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New quantitative methods for measuring plasmid loss rates reveal unexpected stability

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Abstract

Plasmid loss rate measurements are standard in microbiology and key to understanding plasmid stabilization mechanisms. The conventional assays eliminate selection for plasmids at the beginning of the experiment and screen for the appearance of plasmid-free cells over long-term population growth. However, it has been long appreciated in plasmid biology that the growth rate differential between plasmid-free and plasmid-containing cells at some point overshadows the effect of primary loss events, such that the assays can greatly over-estimate inherent loss rates. The standard solutions to this problem are to either consider the very early phase of loss where the fraction of plasmid-free cells increases linearly, or to measure the growth rate difference either by following the population for longer time or by measuring growth rates separately. Here we mathematically show that in all these cases, seemingly small experimental errors in the growth rate estimates can overshadow the estimates of the loss rates. For many plasmids, loss rates may thus be much lower than previously thought, and for some plasmids, the estimated loss rate may have nothing to do with actual loss rates. We further modify two independent experimental methods to separate inherent losses from growth differences and apply them to the same plasmids. First we use a high-throughput microscopy-based approach to screen for plasmid-free cells at extremely short time scales – tens of minutes rather than tens of generations – and apply it to a *par*[−] version of mini-R1. Second we modify a counterselection-based plasmid loss assay inspired by the Luria-Delbrück fluctuation test that completely separates losses from growth, and apply it to various R1 and pSC101 derivatives. Concordant results from the two assays suggest that plasmids are lost at a lower frequency than previously believed. In fact, for *par*[−] mini-R1 the observed loss rate of about 10^{−3} per cell and generation seems to be so low as to be inconsistent with what we know about the R1 stabilization mechanisms, suggesting these well characterized plasmids may have some additional and so far unknown stabilization mechanisms, for example improving copy number control or partitioning at cell division.

Keywords

plasmid loss; fluctuation test; counterselection

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1.0 Introduction

Plasmids are extra-chromosomal genetic elements commonly found in bacteria but also in higher organisms. They are responsible for the majority of antibiotic resistance outbreaks in hospitals, are widely used in their domesticated form – cloning vectors – in research and biotechnology, and are responsible for much of the horizontal gene transfer between distantly related species (De la Cruz and Davies, 2000; Thomas and Nielsen, 2005). Plasmids have therefore been studied in great detail for many decades, and several types of plasmids are almost as well characterized as the *lac* operon or lambda phage. They have also been model systems for control at the level of single cells. In fact, many stochastic effects that now are being studied in great detail, such as intrinsic versus extrinsic noise, feedback noise suppression, and random partitioning of molecules at cell division, were first described in a plasmid context (Nordstrom and Austin, 1989; Paulsson and Ehrenberg, 2001).

A major evolutionary force shaping plasmid mechanisms is the constant threat of extirpation from the host cells, which drives plasmids towards effective control mechanisms that reduce the likelihood of creating plasmid-free competitors at cell division (Summers, 1991; Sengupta and Austin, 2011). All plasmids studied to date have been shown to encode an active mechanism for negative feedback control of the initiation of replication (Summers, 1996; del Solar et al., 1998; Chattoraj, 2000; Brantl, 2002). This narrows the distribution of plasmid copy numbers across single cells (Løbner-Olesen, 1999; Paulsson and Ehrenberg, 2001) and thereby reduces the risk of plasmid loss at cell division, where all copies segregate to one daughter and the other daughter becomes plasmid-free. Many plasmids also encode systems for actively partitioning copies between the two daughters (Gerdes et al., 2010; Salje et al., 2010) which further reduces the inherent plasmid loss rate. A variety of other plasmid-encoded systems instead help plasmids reduce the deleterious consequences of loss. For example, post-segregation killing systems (Gerdes et al., 1997; Hayes, 2003) eliminate plasmid-free progeny that otherwise could outgrow the plasmid-containing cells, and various genes can confer a selective advantage in the form of antibiotic resistance or metabolic enzymes. Despite the variety of maintenance mechanisms, plasmids are still lost from cells, and if plasmid-borne genes confer a metabolic burden on the host, plasmid-free progeny can rapidly outgrow plasmid-containing cells. Accurate plasmid loss rate assays have therefore been central to analyzing plasmid control mechanisms, but losses are also a nuisance to a much broader community of molecular biologists, forcing researchers to use plasmid-encoded drug resistance cassettes and growth media containing antibiotics to maintain expression of genes cloned onto plasmids.

The classic loss rate assay starts with a population of plasmid-containing cells and measures the accumulation of plasmid-free cells over time (Bernard and Payton, 2001). For long time courses, the differential growth rate between plasmid-containing and plasmid-free cells can then significantly interfere with measurements, making losses appear orders of magnitude more frequent than they really are. This problem has been recognized for decades in plasmid biology, but is still frequently overlooked. More careful experiments either account for the difference in growth rates or consider the early phase of the experiment where differences in growth rate have less influence and the accumulation of plasmid-free cells appears to be approximately linear (Boe et al., 1987, 1994; Boe, 1996; Boe and Rasmussen, 1996; Olsson et al., 2004).

However, here we mathematically and experimentally show that both these more careful approaches can still greatly over-estimate loss rates and may even produce results that are independent of actual plasmid losses. The reason is partly that loss rates can be small even compared to the estimation error of growth rate differences, and partly that only the

extremely early phase of the experiment truly reflects loss rates – so early that it typically cannot be analyzed with classic methods.

We also present modified experimental approaches to address this challenge. Because relying on a single assay for reporting important properties can hide experimental errors, we developed two independent assays that measure loss rates in different ways: a modification of the Luria-Delbrück fluctuation test, and a microscopy-based approach that makes it possible to measure loss rates in the extremely early phase of a plasmid loss time course experiment. We then measured the loss rates of several common plasmids with various stabilization mechanisms. The results show that plasmid loss rates may be lower than previously reported. In some cases, as for a *par⁻* mini-R1, they are in fact so low as to suggest the existence of unknown mechanisms that help stabilize plasmids.

2.0 Materials and Methods

2.1 Strains and plasmids

All strains are *E. coli* MC4100 derived and contain a P_{LacO1} -driven (Lutz and Bujard, 1997) chloramphenicol resistance cassette integrated at the Tn7 attachment site (McKenzie and Craig, 2006). All plasmids contain *lacIq-ssrA*, which produces higher levels of a LacI repressor mutant with a shorter half-life. A full list of plasmids is presented in Table 1.

2.2 Semi-automated plasmid loss screening

Strains were cultured overnight to saturation in LB medium at 37°C under antibiotic selection. The culture was then diluted by a factor of 20,000 into selective LB medium and cultured to an OD_{600} of approximately 0.3. This is early enough in exponential phase to ensure roughly balanced growth between generations. Very early exponential phase, e.g. at $OD_{600} < 0.1$ would be even more ideal, since copy numbers then are more stable from generation to generation, but this instead decreases the yield due to the reduced density of microcolonies after resuspension. 1 mL of the suspension was then centrifuged at 4,000g and resuspended in nonselective LB medium. The culture was then immediately diluted tenfold into 37°C nonselective LB medium.

At five minute intervals, 5 μ L of this dilution was spotted onto a 2% w/v selective low-melt agarose pad (MP Biomedical) surrounded by a thin double-sided adhesive (Bio-Rad) on a microscope slide. The agarose pads were then sealed with a coverslip, incubated for 30 minutes at 37°C, cooled to room temperature for 30 minutes, and stored at 4°C before imaging. Individual microscope slides were imaged at 100 $\times$ magnification on an Axiovert 200M (Carl Zeiss) inverted microscope with an automated stage. For each time point, the number of microcolonies and single lysed cells corresponding to plasmid-free cells were manually counted (Figure 2B inset; additional sample micrographs are provided in Supplementary Information). We ensured that the total number of microcolonies counted should be at least ten times higher than the inverse frequency of plasmid-free cells (see Supplementary Information for further protocol details).

2.3 Fluctuation-based test for measuring plasmid loss

Strains were cultured overnight to saturation in LB medium at 37°C under antibiotic selection. This culture was then diluted by a factor of $\sim 10^8$ into nonselective LB medium and pipetted into 96-well plates with each well containing 100 μ L of culture. The diluted cells were incubated with vigorous agitation at 37°C for several hours. If the founding populations are too small, numbers will vary from well to well and the final population sizes will vary accordingly. The method is not particularly sensitive to such fluctuations (see Section 4), but several controls are required to ensure accuracy. First we separately test that

all wells are seeded with cells, or at least the great majority, by sampling small fractions at later time points (see Supplementary Information for further protocol details). Second we separately determined that the founding populations very rarely contain plasmid-free cells by selecting for them in a separate 96-well plate with the same drug concentrations described below. Finally, in order to estimate the final population sizes, a small volume was pipetted from eight wells, individually diluted and spread on LB agar. Under our conditions the fluctuations in population size were so small (a standard deviation of <20%) that this procedure was not necessary for all wells, and we instead used the average population size obtained from the eight wells. If fluctuations are large, either all wells must be individually sampled or the starting population size should be increased. 50 μ L of LB medium containing 180 μ g/ml of chloramphenicol was then pipetted into each well to select for plasmid loss at a final concentration of 60 μ g/ml. The 96-well plate was covered with a breathable silicone film (Sigma Aldrich) and incubated overnight at 37°C with vigorous shaking. Wells in which a plasmid loss occurred grew to saturation, while the remainder did not. The proportion of clear wells p_0 were then counted and the loss rate is calculated as described in Section 4. One potential problem with such assays is that the cells that lose the plasmids inevitably take some time to express the resistance cassettes. We therefore added a degradation tag to the *lac* repressor, such that most repressor should be depleted in less than a generation. If higher accuracy is needed, it may be possible to also treat cells with cephalixin for one generation before selecting for plasmid-free cells in order to give LacI even more time to degrade without additional cell divisions that would alter N .

3.0 Theory

Plasmid stability is commonly measured by quantifying the accumulation of plasmid-free cells over time in a freshly inoculated culture of plasmid-containing cells. The experimental challenge is that the number of plasmid-free cells at any given time reflects both plasmid losses and any subsequent growth advantage of plasmid-free cells. Although initially this population is small, the increase is compounded every generation and, if plasmid-free cells do grow with a higher rate, quickly over-shadows the contribution from actual plasmid loss events. This effect has been described in studies that mathematically predict plasmid loss rates from models that account for both direct losses and differential growth. Many of these have been both sound and careful, particularly the works of Boe and coworkers, and if the experimental assays were completely accurate, we believe they would be satisfactory in most cases. However, below we show how seemingly insignificant experimental imperfections can distort loss rate estimates for a wide range of plasmids, and how this problem is effectively concealed in conventional assays.

3.1 Describing plasmid losses mathematically

The exponential growth of asynchronous populations of plasmid-containing (x) and plasmid-free (y) cells are often described assuming that both cell types double with some inherent generation times $T_{x,y}$, and that, at each division, there is a probability p_{loss} that a plasmid-containing cell produces one plasmid-containing and one plasmid-free cell. In plasmid population biology, this has been captured using differential equations that also account for e.g. horizontal gene transfer (Stewart and Levin, 1977; Levin and Stewart, 1980; Paulsson, 2002). In loss rate experiments, horizontal gene transfer generally makes no appreciable contribution, and the equations used then simplify to

$$\frac{dx}{dt} = k_x(1 - \mu)x(t) \quad \frac{dy}{dt} = k_x\mu x + k_y y$$

Parameter μ is the plasmid loss rate and $k_{x,y} = \ln(2)/T_{x,y}$ are the exponential growth rate constants. This can serve as an operational definition of loss rates in such studies, but is in fact not exactly the same as the probability of loss per cell division. The reason is that plasmid losses change the age structure of the plasmid-carrying population: when plasmids are lost, one rather than two plasmid-containing newborn cells are created, which slightly changes the relative frequencies of newborn versus dividing cells in the population of plasmid-containing cells. Because plasmids tend to be lost very rarely this effect is negligible for most purposes. For determining plasmid loss rates, however, the effects are still small, but surprisingly not insignificant regardless of how low the loss rates are. More precisely, the age distribution of plasmid-carrying cells, ignoring stochastic effects due to fluctuations in growth rate, cell division, etc. follows (see Supporting Information)

$$g(t) = \frac{\ln(2-p)}{T_x(1-p)} (2-p)^{1-t/T_x}$$

where t is the time in the cell cycle, and letting p go to zero recovers the conventional age structure. The exact differential equation for plasmid-carrying cells then follows

$$\frac{dx}{dt} = (1-p)g(T_x)x = \frac{\ln(2-p)}{T_x}x$$

with solution

$$x(t) = x(0)(2-p)^{t/T_x}.$$

This result can also be obtained directly from first principles by accounting for the fact that cells do not double each division, but on average increase by a factor $2-p$, as previously noted (Boe and Rasmussen, 1996). However, the approach above additionally allows us to exactly calculate the dynamics of the plasmid-free cells y , without any approximations. In short, the rate of change in y depends on primary plasmid losses from plasmid-containing cells as well as the subsequent growth of such cells. The primary production of plasmid-free cells from plasmid-containing cells occurs with rate

$$pg(T_x)x(t) = \frac{p}{1-p} \frac{\ln(2-p)}{T_x} x(t).$$

To in turn determine the effect of growth, we note that plasmid losses at time $t - iT_y$ would add a net of $2^i - 2^{i-1} = 2^{i-1}$ cells at time t . For example, the descendants of a plasmid loss event three generations ago, i.e., at time $t - 3T_y$, would create $8 - 4 = 4$ more plasmid-free

cells at time t . If we define $k = \frac{p}{1-p} \frac{\ln(2-p)}{T_x}$, the exact differential equation under these conditions can thus be written as

$$\begin{aligned} \frac{dy}{dt} = & \underbrace{kx(t)}_{\text{rate of creating plasmid-free cells at time } t} \\ & + \sum_i \underbrace{kx(t - iT_y)}_{\text{rate of creating plasmid-free cells } i \text{ generations earlier}} \underbrace{2^{i-1}}_{\text{additional cells at time } t \text{ descending from cells born } i \text{ generations ago}} \end{aligned}$$

where i is an integer such that $iT_y < t$. This equation can be solved analytically and the results were double-checked against numerical simulations of the same system (Supporting Information). The effects of having plasmid-free cells before the start of the experiment can also be directly added, e.g. assuming that those cells grow exponentially from some starting population $y(0)=y_0$.

Though novel and exact, we do not believe the equations above capture anything importantly different from previous approaches (Brown, ME, Cooper, NS, Caulcott, 1987; San and Weber, 1989; Boe, 1996; Boe and Rasmussen, 1996). In fact, the approach by Boe and colleagues, whose mathematical methods may seem very different, produces virtually indistinguishable predictions for all parameters we tested (Supporting Information). We primarily include the analysis to illustrate the sensitivity to experimental errors. One minor point worth mentioning is that since $\ln(2 - p_{loss}) \approx \ln(2) - p_{loss}/2$ is an almost perfect approximation for loss rates of less than 10%, the standard definition of loss rates in the population biology literature (μ above) is slightly higher than the single-cell definition, i.e., $\mu \approx 2\ln(2)p_{loss} \approx 1.38p_{loss}$. Here we use the latter because it has a more straightforward interpretation as the actual probability of loss per cell and division, but also note that a factor 1.38 is not particularly significant in most experiments.

3.2 The dynamics of plasmid loss

The decrease in plasmid-containing cells is initially dominated by actual losses, but as more and more plasmid-containing cells accumulate it instead becomes increasingly dominated by differences in growth rates between plasmid-containing and plasmid-free cells. With $f(t)$ as the fraction of cells that contain plasmids at time t , the average negative slope of decline over any single generation of growth approximately follows

$$p_{obs} = -\frac{1}{T_x} \int_{t_0}^{t_0+T_x} \frac{df}{dt} dt = f(t_0) \left(\frac{p_{loss}}{2} + \Delta s(1 - f(t_0))\ln(2) \right)$$

where $\Delta s = T_x/T_y - 1$ represents the growth differential between plasmid-free and plasmid-containing cells. This expression is highly accurate throughout when reevaluating f along the curve, where the local plasmid loss rate follows $f(t) \approx f(t_0) - p_{obs}(t - t_0)$ (Fig. 1D). If plasmid-free cells exist before the start of the experiment, which is hard to avoid for many combinations of plasmids and selective markers, f will initially change slowly and the rate of decline will be constant. Thus even an excellent fit to a linear initial decrease does not suggest that burdens can be avoided.

At all time points, the rate of decrease in the fraction of plasmid-containing cells thus has two terms, one proportional to the inherent loss rate and one proportional to the product of the growth differential and the fraction of plasmid-free cells. The problem lies in inferring the first term if the second dominates. For example, if plasmids are lost at a rate of 10^{-5} per cell and generation, impose a 10% growth disadvantage, and the slope is estimated with 90% plasmid-containing cells, the contribution from growth would be more than 1,000 times greater than from actual losses – completely overshadowing the actual loss rate unless growth differences are separately measured with three or four significant digits. Plasmid parameters vary enormously. Some natural plasmids are lost less than once every million cell cycles, while other plasmids are lost every few hundred cell cycles. Many plasmids in turn impose growth burdens of a few percent, while some are much more burdensome and yet others have burdens of less than 0.5%. The challenges above thus vary greatly between experiments and next we investigate how current experimental approaches deal with them.

3.3 Inferring loss rates from measured curves

One approach to estimating plasmid loss rates is to ignore differential growth and focus on the very early phase where its impact is the smallest, which also has the additional benefit of simplifying the curve fitting. However, the analysis above shows that growth differences only can be ignored when $1 - f < p_{loss}/\Delta s$, which can be an exceedingly small fraction: for loss rates 10^{-5} and burdens of 1%, the fraction of plasmid-free must be less than 0.1% for this method to work, which would make it exceedingly difficult to screen for plasmid-free cells.

This problem could in principle be resolved by accounting for the entire loss curve (Brown, ME, Cooper, NS, Caulcott, 1987; San and Weber, 1989; Boe and Rasmussen, 1996). However, if plasmid-free cells have a growth advantage, growth rate differences increasingly overshadow primary losses along the curve. Continuing to measure loss rates at later time points then effectively only serves to measure growth rate difference. By fitting the theoretical curves to data, small experimental errors can then become magnified into enormous errors in the loss rate estimates (Fig. 1E).

Some improvements can come from combining the two methods, by measuring burdens either separately or by following the plasmid loss curve for long periods of time, and then separately analyzing and frequently sampling the very early time period where differential growth contributes the least. The problem is that even in this ideal scenario, the *error* in the growth rate estimate may overshadow losses. For example, if the absolute error in the estimate of plasmid growth differences is about one percentage point, and plasmid-free cells make up more than 1% of the population in the early phase, then loss rates lower than 10^{-4} are effectively inseparable from any lower loss rates. Thus even quite rigorous analyses of plasmid loss dynamics are extremely sensitive to experimental errors, possibly causing plasmid loss rates to be overestimated by many orders of magnitude because of small sample errors or other seemingly minor experimental imperfections (see Supplementary Information). When using such methods for stable plasmids with substantial growth burdens, samples must therefore be taken extremely early and frequently to reduce the impact of plasmid-free growth advantages, sampling with enough statistical power to reliably count rare events despite small losses. In fact, even in the absence of experimental sampling errors, methods that effectively attempt to infer a small term from a much larger sum are exceedingly sensitive to slight systematic errors. For example, if the growth difference between plasmid-free and plasmid-containing cells changes slightly along the loss curve, it may not matter how accurate the experiments are, the inferred loss rate may still be off the mark by several orders of magnitude.

This does not mean that the method never works. Because the rate of decline is a sum of two independent terms, depending on which term dominates the results can be extremely misleading but also very accurate. However, it is important to note that it also has a tendency to conceal its own shortcomings. Plasmids with low loss rates and large burdens would often be reported as having much higher loss rates than they actually do, which in turn makes it appear as if the method was more appropriate than it really was. This means that the method cannot be *a priori* trusted even when actually it does work, which greatly reduces the parameters for which it can be used to make scientific claims. Next we develop experimental methods to reduce these problems, and further separate losses from growth rate differences. All our methods are also imperfect and are used here primarily to illustrate the challenge and suggest solutions.

4.0 Results

4.1 Plasmid loss rate measurements over short time periods

The analysis above suggests that the main source of error in measuring plasmid loss rates comes from the exponential accumulation of plasmid-free cells after the initial loss events – even in the early linear regime. The experimental challenge is thus to separate these effects. One approach is to apply an extreme version of tracking the initial decline of the plasmid-containing fraction of cells, where the analysis is restricted to a much shorter interval following removal of antibiotic selection for plasmid-containing cells – ideally only the first generation or so in an asynchronous population. For common plasmid loss rates, plasmid loss events in this phase will be so rare that large numbers of cells must be screened for accurate determination of the fraction of plasmid-containing cells. In order to increase the sampling power towards screening for plasmid loss events, we developed a strategy where we grow *E. coli* in liquid culture and take samples every few minutes for about one generation. The cells are immediately spotted on agarose pads containing the antibiotic for which the plasmid encodes resistance, and the cells are allowed to grow until they form small microcolonies. This short growth period is used to determine if the founding cell of each microcolony contains a plasmid: only plasmid-containing cells are capable of forming microcolonies, and because the antibiotic level is low, copy number fluctuation or subsequent plasmid losses in the microcolony should only cause minor variation in colony size. In contrast, single plasmid-free cells respond to the antibiotic in the agarose pad, in the case of ampicillin elongating without division and adopting a distorted morphology that can be distinguished from filamentous growth (Rolinson et al., 1977). Control experiments on a plasmid-free population indeed show that no plasmid-free cells are capable of growing into microcolonies, but rather stay as single elongated cells with bulging centers, while at the same time the antibiotic concentration is low enough to prevent spontaneous cell death.

We then use automated microscopy to estimate the fraction of plasmid-free cells for many thousand cells at every time point on a scale at which manual replica plating would be prohibitively time consuming. Using this method we observed an apparent total loss rate of $p_{obs}=1.5 \times 10^{-3}$ per cell for plasmid *par⁻* mini-R1, which is almost an order of magnitude lower than previously reported rates of 1–5% per cell and generation. (Nordström and Aagaard-Hansen, 1984; Boe et al., 1987; Dam and Gerdes, 1994; Boe, 1996), though it of course cannot be excluded that the different estimates reflect slight differences in experimental conditions or plasmid burdens. The loss rate curve also shows that the fraction of plasmid-free cells was in the range of 0.5–0.7% during the whole experiment, while separately measured growth rate differences on an incubated plate reader produced an estimated $\Delta s=7\pm 3\%$ across multiple independent trials (Supporting Information). This shows that we are indeed in a regime where primary losses dominate over growth, though not by a very large margin, and thus correcting for differential growth produces a similar estimate for the actual loss rate to be 2.4×10^{-3} . If loss rates had been lower, or the starting fraction of plasmid-free cells had been higher, the growth rates estimates would have to be more accurate. This is in fact one of the strengths of the classical approach, that it determines burdens in the same experiment as losses, and inherently uses the more accurate competition assays. However, since such assays also have errors, and the growth rate differences could be slightly different at the start of the experiment where *de novo* losses can be evaluated, we believe it is generally a better strategy to perform the loss experiments in a regime where losses have a minimal effect, than try to accurately subtract their influence in a regime where they dominate.

These results show that, for this type of plasmid, the microscopy based method indeed enables us to estimate the loss rate of a *par⁻* mini-R1, though loss values may still be slightly exaggerated due to the persistent differential growth effects. Thus despite only

tracking losses during the first generation of cell growth, we were not far from the regime where the influence of differential growth could lead to artifactual results, as we indeed observed when we measured the plasmid-containing fraction with standard plating over a few more generations (Supporting Information). This suggests that the same method would not work for a *par*⁺ mini-R1 where losses would be much rarer and thus likely overshadowed by growth rate differences, unless it was possible to reduce burdens or eliminate more of the initial plasmid free cells as well as sample cells at such high numbers that only the first few minutes of growth could be considered. Another method is therefore needed.

4.2 Fluctuation-based measurement of plasmid loss frequencies

An ideal method for measuring plasmid loss rates would completely separate the effect of initial loss events and subsequent exponential growth. Here we describe such an approach inspired by the Luria-Delbrück fluctuation test (Luria and Delbrück, 1943), which is typically used to estimate mutation rates in a population of cells. It involves inoculating a large number of growth vessels with a small number of cells (precise number is unimportant) and letting them grow into a larger population of size N . Each population is then evaluated to test if it contains at least one plasmid free cell. Starting from a small population and ending with a much larger population of N cells means that there have been close to N cell divisions in total, with most divisions in the last few generations. Because losses are so rare and fluctuations in copy number are corrected on the order of a few generations or less, individual loss events should be effectively independent. Therefore the probability of not losing the plasmid in any of the N divisions should be $p_0 = (1 - p_{\text{loss}})^N$, which can be almost perfectly approximated by $p_{\text{loss}} = -\ln(p_0)/N$. By repeating the assay a large number of times using multiple 96-well plates, and estimating the population size N , the probability p_0 can be estimated experimentally (see Materials and Methods). The exact value of N in each well, which ideally should be on the order of the inverse estimated loss rate, is determined by plating a small fraction of the population (see Section 2.3 for details). For increased accuracy these sub-populations are also screened for plasmid-loss events using standard replica plating, which also would reveal the rare cases where the founding cells are plasmid-free.

Calculating event probabilities under the Luria-Delbrück assay has been extensively discussed in the literature. Many of the analyses, including the initial study, extract information not merely from the existence of the assayed event – in this case plasmid losses – but also use the actual number of such cells to infer when the event occurred. Although this approach can improve the rate estimates (Lea and Coulson, 1949; Ryan, 1952; Boe et al., 1994) it comes at the cost of being influenced by errors in growth rate differences. We therefore ignore that information, which simplifies the experimental assay, and only consider how many of the populations have at least one loss event.

Similar methods have been introduced in the past to measure plasmid loss rates (Tolker-Nielsen and Boe, 1994), but have met with difficulties because screening for plasmid-free cells is technically challenging. Replica plating would be prohibitively time-consuming, and thus our method instead relies on developing a reliable counterselection scheme, such that only plasmid-free cells grow under certain conditions. Previous analyses have introduced the induction of plasmid-borne suicide systems (Tolker-Nielsen and Boe, 1994), but leaky expression of such genes can create systematic overestimates of the plasmid loss rate; however, the magnitude of this effect is unknown. Other assays use indicators such as X-Gal to assay for plasmid presence but suffer from lack of resolution at high colony densities (Wang and Silva, 1993), while genes such as *sacB* confer sensitivity to sucrose but suffer from a high rate of spontaneous mutants (Thomason et al., 2007). Given this dearth of efficient counterselection systems, fluctuation-based assays have not gained traction as

methods to measure plasmid loss rates. We therefore developed a simple synthetic genetic circuit in *E. coli* whereby antibiotic resistance is induced upon plasmid loss.

While many counterselection systems rely on selecting against genes located in the plasmid, we chose to create chromosomal operons that are conditionally activated and can be selected for upon plasmid loss. This method is attractive because any leakiness in expression of the antibiotic resistance cassette can be addressed by tuning the drug dosage. Furthermore, the system can be easily extended such that any arbitrary gene can be activated upon plasmid loss. Here we constitutively express a short-lived version of the lac repressor from the plasmid (*lacIq-ssrA*), which inhibits expression of a chromosomal P_{LacO-1} -driven (Lutz and Bujard, 1997) chloramphenicol resistance gene. Upon plasmid loss, rapid degradation of the repressor leads to the induction of antibiotic resistance, which is important to ensure that the plasmid free cells are not killed before they express the resistance cassettes. After a short period of growth in nonselective medium, addition of antibiotic is used to select for plasmid-free cells. We calibrate the counterselection concentration of chloramphenicol to be 60 $\mu\text{g}/\text{ml}$ (Supporting Information). In contrast to inducible plasmid-borne counterselection systems, problems with leaky expression of a suicide gene are effectively eliminated. For this particular assay, leakiness is also less of an issue since it would have to persist at a high level and for a great number of generations in order to create a population of cells that is large enough to have turbidity at the end of the assay.

Using this method, we measure the plasmid loss rate of several types of plasmids, including a pSC101-derived plasmid with and without the *hok/sok* toxin-antidote module of plasmid R1 (Gerdes et al., 1986), as well as an R1 plasmid with (*par*⁺) and without (*par*⁻) an active plasmid partitioning system. Furthermore, we observe that plasmid loss rates are significantly lower when measured by the fluctuation-based method, as expected given that the mathematical analysis predicts that the commonly used methods greatly over-estimate loss rates due to differential growth of plasmid-free and plasmid-containing cells. We also confirm the results for *par*⁻ mini-R1 obtained above: both methods show an inherent loss rate of about 10^{-3} , an order of magnitude less frequent than previously reported rates of 1–5% for *par*⁻ mini-R1 (Nordström and Aagaard-Hansen, 1984; Boe et al., 1987; Dam and Gerdes, 1994; Boe, 1996). Neither the plasmids nor the conditions are perfectly identical, but even if the comparison is not perfect we find the 10–50 fold lower rates are quite striking. Our results also suggest a lower-than-expected loss rate for pSC101 when assuming binomial partitioning of previously reported copy numbers (Cabello et al., 1976; Tucker et al., 1984), although this rate is too low to be feasibly cross-validated with our microscopy-based method described above. Given the low average copy number of this plasmid, this indicates that additional unknown stabilization mechanisms may be present.

5.0 Discussion

We show here that the traditional methods for measuring plasmid loss rates could greatly over-estimate inherent losses by failing to separate actual losses from the contributions of faster growth of the plasmid-free cells – even when done carefully and when specifically designed to separate these effects. We then modify two methods that can substantially increase accuracy while still being experimentally practical, one using microscopy to assay the creation of plasmid-free cells over one or two generations of growth rather than over tens of generations, and one based on a modification of the Luria-Delbrück fluctuation test. The latter uses a new counter-selection system where the marker can be easily changed, and may thus be useful also in other assays to conditionally trigger other operons upon plasmid loss. The microscopy-based method reduces the confounding impact of differential growth, while the fluctuation-based assay is completely agnostic to both the plasmid growth burden and the loss rate itself. Applying two separate methods to the same plasmids also allows for

independent validation. We expect applying these methods will show that many plasmids have much lower loss rates than previously believed, but since the total loss rates depend on the sum of two terms – one reflecting primary losses and one reflecting growth differences – we do not suspect small errors for all plasmids, but rather that some previous measurements are near perfect while others could be off by many orders of magnitude.

Using these methods we observe that the plasmids evaluated are lost at a significantly lower rate than previously reported. For example, we estimate that *par⁻* mini-R1 is lost at a rate of $\sim 10^{-3}$ per cell division – ten times less frequently than the previous observed rates of 1–5% (Nordström and Aagaard-Hansen, 1984; Boe et al., 1987; Dam and Gerdes, 1994; Boe, 1996). With an expected average copy number of around 6–7 per cell, and thus probably close to 10 copies per average dividing cell, this at first glance seems to make perfect sense, since binomial partitioning implies a loss rate of $2 \times (1/2)^{10} \approx 2 \cdot 10^{-3}$. However, mini-R1 has also been observed to have rather weakly corrective feedback loops that have been predicted to create a roughly Poisson distributed number of copies per cell division (Paulsson and Ehrenberg, 2001), or even much broader than Poisson if the plasmid is influenced by stochastic fluctuations in the components that determine its replication control (Paulsson, 2004). Previous analyses show that a loss rate lower than 10^{-3} then requires closer to 15 copies per average dividing cell. This inconsistency may suggest that plasmids employ other mechanisms to reduce their loss rates, such as unknown mechanisms to tighten the copy number distributions or generating ‘better than binomial’ partitioning despite being *par⁻*. Measurements of plasmid losses are insufficient to separate between these effects since they depend on the convoluted contributions of many stabilization systems. Pinpointing how these various mechanisms contribute to stability will instead require quantitative single-cell measurements of plasmid copy numbers and their partitioning statistics at cell division, which are challenging to develop (Tal and Paulsson, 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- State of the art plasmid loss rate measurements are often affected by growth rate differences between plasmid-free and plasmid-containing cells.
- We introduce two new quantitative methods for measuring plasmid loss rates.
- The new methods show that inherent plasmid loss rates can be much lower than previously thought.

Even state-of-the art plasmid loss rate measurements can be confounded by small plasmid burdens.

Figure 1.

Problems with measuring plasmid loss rates. (A) After a plasmid loss event, exponential growth of plasmid-free cells results in a higher apparent loss rate. (B) The compounded exponential growth of plasmid-free cells results in substantial errors between the measured and true loss rates. Parameters: $p_{loss}=0.00001$, $f(0)=0.995$, $\Delta s=0.01$. (C) Compounded growth errors can be minimized by measuring at earlier time points and only with plasmids that have loss rates comparable to the contribution from the differential growth of plasmid-free cells. Parameters: $p_{loss}=0.005$, $f(0)=0.995$, $\Delta s=0.01$. (D) Although plasmid losses can be accurately accounted for using piecewise averaged slopes, the majority of contributions to the changing fraction of plasmid-containing cells comes from differential growth. Parameters: $p_{loss}=0.00001$, $f(0)=0.995$, $\Delta s=0.01$. (E) Small fluctuations in simulated data results in unreliable parameter estimation. Parameters: $p_{loss}=0.00001$, $f(0)=0.995$, $\Delta s=0.05$.

Figure 2.

Rapid measurement of plasmid loss rates can reduce compounded growth errors. (A) Multiple measurements taken within a single generation can provide insight into loss rates while minimizing the exponential growth of plasmid free cells. (B) Plasmid loss rates for a mini-R1 plasmid measured using rapid screening. Error bars represent standard deviations ($N=3$). Inset: sample micrograph of microscopy-based plasmid-loss screening on a selective agarose pad. A cell affected by ampicillin is shown by the red arrow; other microcolonies otherwise appear healthy.

Figure 3.

Plasmid loss rates measured with a digital fluctuation-based assay. (A) Counterselection is achieved using P_{lac} -driven antibiotic cassette integrated into the chromosome, which is repressed by the expression of an *ssrA* degradation-tagged LacIq on the plasmid. Upon a plasmid loss event, a cell becomes resistant to an antibiotic of choice. (B) Overview of the digital plasmid loss assay. Parallel cultures are grown in replicate for several hours, upon which some wells are sacrificially taken for measuring the average population size. Antibiotic is added to the remaining wells to counterselect against the plasmid and identify wells where a plasmid loss event occurred. (C) Plasmid loss rates measured for various plasmids and stabilization operons. Error bars represent standard deviations calculated using standard propagation methods; see Supplementary Information (Replicate 96-well plates: 5; Replicates for population estimation: 4).

Table 1

Plasmids used in this study.

Plasmid name	Plasmid origin	Plasmid resistance	Stabilization mechanisms
pBTCL74	pSC101	Kanamycin	None
pBTCL75	pSC101	Kanamycin	<i>hok/sok</i>
pBTCL89	R1	Ampicillin	None
pBTCL90	R1	Ampicillin	<i>parMRC</i>