°C, vortex-mixed and sampled after 0.75, 1.5, 5, 19 and 25 h during the conjugation assay. We chose to use static rather than shaken conditions to maximize the number of successful conjugation events, as D-R mating pairs may be broken with agitation (Xue et al., 2010). This represents a departure from the model assumption that the culture is well-mixed throughout the experiment. Only after 19 and 25 h we observed some cell settlement prior to mixing, which may have affected the number of cell-cell contacts formed. Following serial dilutions, CTX plates (cefotaxime, 1 mg/L) were used to select for strain D and transconjugants, CAM (chloramphenicol, 32 μg/mL) for strain R and transconjugants and CTX + CAM for transconjugants only. Cell densities (CFU/mL) were calculated based on the dilution factors and transconjugant counts were subtracted from total counts on the CTX and CAM plates. We assume

that the transconjugant counts on double selective plates reflect the formation of transconjugants in liquid culture, however we did not control against the possibility of 'on-agar' conjugation events on the double-selective agar plates. Such 'on-agar' conjugation could have increased transconjugant counts (Benz et al., 2021; Bethke et al., 2020; Philipsen et al., 2010), and would require further study. We verified that the CTX and CAM agar types were selective for strain D and strain R respectively by plating a monoculture of each strain on the two agar types. We did not see any colony growth on the opposite selective agar for each strain. The mixed culture experiment was repeated in three biological replicates (separate experimental runs) with three agar plate replicates per time point where possible. Cell densities across replicate agar plates were averaged for each biological replicate per time point.

We then performed a growth assay of strains D and R, and three transconjugant clones (T). A single transconjugant clone from each of the three replicate conjugation assays was selected and strains were grown overnight in LB medium. Three biological replicate cultures of strains D and R were prepared. The growth assay was set up in a 96-well microtiter plate. Note that these growth conditions (96-well microtiter plate) differ from the conjugation assay described (culture tubes) and therefore might have caused the growth rates to differ in the conjugation assay. Because the growth rates of D and R strains were both measured in microtiter plates, we do not expect large effects on the measure of relative fitness, although it may affect the quantitative value of the estimated conjugation rate. From overnight cultures, a 1:100 dilution was performed by adding 2 μL cells in 200 μL of LB medium in each of six (technical) replicate wells within a column of the 96-well microtiter plate. This resulted in initial cell densities of approximately 10^7 CFU/mL. OD_{600} readings were measured in a Victor3 plate reader (Perkin and Elmer, Massachusetts, US) every 16 min over 24 h during incubation at 37°C with orbital shaking prior to each reading. Growth rates were estimated per strain, per biological replicate by pooling data from each set of six replicates. OD data was clipped after 8 h (when strains entered stationary phase) and a logistic growth model without lag phase was fitted to each set of data as described in (Fischer et al., 2014) using non-linear least squares fitting in R version 4.0.3.

2.2.3. Full protocol

To illustrate the full protocol, we ran DRT and TRT conjugation assays with strains D and R, and D′ and R respectively. Conjugation and growth assays were run in a single 96-well microtiter plate, including growth profiling of the mixed conjugation cultures (N). To measure growth of a transconjugant strain (T), a transconjugant was isolated from a prior conjugation assay in which the IncI1 plasmid was transferred from strain D to strain R. The assays were repeated in three biological replicates (separate days). The methods described below for the growth and conjugation assays are similar to those described by Duxbury et al. (Duxbury et al., 2021). The TRT (D'RT) assay performed here matches one of the control assays performed by Duxbury et al. (Duxbury et al., 2021) (1.0x_VL medium condition) in which monoculture growth rates were measured alongside conjugation rates after 4 h.

Overnight cultures were prepared in VL (Viande-Levure) medium (Lei et al., 2012). A separate culture per strain was then diluted by 1:100 and grown into exponential phase at 37 °C and 250 rpm until OD_{600} nm (measured via a spectrophotometer) reached 0.4 (approximately 1.5 h). Each strain was then diluted to $2 \cdot 10^6$ CFU/mL in VL medium (approximately 100-fold dilution). Monocultures and mixed cultures were prepared with the same starting density per strain. For each monoculture, the $2 \cdot 10^6$ CFU/mL culture was diluted two-fold and 200 μL aliquots were added in two replicate microtiter wells. For mixed cultures, strain cultures ($2 \cdot 10^6$ CFU/mL) were mixed in 50:50 ratios and a single 200 μl aliquot of each was added to the microtiter plate. OD_{600} readings were measured in the Victor3 plate reader at 37°C under static conditions every 6 min up to 24 h. OD_{600} data was blank corrected with the minimum OD reading of a medium control well. Maximum growth rates were estimated from the best-fit maximum gradient of natural logarithm-transformed OD values during sliding 1.5-h windows in the exponential phase (first four hours of growth), similarly to the methods of (Hall et al., 2014), using Python version 3.6.3. To enumerate strain densities, the mixed cultures were serially diluted appropriately and plated on selective VL agar at $t = 0$, 4 and 24 h. Two agar plate replicates were included for each agar type per strain, per time point. Cell densities across replicate agar plates were averaged for each biological replicate per time point. Strain D was selected on CTX and strain D′ was selected on CTX + NAL (cefotaxime, 1 mg/L + nalidixic acid, 20 μg/mL). Strain R was selected on CAM and transconjugants were selected on CTX + CAM. Transconjugant counts were subtracted from counts on CTX and CAM plates. Plates were incubated at 37°C for 24 h. Selectivity of the agar types was confirmed by plating of monocultures at the start and end point of the assay.

2.2.4. Data availability

Raw and processed datasets are available via the github repository at https://github.com/JSHuisman/conjugator_paper/blob/master/data/.

3. Theory and calculations

3.1. The Simonsen Model (SM)

Simonsen et al. (Lone et al., 1990) developed a model (called the SM in the following) that estimates the conjugation rate from end-point measurements of the population densities (D, R, T), the initial population density ($N_0 = D_0 + R_0$), as well as the joint growth rate (ψ_{max}) of these populations. This model accounts for resource competition between the populations, and the elegant mathematical solution critically requires the assumption that both growth and conjugation have the same functional dependency on the resource concentration. The SM implicitly assumes that conjugation does not occur during the stationary phase. The dynamical equations are given by:

$$\dot{D} = \psi_c(C)D \tag{3.1}$$

$$\dot{R} = \psi_c(C)R - \gamma_c(C)(T+D)R \tag{3.2}$$

$$\dot{T} = \psi_c(C)T + \gamma_c(C)(T+D)R \tag{3.3}$$

$$\dot{C} = -\psi_c(C)(D+R+T)e \tag{3.4}$$

where the designations D, R, T stand for donors, recipients, and transconjugants respectively, $\psi_c(C) = \psi_{max}\frac{C}{C+Q}$ is the growth rate, $\gamma_c(C) = \gamma_{max}\frac{C}{C+Q}$ is the conjugation rate, C is the resource, and e is the conversion factor of resource into cells.

From this model, Simonsen et al. (Lone et al., 1990) derived that at any time point during the experiment the following relation holds:

$$\gamma_{max} = \psi_{max} ln\left(1 + \frac{TN}{RD}\right)\frac{1}{(N - N_0)} \tag{3.5}$$

where $N = D + R + T$ is the total population density at the measurement time point, N_0 is the initial population density, and the growth rate ψ_{max} should be determined from the conjugating population during the phase of exponential population growth.

3.2. The Extended Simonsen Model (ESM)

The SM makes two implicit simplifying assumptions. First, it assumes that donors, recipients and transconjugants all have the same growth rate. Second it assumes that the conjugation rate from donors to recipients (γ_D) and from transconjugants to recipients (γ_T) is the same. Both of these assumptions may not be justified. We thus extend the SM to reflect population specific growth rates (ψ_D, ψ_R, ψ_T) and conjugation rates (γ_D, γ_T). This models is called the extended Simonsen model (ESM),

and its dynamical equations are:

$$\dot{D} = \psi_{D_c}(C)D \tag{3.6}$$

$$\dot{R} = \psi_{R_c}(C)R - \left(\gamma_{T_c}(C)T + \gamma_{D_c}(C)D\right)R \tag{3.7}$$

$$\dot{T} = \psi_{T_c}(C)T + \left(\gamma_{T_c}(C)T + \gamma_{D_c}(C)D\right)R \tag{3.8}$$

$$\dot{C} = -\left(\psi_{D_c}(C)D + \psi_{R_c}(C)R + \psi_{T_c}(C)T\right)e \tag{3.9}$$

where $\psi_{X_c}(C) = \psi_X \frac{C}{C+Q}$ are the population specific growth rates (subscript X stands for D, R, T), and $\gamma_{Z_c} = \gamma_Z \frac{C}{C+Q}$ are the conjugation rates from donors or transconjugants (subscript Z stands for D, T).

3.3. The Approximate Extended Simonsen Model (ASM)

We can simplify the equations for the ESM (Eqs. (3.6)–(3.9)) by assuming that the growth and conjugation rates are constant until the resource C is gone and switch to zero in stationary phase. This assumption allows one to drop the equation for the resource C as long as the stationary phase has not yet been reached. The dynamical equations of the Approximate Extended Simonsen Model (ASM) then become:

$$\dot{D} = \psi_D D \tag{3.10}$$

$$\dot{R} = \psi_R R - (\gamma_T T + \gamma_D D)R \tag{3.11}$$

$$\dot{T} = \psi_T T + (\gamma_T T + \gamma_D D)R \tag{3.12}$$

Assuming that initially the dynamics of the recipient population are dominated by growth, i.e. $\psi_R R > > \gamma_T TR + \gamma_D DR$, and that the transconjugant population is not yet dominated by conjugation from transconjugants, i.e. $\psi_T T + \gamma_D DR > > \gamma_T TR$, we obtain that the conjugation rate γ_D at a time point t is given by (see Supplementary Materials for detailed derivation):

$$\gamma_D = \begin{cases} (\psi_D + \psi_R - \psi_T)\dfrac{T(t)}{D(t)R(t) - D_0R_0e^{\psi_T t}} & \text{for } \psi_D + \psi_R \neq \psi_T \\ \dfrac{T(t)}{D_0R_0e^{\psi_T t}t} & \text{for } \psi_D + \psi_R = \psi_T \end{cases} \tag{3.13}$$

This estimate of the conjugation rate based on the ASM can be used instead of the Simonsen endpoint formula (Eq. (3.5)) when the growth rates and conjugation rates differ between populations. It is valid as long as the approximate solutions are good approximations to the full ODE. In

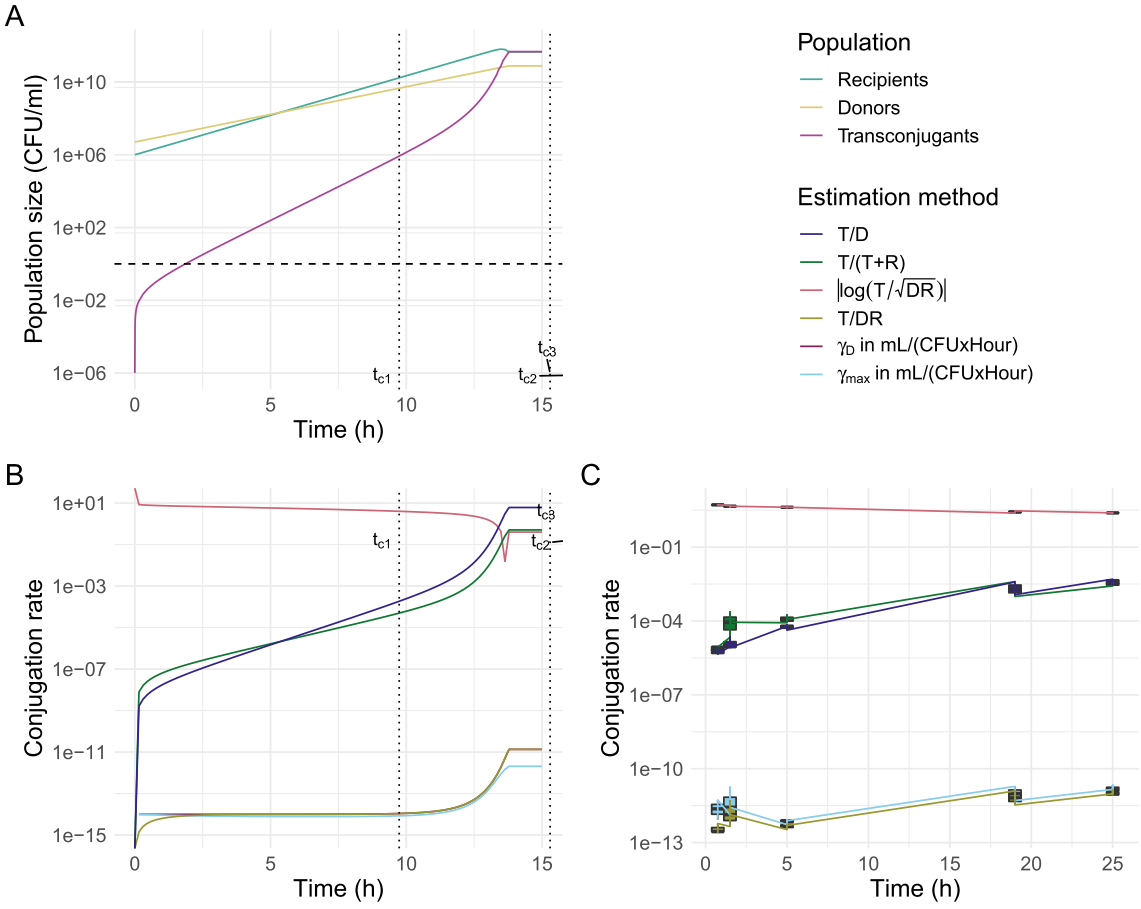

Fig. 1. Impact of the time point of measurement on the magnitude of conjugation rate estimates. Panel **A** shows the simulated population dynamics; panel **B** shows the corresponding conjugation proficiency according to several population density and population dynamics based methods; panel **C** shows some of the same methods applied to data from a time course conjugation experiment. In panel **A,B** the simulation parameters were chosen to illustrate a cost of plasmid carriage, and a higher rate of conjugation from transconjugants to recipients than from donors. The SM estimate is denoted by γ_{max} and the ASM estimate by γ_D. The T/DR and γ_D methods are partially overlaid. The vertical dotted lines indicate the first critical time, t_{c1} (at which the contribution of conjugation events from transconjugants becomes substantial), and the third critical time t_{c3} (see Supplementary Materials). In panel **A**, the horizontal dashed line indicates a single cell. The simulation parameters were: initial population densities $R_0 = 1 \cdot 10^6$ CFU/mL, $D_0 = 5 \cdot 10^6$ CFU/mL; initial resource concentration $C_0 = 10^{12}$ μg/mL; growth rates $\psi_T = \psi_D = 0.7$, $\psi_R = 1.0\ \mathrm{h}^{-1}$; conjugation rates $\gamma_D = 10^{-14}$ mL · CFU^{-1}h^{-1}, $\gamma_T = 10^{-11}$ mL · CFU^{-1}h^{-1}; approximation factor $f = 10$. In panel **C**, we incubated a 1:1 mixture of donor D with recipient R in LB medium ($n = 3$), starting from an initial density of $1.5 \cdot 10^7$ CFU/mL. Note that $n = 2$ at $t = 19$, due to an error in selective antibiotic plating. The measured growth rates were $\psi_D = 1.46$, $\psi_R = 1.43$, and $\psi_T = 1.40$.

the Supplementary Materials we derive the critical times (Eqs. (9.24), (9.25), (9.26)) beyond which different aspects of the approximation are not met anymore, and the ASM formula starts to break down. With 'the' critical time t_{crit}, we refer to the minimum $t_{crit} = \min(t_{c1}, t_{c2}, t_{c3})$ of these three time points.

4. Results

4.1. Population based methods depend sensitively on the experimental conditions

To study the merits of different measures used to quantify conjugation, we test the behaviour of the most common measures on simulated bacterial population dynamics. To this end, we simulate the population dynamics using the extended Simonsen model with resource dynamics (ESM) to include a maximum of biologically relevant detail (see Fig. 1A; more scenarios can be investigated on the Shiny app). The population density based measures vary over many orders of magnitude, depending on when the population densities are measured (Fig. 1B). Given the simulated cost of plasmid carriage, the T/D estimate is higher than $T/(R + T)$, although both would give (approximately) the same result if the growth rate of the D and R populations were the same. The measure $log\left(T/\sqrt{DR}\right)$ is relatively stable as a function of the measurement time. However, it is negative as long as T is smaller than D and R, and one has to take the absolute value to allow comparison with the other conjugation measures. The measure T/DR performs almost as well as the populations dynamics based measures (SM / ASM), as it approximates the same mass action kinetics for short time frames. One can also see that the dimensionless population density based measures are many orders of magnitude larger than conjugation rates estimated using population dynamic models, as the latter are typically reported in mL · CFU^{-1}h^{-1}. Similar biases are found when applying the same methods to experimental data (Fig. 1C).

As an example of the population-density based measures, we investigate the behaviour of the T/D method on a wider range of simulated data. Fig. 2 shows that T/D varies multiple orders of magnitude as a function of the initial population densities and donor to recipient ratios. This variation occurs regardless of the measurement time point. If the initial population densities are manipulated, but the ratio of D:R is kept constant at 1:1 (Fig. 2A), the T/D measure increases roughly proportional to the increased initial population density. Instead, if the total population density is kept constant, but the relative ratios of recipient and donor densities are varied (Fig. 2B), the T/D measure declines roughly proportional to the change in initial recipient population density. The sensitive dependence on initial population densities, donor to recipient ratios, and time of measurement complicate the interpretation of population density based measures such as T/D. It also means that experimental condition that affect the initial donor and recipient population densities or ratios, will affect T/D independent of any true effects of the experimental condition on the plasmid conjugation rate.

Similarly, an experimental treatment that affects growth rates may confound the conclusion of the effect of treatment on conjugation rates, depending on the method used to estimate these rates (Fig. 3). A researcher may for instance wish to study the effect of sublethal concentrations of antibiotics, temperature, or nutrient conditions on plasmid conjugation rates, or establish whether a plasmid transfers with a higher rate to strain A than strain B. In each of these cases, the growth rate of donors, recipients, or transconjugants may be different in the treatment than the control. Especially population density based methods will conflate these growth rate differences with conjugation and may find significant effects of treatment on plasmid conjugation rates in the absence of any real effect (Fig. 3).

4.2. Extending the Simonsen method

We have seen that population-based measures are not robust to variation in (i) the time-duration of the assay (Fig. 1B), (ii) initial population densities (Fig. 2A), (iii) and donor to recipient ratios (Fig. 2B). The 'end-point' method based on the Simonsen model (SM), which has been around for 30 years, is robust to these factors. However, this method is not applicable to populations with differing growth rates (Fig. 3), nor differences in conjugation rates from donors and transconjugants. Thus, we extended the SM for differing growth and conjugation rates (see Materials and Methods and Supplementary Materials), and derived a similar 'end-point' formula for this new model (the ASM), which is easily computed on experimental data.

In deriving the ASM estimate, we make some assumptions about the relative size of different processes contributing to the overall dynamics of D, R and T populations. Some of these assumptions are also tacitly made in the SM estimate. Most prominently, this includes the assumptions that (i) the recipient population is not substantially reduced due to transformation to transconjugants, and (ii) no conjugation takes place in stationary phase. If the rates of conjugation from donors and transconjugants differ, both the SM and ASM further require that (iii) the populations were measured at a time where the dynamics are still dominated by conjugation events between donors and recipients rather

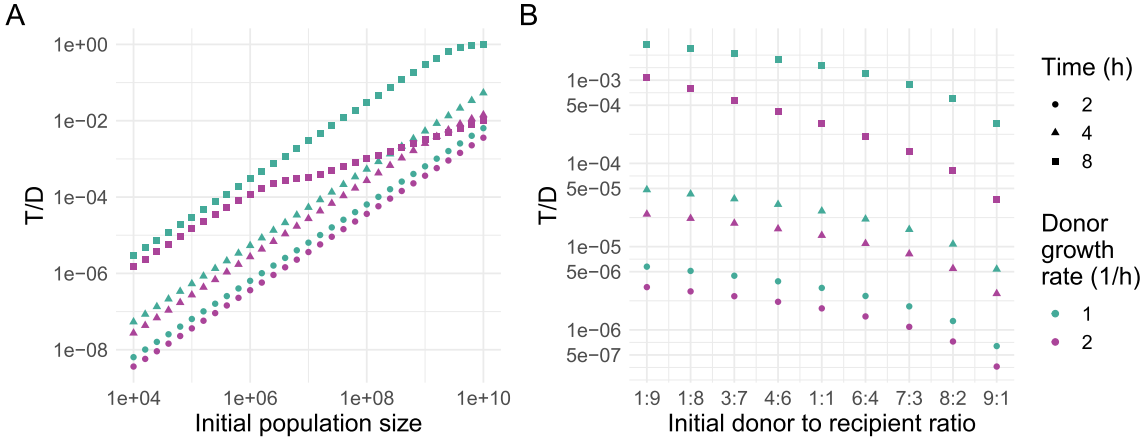

Fig. 2. Impact of initial population density **(A)**, and donor-recipient ratio **(B)** on the T/D conjugation frequency estimate. The estimate varies over several orders of magnitude as a function of the initial population densities, relative population densities, and measurement time point. Parameters: initial resource $C_0 = 10^{14}$ μg/mL; growth rates $\psi_T = \psi_D = \psi_R = 1.0$ h^{-1}; conjugation rates $\gamma_{T/D} = 10^{-13}$ mL · CFU^{-1}h^{-1}. For panel **(A)**, the initial population densities are D_0, $R_0 \in [10^4, 10^8]$ CFU/mL. Recipient and donor populations are kept at the same density. For panel **(B)**, the ratio between initial population densities is $D_0 : R_0 \in [9:1, 1:9]$, with the total population density constant at 10^7 CFU/mL.

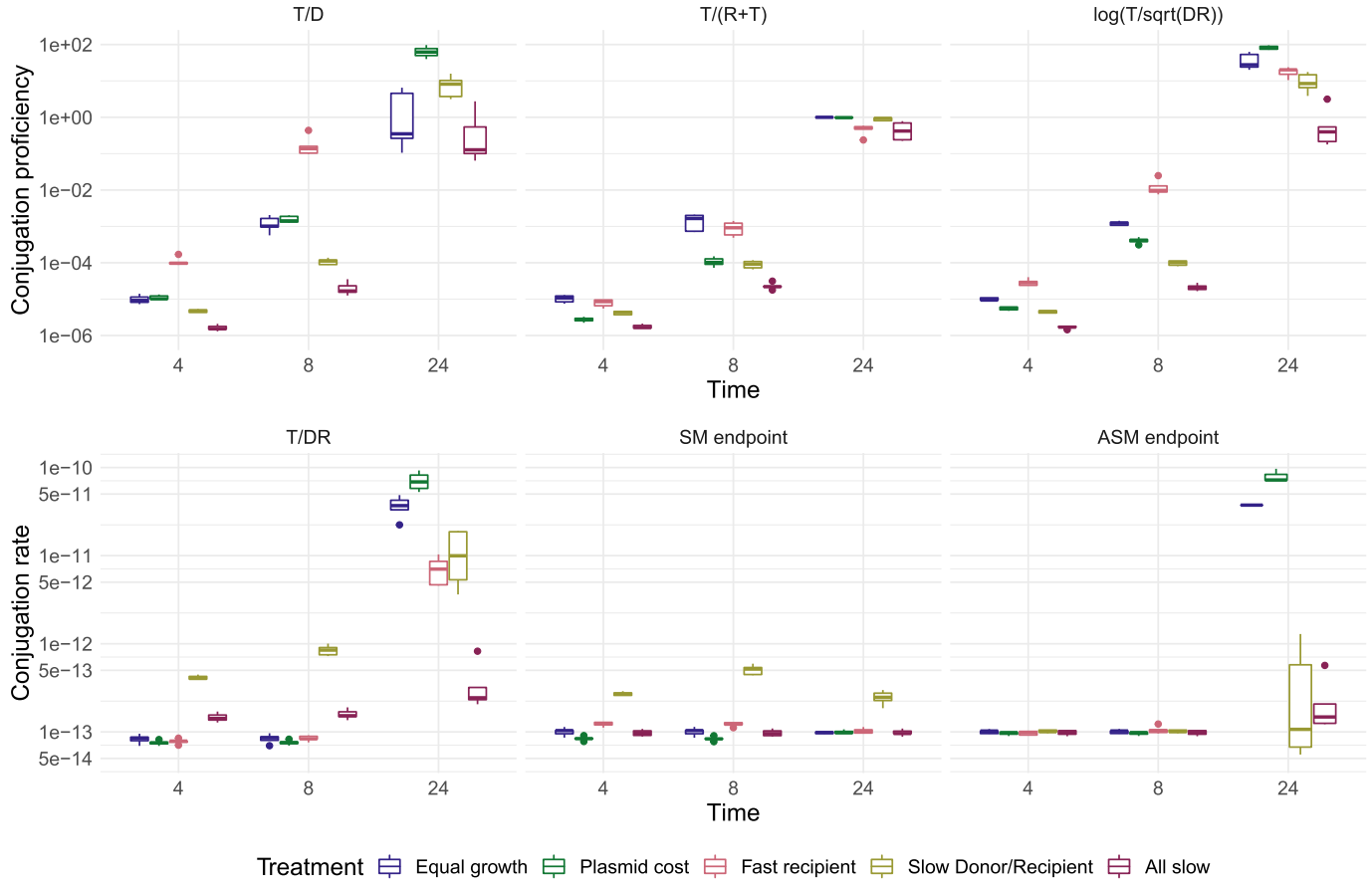

Fig. 3. The effect of experimental treatments that affect growth rates, on conjugation rates estimated from simulated data. The true plasmid conjugation rate is drawn from a normal distribution to simulate observation errors, but otherwise kept the same across all panel and treatments ($\gamma_D = \gamma_T = \mathcal{N}(10^{-13}, 5{\cdot}10^{-15})$ mL · CFU^{-1}h^{-1}). We compare a control (i) and 4 experimental scenarios (ii-v). (i) Equal growth: all strains grow equally fast at $\psi = 1.2$ h^{-1}. (ii) Plasmid cost: donors and transconjugants have a 25% growth cost due to plasmid carriage. (iii) Fast recipient: the recipients and transconjugants grow 50% faster, e.g. because they are a different species. (iv) Slow Donor/Recipient: the growth rate of donor and recipient strains is reduced by 50%, e.g. due to antibiotic pre-treatment. (v) All slow: all populations grow 50% slower than in the control scenario (i), e.g. due to a different temperature or growth medium. The critical time of treatment (i) is 11–12 h. We added 5% normal distributed noise to the model parameters to simulate experimental variation. For all treatments, the initial resource concentration $C_0 = 10^{14}$ μg/mL, and initial densities $D_0, R_0 \in \mathcal{N}(10^6, 5{\cdot}10^4)$ CFU/mL.

than between transconjugants and recipients. When these assumptions are no longer valid, we expect the SM and ASM estimates for the donor conjugation rate to fail. By making these assumptions explicit, we can derive the critical time t_{crit} beyond which the approximations break down (see the Supplementary Materials). Importantly, this critical time t_{crit} is the minimum of three different time points, reached when one of the approximations (i) or (iii) fails. Which of these time points is reached first, and thus which dictates the latest possible measurement time point, depends on the relative magnitude of the growth rates (ψ_D, ψ_R, ψ_T), conjugation rates (γ_D, γ_T), as well as the initial population densities (D_0, R_0, see Supplementary Materials Eqs. (9.24), (9.25), (9.26)). There is some circularity in the expressions, since knowledge of the conjugation rates is required to determine when to measure the conjugation rates. This should become part of the routine of testing and setting up a new conjugation assay, and will not change much for strains with similar growth and conjugation rates. With species like *E. coli*, we generally recommend to measure early (e.g. after 4–8 h) rather than to wait for overnight cultures.

We use simulated data to investigate whether the ASM estimate improves the conjugation rate estimate in the face of differing (i) growth, and (ii) conjugation rates. Here, we use the fold change, i.e. the ratio between the estimated value and the true value of the conjugation rate γ_{max}, to quantify the error made during estimation.

4.3. Growth

As Fig. 4 shows, the SM estimate varies as a function of the donor and recipient population growth rate. The SM overestimates the conjugation rate if donor and/or recipients populations grow more slowly than the transconjugant population (lower left corner of Fig. 4C). If the transconjugants grow more slowly than D and/or R, the SM underestimates the conjugation rate (upper right corner of Fig. 4C). This is the case for all measurement time points, although the effect is exacerbated for measurements that are made after a longer conjugation time (Fig. 4A). In contrast, the new ASM estimate γ_D is valid until the critical time t_{crit}, i.e. the time point for which the approximations of the model break down (Fig. 4E). The critical time window grows shorter as the absolute magnitude of the growth rates increases (Figs. 4B, D, and S1). Because the critical time is determined as the minimum of three different processes, all of which depend on the growth rates in different ways, the process dictating the critical time changes as a function of the growth rate. In Fig. 4B the limiting process for low donor growth rates is the early onset of substantial conjugation from transconjugants (time t_{c1}, see Supplementary Materials) and at higher donor growth rates the substantial reduction of the recipient population due to conjugation events (time t_{c2}, see Supplementary Materials).

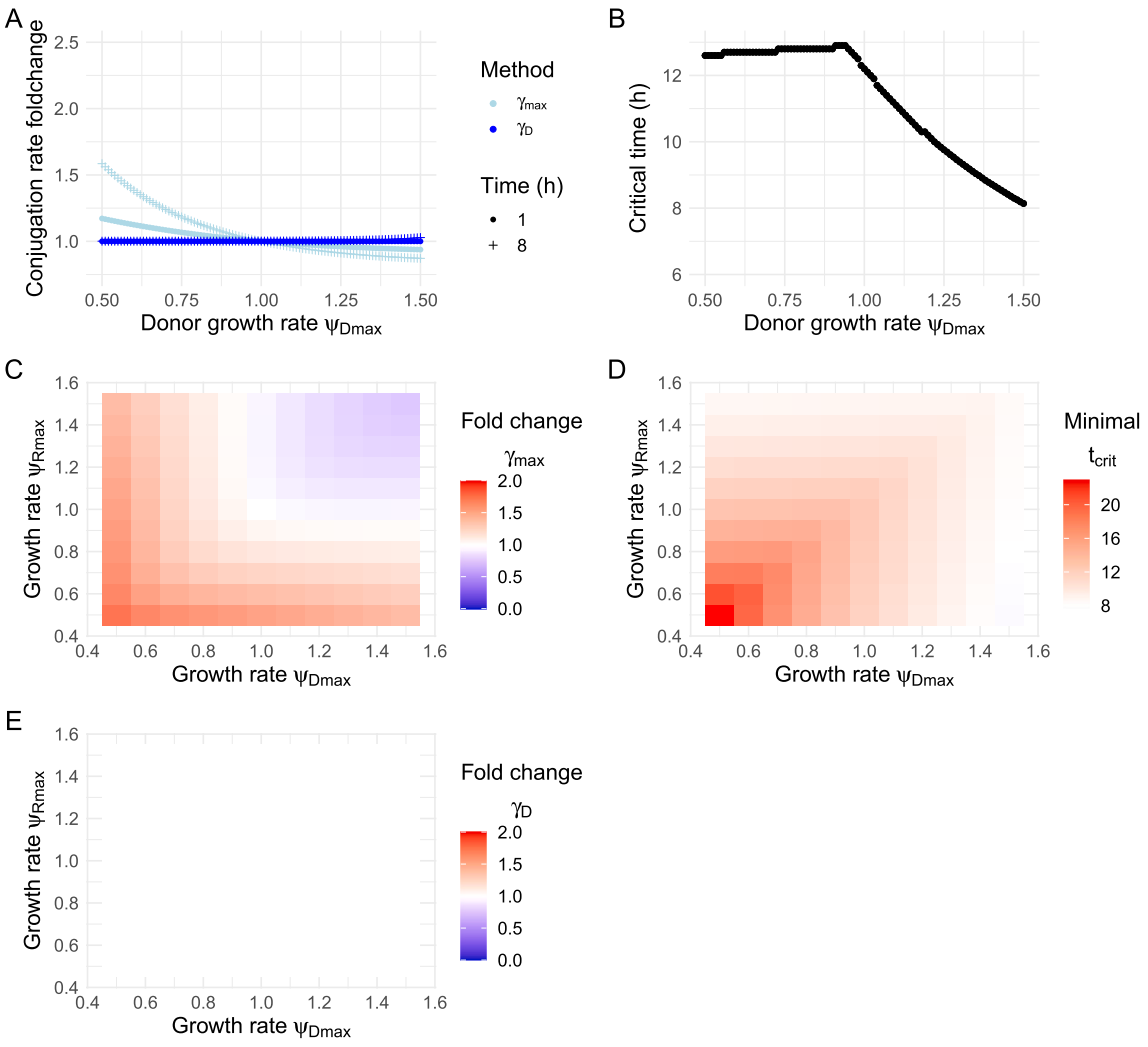

Fig. 4. The effect of growth rate differences on the accuracy of the SM and ASM conjugation rate estimates. Panel **A** shows the deviation of the estimated conjugation rate from the true value (fold change 1) in the simulation as a function of the donor growth rate, where γ_{max} denotes the SM estimate and γ_D the ASM estimate (symbols denote different measurement time points). Panel **B** shows the corresponding critical time. Panels **C** and **E** show the same comparison to the true conjugation rate as panel **A**, but with additionally varying recipient growth rates and assuming measurement after 8 h. Panel **E** seems empty because the ASM estimate is so close to the true value. Panel **D** shows the critical time corresponding to panels **C** and **E**. For deviating growth rates, the SM always shows a minor estimation error. Faster donor or recipient growth reduces the critical time, which is mirrored by the greater deviation of the estimated conjugation rates from the true value (especially for later measurement time points, as in **A**). Fold change is defined as the ratio between the estimated value and the true value. Parameters: initial population densities $R_0 = D_0 = 5 \cdot 10^6$ CFU/mL; initial resources $C_0 = 10^{14}$ μg/mL; growth rate $\psi_T = 1.0$ h^{-1}; conjugation rates $\gamma_D = \gamma_T = 10^{-13}$ mL $\cdot$ CFU^{-1}h^{-1}; approximation factor $f = 10$ are the same for all panels. For panels **A, B**, growth rate $\psi_D \in$ [0.5,1.5] h^{-1}, $\psi_R = 1$ h^{-1}. For panels **C, D, E**, growth rates ψ_D, $\psi_R \in$ [0.5,1.5] h^{-1}.

4.4. Conjugation rates

If the rates of conjugation from donors and transconjugants differ, both the SM and the ASM estimates accurately estimate the donor to recipient conjugation rate, as long as D, R, T are measured sufficiently early (Fig. 5A/C/E). This is because the contribution of TRT conjugation events will be small as long as the transconjugant population is still small. For later times, the estimated SM conjugation rate γ_{max} will interpolate between γ_D and γ_T. The estimated time at which the approximations break down (t_{crit}) is the same for both methods (Fig. 5B/D). As can be seen in Fig. 5A and C/E, this means that the magnitude of the misestimation of SM and ASM estimates depends strongly on the measurement time point. This shows that it is critically important not to measure too late.

4.5. Protocol

These theoretical considerations have led us to propose the following protocol to perform conjugation assays. In its most complete form the protocol requires two conjugation experiments: a first one starting from a $D + R$ mixed culture, and then a second one with $T + R'$. The dash is to indicate that the recipients of the second experiment (R'; or the transconjugants from the first experiment) need to be provided with an additional selective marker such that the transconjugants of the second experiment (T') can be distinguished from those of the first (T).

As pointed out in the previous section, it is important that the population densities of D, R and T are measured before the critical time is reached. Strictly speaking, this critical time can only be determined after both conjugation experiments are completed, as they require an estimate of both conjugation rates (γ_D, γ_T), as well as all growth rates (ψ_D,

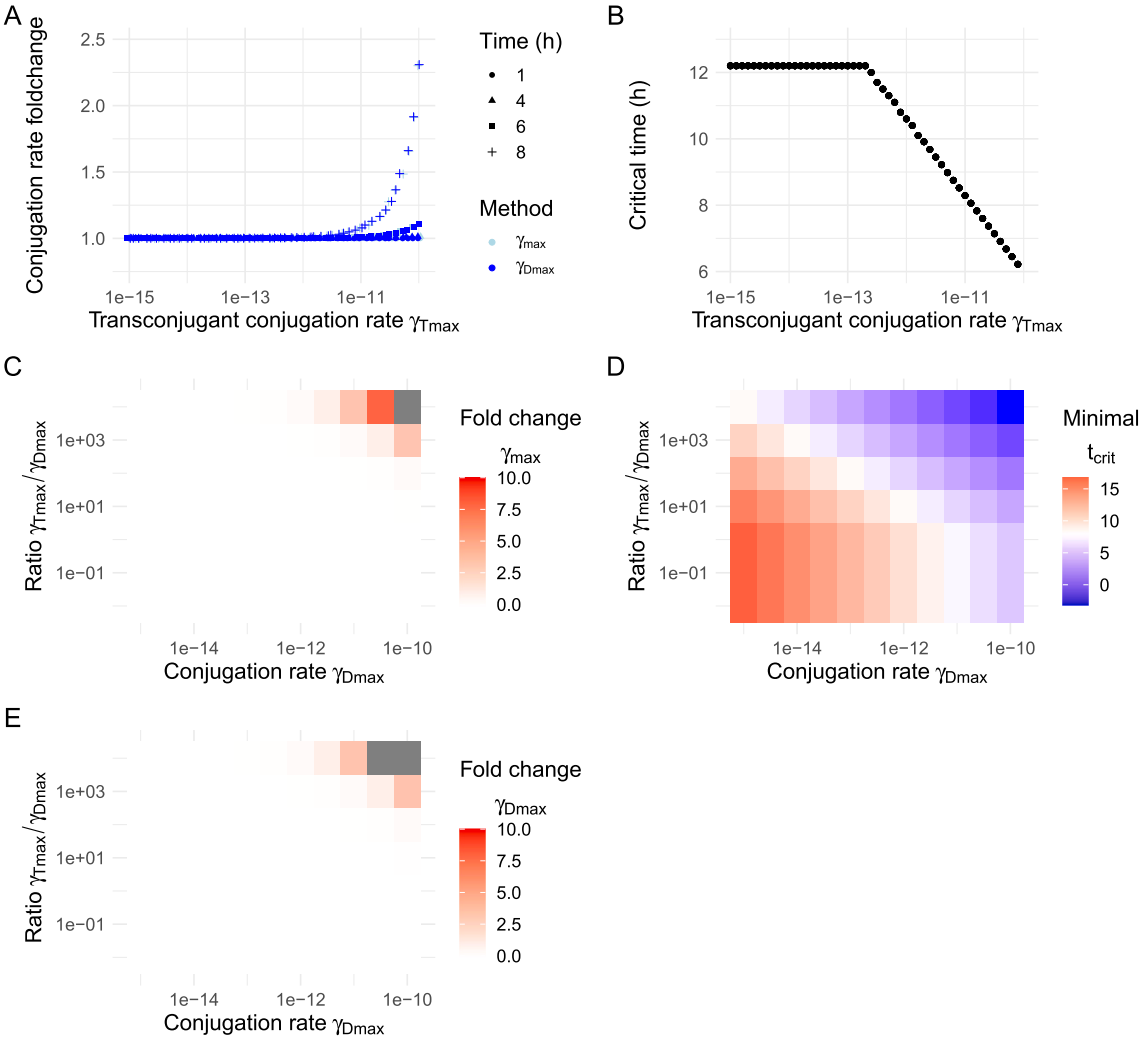

Fig. 5. The effect of conjugation rate differences on the accuracy of the SM and ASM conjugation rate estimates. Panel **A** shows the deviation of the estimated conjugation rate from the true value (fold change 1) in the simulation as a function of the transconjugant conjugation rate, where γ_{max} denotes the SM estimate and γ_D the ASM estimate (symbols denote different measurement time points). Panel **B** shows the corresponding critical time. Panels **C, E** show the same comparison to the true conjugation rate as panel **A**, but with additionally varying donor conjugation rates and assuming measurement after 8 h. Panel **D** shows the corresponding critical time. The ratio γ_T/γ_D **C, D, E** indicates how much bigger the rate of conjugation from transconjugants is than that from donors. For deviating transconjugant conjugation rates both methods are correct within the critical time **A** (the methods are partially overlaid). Faster conjugation rates reduce the critical time, which is mirrored by the greater deviation of the estimated conjugation rates from the true value (especially for later measurement time points, as in **A**). Fold change is defined as the ratio between the estimated value and the true value. Both the SM and ASM result in numerical errors when measuring substantially above the critical time, upper right corner of panels **C, E**. Parameters: initial population densities $R_0 = D_0 = 5 \cdot 10^6$ CFU/mL; initial resources $C_0 = 10^{14}$ μg/mL; growth rates $\psi_D = \psi_T = \psi_R = 1.0\ h^{-1}$; approximation factor $f = 10$ are the same for all panels. For panels **A, B**, conjugation rate $\gamma_D = 10^{-13}$ mL · CFU^{-1}h^{-1}, and conjugation rate $\gamma_T \in [10^{-15}, 10^{-10}]$ mL · CFU^{-1}h^{-1}. For panels **C, D, E**, conjugation rates $\gamma_D \in [10^{-15}, 10^{-10}]$ mL · CFU^{-1}h^{-1}, $\gamma_T \in [10^{-17}, 10^{-6}]$ mL · CFU^{-1}h^{-1}.

ψ_R, ψ_T, see Supplementary Materials Eqs. (9.24), (9.25), (9.26)). To optimise the chance of measuring below the critical time, we recommend to measure as soon as a measurable number of transconjugants has been formed.

Note, if one can assume that the difference between γ_D and γ_T is negligible, then the second conjugation experiment with $T + R'$ is not necessary. This protocol also does not capture the effect of transitory derepression and thus implicitly assumes that this state has a small effect on the conjugation rate from transconjugants compared to genetic factors. The general effect of transitory derepression would be to increase γ_T, and thus reduce the critical time further. The R package and Shiny app we developed contains a function to determine how sensitively the minimal critical time depends on the presumed values of the conjugation rate from transconjugants γ_T.

Run 1st experiment with D and R:

- Grow overnight cultures of D and R. We recommend diluting overnight cultures appropriately and growing strain cultures into early exponential phase before the start of the assay (Lopatkin et al., 2016).
- The ASM method requires the initial densities of D and R, but is not sensitive to the exact value as long as the order of magnitude is correct (necessary to determine the critical time). Plating at t_0 is therefore mostly optional.
- Incubate cultures of D and R in isolation and as a mixed culture of $D + R$. Measure the growth rates of all cultures in exponential phase. It can be convenient to set this experiment up in a plate reader and measure optical density through time. This yields estimates for the growth rates ψ_D and ψ_R (in h^{-1}) from the single cultures, as well as ψ_{max} from the mixed culture.
- Plate the mixed culture on selective plates at a time t_1, to estimate the population densities of D, R and T (in CFU/mL). This time point

should be early enough, such that there is a high chance that it is below the critical time $t_{crit,\ 1}$ for the 1st experiment. We recommend the inclusion of appropriate controls to test whether conjugation occurs on the double selective agar plate, rather than in liquid culture (Benz et al., 2021; Bethke et al., 2020; Philipsen et al., 2010).

- Calculate the ASM estimate for the conjugation rate from donors γ_D. This requires the initial population densities D_0, R_0; the growth rates ψ_D, ψ_R, ψ_T; the time of measurement t_1; and the measured population densities D_{t1}, R_{t1}, T_{t1}.
- In case you are considering not to perform the 2nd conjugation experiment, you can use the R package or Shiny app to determine how sensitively the minimal critical time depends on the presumed values of the conjugation rate from transconjugants γ_T.

Run 2nd experiment with T and R':

- Isolate single transconjugant clones T from the 1st experiment, to use as plasmid donors in the 2nd experiment. Either these clones or the recipients used in this 2nd experiment need to be provided with an additional selective marker such that the transconjugants of the 2nd experiment (T') can be distinguished from those of the 1st experiment (T).
- Grow overnight cultures of T and R'. We recommend diluting overnight cultures appropriately and growing strain cultures into early exponential phase before the start of the assay (Lopatkin et al., 2016).
- Incubate cultures of T and R' in isolation and as a mixed culture of $T + R'$. Measure the growth rates of all cultures in exponential phase. This yields estimates for the growth rates ψ_T from the single cultures, as well as ψ_{max} from the mixed culture.
- Plate the mixed culture on selective plates at a time t_2, to estimate the population densities of T, R' and T'. This time point should be early enough, such that there is a high chance that it is below the critical time $t_{crit,\ 2}$ for the 2nd experiment.
- Estimate the conjugation rate from transconjugants γ_T.
- Check whether $t_2 < t_{crit,\ 2}$ for the 2nd experiment.
- If the 2nd experiment is within the critical time, check whether $t_1 < t_{crit,\ 1}$ for the 1st experiment.

If either t_1 or t_2 are too large, the experiments will need to be repeated, choosing times smaller than t_{crit}.

In Fig. 6 we show the results of such a full protocol for a conjugation experiment between two *E. coli* strains with similar growth rates. The conjugation rate from transconjugants was about one order of magnitude higher than from donors. The ASM estimate could not be computed based on the measurements taken after 24 h, since they were past the critical time (9.3 h). The SM estimate shows slightly higher γ_T estimates (from the TRT experiment) after 24 h than after 4, but this difference is not significant. The reason these estimates do not differ more strongly is likely because the stationary phase was reached relatively early (after 6 h).

5. Tools for the scientific community

We present an R package called *conjugator* (https://github.com/JSHuisman/conjugator) which allows researchers to calculate various plasmid conjugation rates from experimental data, and check whether a given experiment was measured within the critical time. Currently the package includes the SM, ASM, T/D, T/DR, $T/(R + T)$, $T/\sqrt{DR}$, and $log(T/\sqrt{DR})$. The package can be extended by other methods in the future, including recently proposed fluctuation test based methods (Kosterlitz et al., 2021).

To further enhance the accessibility of these functions, we added a graphical user interface to the package in the form of a Shiny web application (https://ibz-shiny.ethz.ch/jhuisman/conjugator/). This app allows researchers to (i) upload their own data, use the functions from the package, and download the results, as well as (ii) simulate bacterial population dynamics with conjugation to get a better feeling for how the different conjugation measures behave depending on different parameters of the experiment.

6. Discussion

There is no gold standard to determine and report conjugation rates, and this has complicated the comparison of experimental values obtained by different research groups or under different (a)biotic conditions (Sheppard et al., 2020; Alderliesten et al., 2020). We have presented an overview of the different methods found in the literature, and exemplified how commonly used methods are affected by initial population densities, donor to recipient ratios, differences in growth rate of the mating strains, or measurement time point. As much as possible, we propose to settle on a single method to describe conjugation proficiency (Sørensen et al., 2005). Ideally, such a measure would allow comparison across experimental conditions, and to parametrise mechanistic models used to explain and predict plasmid dynamics. Both the SM and ASM methods are reasonable for this purpose.

If the donor, recipient, or transconjugant populations differ in their growth rates, the SM makes a minor estimation error that is corrected by using our new ASM estimate. When the conjugation rate from transconjugants differs substantially from the donor conjugation rate, both

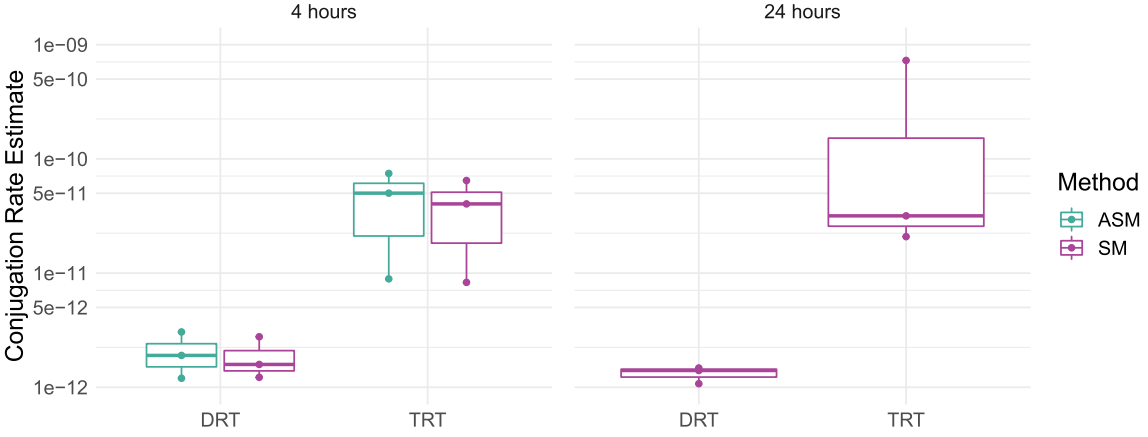

Fig. 6. Full protocol measured after 4 and 24 h, and conjugation rates estimated with the SM and ASM. The conjugation rate from transconjugants was about an order of magnitude higher than from donors. For these strains the minimal critical time was 9.3 h, but stationary phase was reached already after 6. Donor D was mated with recipient R in VL medium ($n = 3$), according to the full protocol (see Methods). For the TRT experiment, we used a previously isolated transconjugant strain with spontaneous nalidixic acid resistance (D′). The Donors and recipients were mixed in a 1:1 ratio, starting at an initial density of $1 \cdot 10^6$ CFU/mL.

methods estimate a correct conjugation rate only during the initial phase of the experiment, before the critical time is reached. Overall, we find that bacteria with large growth rate differences, high absolute growth rates, and high absolute conjugation rates are most likely to lead to problems in conjugation rate estimation, as these factors speed up the population dynamics and reduce the critical time.

To encourage 'best practices' in the estimation and reporting of conjugation rates, we developed an R package and web application that compute these values from experimental data. A further clear conclusion of this work is that one should measure the outcome of conjugation assays early, before the dynamics become dominated by conjugation from transconjugants. Our critical time estimates give an indication of how early this should be.

Several caveats remain for both the SM and the ASM. First, these models are in principle not suitable for application to mating assays on solid surfaces, as they assume well-mixed conjugating populations. However, the conjugation rates in high-density, well-mixed surface mating experiments are comparable to liquid mating, provided they are measured sufficiently early (Xue et al., 2012). Second, the ASM assumes that the growth rates in monoculture are predictive for the same strains in mating populations, and thus disregards competitive effects. Direct measurements of the individual mixed culture growth rates would require in situ tracking of donors, recipients and transconjugants. Last, these methods are based on population dynamic models that assume no dependence of conjugation on cell density, no induction in stationary phase, and no segregational loss. These assumptions may hold for IncF plasmids, but do not extend to all plasmid families (Sysoeva et al., 2020; Turner, 2004). Future work should establish how common such 'atypical' plasmids are, and develop methods to quantify the conjugation rate also in these cases. Until then, these concerns could be addressed by constructing a more complex conjugation and growth model and fitting it to time course data of the different mating populations (Kneis et al., 2019; Fischer et al., 2014; Xue et al., 2010). Further methods can be added to the conjugator R package in the future.

Author contributions

JSH: Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing - review & editing

FB: Conceptualization, Validation, Writing - review & editing

SJND: Investigation, Validation, Writing - review & editing

JAGMdV: Funding acquisition, Resources, Supervision, Writing - review & editing

ARH: Conceptualization, Funding acquisition, Supervision, Writing - review & editing

EAJF: Funding acquisition, Methodology, Software, Supervision, Writing - review & editing

SB: Conceptualization, Funding acquisition, Methodology, Resources, Software, Supervision, Writing - review & editing

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2022.102627.

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