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Guidelines for the estimation and reporting of plasmid conjugation rates

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ABSTRACT

Conjugation is a central characteristic of plasmid biology and an important mechanism of horizontal gene transfer in bacteria. However, there is little consensus on how to accurately estimate and report plasmid conjugation rates, in part due to the wide range of available methods. Given the similarity between approaches, we propose general reporting guidelines for plasmid conjugation experiments. These constitute best practices based on recent literature about plasmid conjugation and methods to measure conjugation rates. In addition to the general guidelines, we discuss common theoretical assumptions underlying existing methods to estimate conjugation rates and provide recommendations on how to avoid violating these assumptions. We hope this will aid the implementation and evaluation of conjugation rate measurements, and initiate a broader discussion regarding the practice of quantifying plasmid conjugation rates.

1. Introduction

The transfer of a plasmid between neighboring bacteria through conjugation plays a central role in bacterial ecology and evolution. However, there is little consensus on how to determine plasmid conjugation rates (Huisman et al., 2022; Sheppard et al., 2020). Indeed, the experimental assays and analytical methods commonly used to quantify conjugation differ widely and are often biased (Huisman et al., 2022; Kosterlitz et al., 2022). In the past year, we have developed new methods to extend the range of biological systems for which conjugation rates can be estimated accurately (Huisman et al., 2022; Kosterlitz et al., 2022). However, across all available methods, the accuracy and precision of conjugation rate estimates strongly depend on the way experiments are designed and implemented. Clear and detailed reporting is essential to assess the quality of published conjugation rate estimates. Unfortunately, many publications lack the necessary detail to evaluate and reproduce the reported experiments. Here, we propose actionable guidelines for experimental design and reporting, and aim to start a broader discussion about best practices when estimating plasmid conjugation rates.

One important source of confusion is the common use of methods that describe conjugation but do not quantify conjugation rates. These methods (hereafter "population ratios") calculate the ratio between two populations involved in conjugation (e.g., the number of transconjugants per donor). Rather than conjugation alone, this composite metric combines plasmid spread through conjugation and clonal growth into a single quantity. As a result, these measures are affected by environmental conditions that influence growth (e.g., nutrient concentrations or antibiotics). They also vary depending on the initial population densities, initial donor-to-recipient ratio, and duration of the conjugation assay (Huisman et al., 2022; Simonsen et al., 1990).

Instead, methods to estimate conjugation rates use explicit models of bacterial population dynamics to derive a measure for conjugation, independent of growth. These methods include the Levin et al. method (Levin et al., 1979), the Simonsen et al. end-point method (Simonsen et al., 1990), the Huisman et al. approximate Simonsen method (Huisman et al., 2022), or the Kosterlitz et al. Luria-Delbrück method (Kosterlitz et al., 2022); see Supplementary Material Table S1. Thus, in contrast to population ratios, these methods are robust to changes in initial population densities and initial donor-to-recipient ratios. However, all existing methods are bound by their underlying assumptions about the dynamics of conjugation (Huisman et al., 2022; Simonsen et al., 1990). An overview of these theoretical assumptions can be found in Supplementary Material I. When assumptions are violated a method may become inaccurate (Huisman et al., 2022; Simonsen et al., 1990). This restricts the range of biological systems that can be explored with a

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given method and further underscores the importance of reporting all relevant experimental and environmental parameters to reproduce an experiment.

We developed a checklist to guide the planning of laboratory experiments and the reporting of plasmid conjugation rates. We found that methods to estimate conjugation rates exhibit sufficient similarity to curate such a general checklist. The goal is to help researchers design experiments that produce accurate conjugation rate estimates. In addition, the checklist can help other researchers assess the data and the conclusions drawn from it. Guidelines in other fields have helped standardize experimental design and reporting practices, sparked new method development, and increased research reproducibility (Bustin, 2010). Standardized reporting of conjugation experiments will improve the ability to compare conjugation rates estimated for different plasmids, bacteria, and environments. Such a standardized approach may help resolve contradictory claims in the literature (e.g., the effects of antibiotics on the conjugation rate (Lopatkin et al., 2016; Shun-Mei et al., 2018; Zhang et al., 2013)) and translate estimates from in vitro settings to more complex environments (e.g., the animal gut (Benz et al., 2021; Loftie-Eaton et al., 2021)).

We first clarify nomenclature to establish terminology for the checklist, and to promote standardization in the literature. Then, we give the general outline and motivation for the checklist, focusing on its four constituent sections: experimental design, verification of assumptions, data analysis, and conclusions. Lastly, we discuss some considerations that may extend beyond the current framework of plasmid conjugation experiments. We hope this piece will prompt a broader discussion regarding best practices for estimating plasmid conjugation rates.

2. Nomenclature

To aid the standardized reporting of plasmid conjugation experiments, it is important to define a few key terms and steps in this process. In the following, we focus on conjugative plasmids; however, these recommendations can be extended to other mobile genetic elements that conjugate, such as integrative conjugative elements (ICEs). Plasmid c*onjugation* is a process in which a plasmid is transmitted via close contact between a *donor* (D) and *recipient* (R) bacterium. These populations may be different strains or species. Upon conjugation, the recipient is turned into a *transconjugant* (T). The transconjugant population generally contains the same chromosome and resident plasmids as the recipient population, as well as the plasmid(s) that are transferred by conjugation from the donor.

The *conjugation rate* (also called *plasmid transfer rate*) describes the number of conjugation events per donor density, per unit of time. It parametrizes the horizontal infective spread of a focal plasmid and the corresponding increase in transconjugants. Rates are typically reported as population-level averages. Importantly, the conjugation rate is independent of the increase in transconjugants due to clonal growth. This is in stark contrast to the ratio-based methods that attempt to describe conjugation proficiency using dimensionless ratios between the different populations involved in conjugation (e.g., T/D or T/R). We propose to call these *population ratios*, as their common name "conjugation frequency" could be easily confused with the term "conjugation rate." Population ratios do not quantify conjugation dynamics, but describe relative success of a plasmid in a new host (i.e., T in the numerator is compared to various populations in the denominator). The conjugation rate is specific to the conjugating donor and recipient populations, the focal plasmid, and the environmental conditions (Sheppard et al., 2020; Benz et al., 2021). Hence a conjugation rate is meaningless without referencing the experimental context and the biological entities involved.

Conjugation rates are measured in the laboratory by performing a *conjugation* (or mating) *experiment*. This typically consists of two parts: (i) an *experimental assay* and (ii) a subsequent *quantification method* used to estimate a conjugation rate from the experimentally measured parameters. The experimental assay typically includes separating the donor, recipient, and transconjugant populations through selection to quantify their population densities (e.g., dilution plating with different antibiotics). The *specificity* of such a selection assay describes its ability to select only the intended cell types, thus decreasing the number of false positives. The *sensitivity* of a selection assay describes its ability to accurately enumerate the intended cell types, thus decreasing the number of false negatives.

Each quantification method requires specific parameters to be measured and thus influences the design of the experimental assay. Although many components of a mating experiment may vary between studies (i.e., quantification method, focal hosts and plasmids, selective conditions), there is sufficient similarity between the various quantification methods that a general checklist can help guide the design and reporting of different conjugation experiments.

3. Reporting checklist

The reporting checklist (Table 1) is intended to help authors plan and report experiments to estimate conjugation rates. The construction of the checklist was guided by current literature, which suggests that certain biological variables and experimental steps affect the accuracy and precision of conjugation rate estimates (Huisman et al., 2022; Sheppard et al., 2020; Kosterlitz et al., 2022; Simonsen et al., 1990; Zhong et al., 2012; Alderliesten et al., 2020). The items in the checklist are organized into four sections: (i) experimental design, (ii) verification of assumptions, (iii) data analysis, and (iv) conclusions. Here we briefly summarize the motivation and rationale for these general sections of the checklist. A more detailed description and an example of each item are provided in the Supplementary Material II.

The first section, experimental design, deals with the study's blueprint for estimating conjugation rates. The six items in this section are motivated by the extensive literature showing that conjugation rates depend on (a)biotic factors such as the identity of donors, recipients, and their plasmids (Sheppard et al., 2020; Benz et al., 2021; Alderliesten et al., 2020; Dimitriu et al., 2019), the physiology of the donors and recipients (Sysoeva et al., 2020), spatial structure (Zhong et al., 2012), and temperature (Rozwandowicz et al., 2019). For instance, the conjugation rate often changes depending on the growth phase of the participating donor and recipient bacteria in a plasmid-dependent manner (Sysoeva et al., 2020). Therefore, item 1f calls for reporting details on the preparation of donor and recipient bacteria for the mating assay. Additionally, item 1 g focuses on the known dependence of conjugation rates on the probability of donor and recipient bacteria to encounter each other. Therefore, the shape and size of the vessels used for the conjugation assay and the shaking speed could affect estimated conjugation rates and should be reported (Zhong et al., 2010). These details are not only crucial for the replication of the study, but ultimately affect the interpretation of the estimated conjugation rates by authors, reviewers, and readers.

The second section focuses on verifying the experimental and theoretical assumptions that underlie the experimental assay and its quantification. Violations of either type of assumption can lead to inaccurate conjugation rate estimates regardless of the chosen quantification method (Huisman et al., 2022; Kosterlitz et al., 2022; Simonsen et al., 1990; Zhong et al., 2012). Notably, the experimental design (checklist section 1) will determine what assumptions to verify. For instance, a common experimental assumption is that the selective conditions quantify only transconjugants that arose in the mating culture. However, in the case of selective plating, it has been shown that donors and recipients often continue to form transconjugants on the transconjugant-selective plates (Jordt et al., 2020; Bethke et al., 2020; Smit and van Elsas, 1990). If this occurs, the transconjugant density is overestimated, and the conjugation rate estimate will be inflated. On the theoretical side, a common assumption is that the donors and transconjugants

Table 1
Overview of the reporting checklist for conjugation experiments. The checklist items are organized into four main sections (1–4) with multiple items per section (e.g., 1.a-1.g). A brief description is provided for all items. A more detailed description and example for each item are provided in Supplementary Material I.

1.	**Experimental design**	
1.a	Purpose	The question, goal, hypothesis, and rationale of the experiment.
1.b	Experimental variables	The experimental conditions used. State the different biological (e.g., bacteria or plasmids) and environmental parameters (e.g., temperature, growth media) used in each assay.
1.c	Biological samples	The donors and recipients used. Report strain identity (e.g., taxonomy, sequence accessions, source) and characteristics (e.g., antibiotic resistance profiles).
1.d	Quantification method(s) to be used	The conjugation quantification method(s) applied (e.g., Simonsen et al. end-point method4) and the corresponding variables measured during the experiment.
1.e	Description of the selective conditions	The chosen quantification method (e.g., dilution plating, flow cytometry, qPCR) and selective agent(s) (e.g., antibiotics, wavelength for fluorescent markers, primers) used. Include the expected results of each selective agent.
1.f	Sample preparation	The preparation of the biological samples for the assays (e.g., freezer conditions, reanimation procedure, enrichment/growth protocol, growth medium, culturing vessel, added selective agents).
1.g	Protocol details	The full conjugation protocol (e.g., preparation of the mating mixtures and the chosen incubation times). Clearly describe each quality control step (e.g., number of biological/technical replicates, equipment calibrations, controls).
2.	**Verification of assumptions**	
2.a	Verification of experimental assumptions	The assays used to verify experimental assumptions such as the specificity and sensitivity of the selective conditions and the absence of post-assay conjugation in the selective conditions.
2.b	Verification of theoretical assumptions	The assays or implementation procedures used to verify that the conjugation protocol abides by the theoretical assumptions of the chosen quantification method(s).
3.	**Data analysis**	
3.a	Raw data	Provide the unprocessed raw data for each assay reported in the study with the appropriate metadata that describes the identity of all variables in the dataset.
3.b	Analysis	The analysis steps used to process the data and to calculate the conjugation rates with the chosen quantification method(s).
3.c	Processed data	Provide the processed data for each figure or analysis reported in the study with the appropriate metadata that describes the identity of all variables in the dataset.
3.d	Results	Report results of the conjugation assay including variance across technical replicates.
4.	**Conclusions**	
4.	Conclusions	Interpret the experimental results in light of the assumptions and limitations of the experimental assay and quantification method (s).

conjugate to recipients at the same rate. However, suppose the donors and recipients are different species. In that case, the donor-to-recipient (i.e., cross-species) conjugation rate will likely differ from the transconjugant-to-recipient (i.e., within-species) rate (Kosterlitz et al., 2022; Benz et al., 2021; Lundquist and Levin, 1986). When an experimental or theoretical assumption is violated, authors may need to adjust the experimental design to obtain accurate conjugation rate estimates. Modifications can include changing the quantification method, the conjugation protocol (to minimize the effects of violations), or the biological samples. We recommend checking some assumptions during the experiment, while it may be advisable to check others beforehand. To produce reliable and accurate conjugation estimates, allowing a feedback loop between the verification of assumptions and the experimental design is key.

The third section of the checklist deals with data sharing and analysis. Good data management and reporting serve three main purposes: it helps catch mistakes in data entry or analysis scripts, helps determine whether the analysis was appropriate, and aids other researchers when using or replicating the reported results (Broman and Woo, 2018; Wilkinson et al., 2016; Hart et al., 2016). Data analysis choices profoundly affect the final results and should be documented in as much detail as the experimental protocol. Ever more research institutions, journals, and funders require data sharing according to the FAIR principles (Findability, Accessibility, Interoperability, and Reusability) (Wilkinson et al., 2016). These dictate best practices in data sharing including the use of standard, machine-readable file formats, and reporting metadata describing data columns and variables (Wilkinson et al., 2016; Hart et al., 2016; White et al., 2013). It is important to share not only processed data and research results but also the raw data from experiments. This helps preserve the original data, evaluate the reliability of density estimates, understand the data analysis steps, and reuse the data in future studies such as meta-analyses (Broman and Woo, 2018; White et al., 2013). In addition to data, all workflows, methods, and scripts that led to the final results should be reported and deposited in publicly accessible repositories. We encourage readers to consult existing guidelines to improve code and data readability (Broman and Woo, 2018; Wilson et al., 2014; Wilson et al., 2017).

The last section of the checklist covers the interpretation of results and conclusions drawn from the experiment. All other items in the checklist should be incorporated into this step. Together they determine the generality of the conjugation rate estimates and their suitability to address the motivating question of the study. Careful consideration of items in the checklist, specifically experimental and theoretical assumptions, may cause the conclusions of the study to be more limited or conditional than intended. However, this should not prevent author's from publishing their findings. Instead, they can use the checklist to inform the discussion and interpretation of the results. Alternatively, if this information is used early, it can help change the experimental design. Thus, it may be useful to envision this item not necessarily as the last step but as an informative step in a feedback loop.

4. Discussion

Conjugation rate estimates are important to understand and predict the ecology and evolution of bacterial communities. Here, we revisited the approaches used to estimate plasmid conjugation rates. We found substantial similarities across existing experimental assays and quantification methods, providing an opportunity for synthesis and general recommendations. First, we propose to unify the nomenclature on plasmid conjugation rates to aid communication in the field. Second, we curated a structured list of things that are important when designing and executing conjugation rate estimation experiments. We recommend adopting this as a reporting checklist while documenting and assessing conjugation experiments. The checklist is a general starting point, but we acknowledge that the content reported for each checklist item will differ substantially between studies. Indeed, there are a large number of possible combinations - including quantification methods, hosts, plasmids, and environments - in the experimental design section of the

checklist. As a result, there is no one-size-fits-all approach to executing conjugation experiments.

The checklist is not meant to define a linear path of execution for a conjugation experiment. If an experimenter needs to adjust a design component, it will likely require revisiting other items in the checklist. For example, one may shorten the incubation time of the mating assay to avoid violating the assumption of exponential growth at a constant growth rate. Yet, this in turn can increase the variance in conjugation rate estimates, and decrease the ability to detect differences between conjugation rates. These feedback loops can complicate troubleshooting because each design choice is intertwined with and dependent on others. A seemingly simple modification may cause a cascade of adjustments to other elements of the experimental design. Although our guidelines do no't offer system-specific modifications (although see Supplementary Material I for suggested modifications), the checklist can be a structured aid for designing, troubleshooting, executing, and reporting.

A clear focus for future development should be to address the limitations of existing quantification methods (an overview of their assumptions can be found in Supplementary Material I). Although new methods have become available this past year, there are still combinations of biological questions, hosts, plasmids, and environments that no available method can address. This opens several important avenues for future method development. For example, conjugation in a spatially structured environment (Zhong et al., 2012; Reisner et al., 2012; Stalder and Top, 2016), including on agar and filters, violates the assumption of well-mixed populations common to all existing methods. As such, no method can accurately assess conjugation rates in the numerous spatially structured environments bacteria inhabit in nature, including highly relevant ones such as biofilms. Novel techniques like single-cell imaging could help develop new models to quantify the effects of spatial mixing and lead to novel ways to estimate bulk conjugation rates in structured environments (Couturier et al., 2023). Overall, we recommend that the available techniques to estimate plasmid conjugation rates be continually evaluated. We hope this will spark new method development and make existing methodologies more robust and reliable.

5. Conclusion

We provided guidelines for estimating and reporting plasmid conjugation rates. These are based on careful consideration of the sources of variability when estimating such rates and constitute what we consider best practices. We hope this will initiate a broader discussion regarding the practice of estimating plasmid conjugation rates and improve both the implementation and evaluation of conjugation measurements. Ongoing method development will extend the number of environments and species in which we can estimate and compare plasmid conjugation rates, and will also feed into this continuing discussion. We encourage researchers to reconsider these guidelines and the checklist as new insights and innovations become available.

CRediT authorship contribution statement

Olivia Kosterlitz: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Jana S. Huisman:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

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Appendix A. Supplementary Material

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