Asymmetric costs benefit the carriage of non-transmissible plasmids

Rafael C. Reding[1*]

[1] Living Systems Institute, University of Exeter, Exeter EX4 4QD, UK.

*Corresponding author: rc_reding@teknik.io

The abundance of non-transmissible plasmids challenges classical models of plasmid persistence. Costly to maintain and unable to self-propagate to avoid extinction, these plasmids should not exist without selection. And yet, they are the most abundant type of plasmid. Why? Plasmid carriage costs is measured exclusively in terms of growth rate. Here I report that plasmids can also change other traits, but not all changes reflect a 'cost'. Using two constructs of *Escherichia coli*, with and without a non-transmissible plasmid with a tetracycline-resistance gene, I found that lag and yield are also sensitive to plasmid carriage. The construct harbouring the plasmid (R) divided at slower rates, but it had longer lag and higher yield. This construct was $\sim 35\%$ less fit than its sensitive counterpart S. However, I co-maintained both constructs, and the plasmid, for more than 80 generations in pairwise competition experiments that favoured yield over growth rate. The introduction of asymmetric costs could explain the seemingly-paradoxical abundance of non-transmissible plasmids. 'Fitness' is widely used to estimate carriage costs and plasmid persistence, but relies solely on growth rate. Its predictions were inconsistent with my data, suggesting that 'fitness' can be unreliable when plasmids alter simultaneously multiple life-history traits.

I. Introduction

Plasmids are independent genetic elements that complement the chromosome of prokaryotes [1, 2] and eukaryotes [3] alike. Unlike other genetic elements, passed down from mother to daughter cells during cell division, plasmids can be *actively* transmitted to nearby cells [2, 4, 5]. This horizontal gene transfer (HGT) mechanism is considered a major evolutionary force for microbial populations [2, 5]. For example, plasmids impose a metabolic cost on their host associated with their upkeep, reducing the host's growth rate [6–11]. Without selection, therefore, plasmids should be lost to purifying selection [2, 4]. But they are not: The rates of horizontal transfer are high enough to avoid extinction [12]. However, despite the advantages of HGT, genomic data show that most plasmids are *non-transmissible* [13]. In other words, they lack the enzymes to self-transfer by conjugation, and cannot be mobilised by a trans-acting conjugative element present in the same cell. How are they maintained?

As I demonstrate below, plasmids influence each phase of microbial growth differently. The Malthusian parameter, widely used to estimate microbial and tumour growth rates [14–23], lies at the heart of classical plasmid persistence models. Therefore, little is known about how plasmids interfere with microbial growth and metabolism [5, 8, 11, 24, 25]. Here, I asked what cost do microbes pay in each phase of growth by comparing the growth dynamics of two constructs of the bacterium *Escherichia coli* [26] in pairwise competition assays. One of them, R in the remainder, harbours a non-transmissible plasmid with a tetracycline-resistance gene. This plasmid reduced substantially the growth rate of R-cells with respect to its sensitive counterpart, S. But it also increased its biomass yield—proxy for metabolic efficiency [27].

Defining plasmid costs as the relative difference in growth rate, in competition against a plasmid-free counterpart, is the 'gold standard' in microbiology [11, 28]. However, a classical concept in ecology, the rK trade-off, where r is growth rate and K is organismal carrying capacity, predicates that selection can favour more traits beyond growth rate [29–31]. Consistently with this idea, here I show that R-cells maintained their non-transmissible plasmid—carrying an antibiotic resistance gene—in competition assays that favoured yield over growth rate. The asymmetry in carriage costs suggests that traits unexpectedly benefited from plasmid carriage, like yield, may play an essential role in the maintenance of non-transmissible plasmids between positive selection events.

II. Results

Growth rate, yield and lag are sensitive to plasmid carriage. It is widely accepted that plasmids reduce the host's growth rate, but the metabolic alterations that they introduce are still unclear [5, 8, 24, 25, 32]. Crucially, growth curves can provide insight into metabolic changes undergoing in bacteria. The transition between efficient and inefficient glucose metabolism, for example, can be detected by analysing them [27]. Therefore, to study the persistence of non-transmissible plasmids I sought changes in the growth curves of two strains of *Escherichia coli* MC4100 (see methods); one of which (R) bears the plasmid pGW155B [33]. This is a 5.4kb artificial plasmid that lacks the origin of transfer *oriT*, the relaxase to catalyse the initial and final stages in conjugation, type IV coupling protein (T4CP), and type IV secretion system (T4SS) required for either self-propagation or mobilisation by a trans-acting conjugative plasmid present in the same cell [13, and references therein] (addgene vector database accession #2853). The distribution of non-transmissible plasmids with respect to their size is multimodal [34], and those around 5kb in size are the second most frequent type. pGW155B also harbours the tetracycline resistance gene *tet(36)*, a ribosome-protection type resistance protein [33] that I used as advantageous, selectable trait. Importantly each construct is tagged with different fluorescence proteins, that I use to identify them in mixed culture conditions. Now, the growth dynamics of each construct in the absence of tetracycline and competition (Figure 1A) show that harbouring pGW155B penalised the growth rate of R

by 38.42% ± 12.39% (mean ± standard error with 8 replicates, Mann-Whitney U-test $p = 1.554 \times 10^{-4}$, ranksum = 100; Figure 1B) compared to its sensitive counterpart, S, as we may expect. But the growth curves also exposed noteworthy differences in other growth parameters.

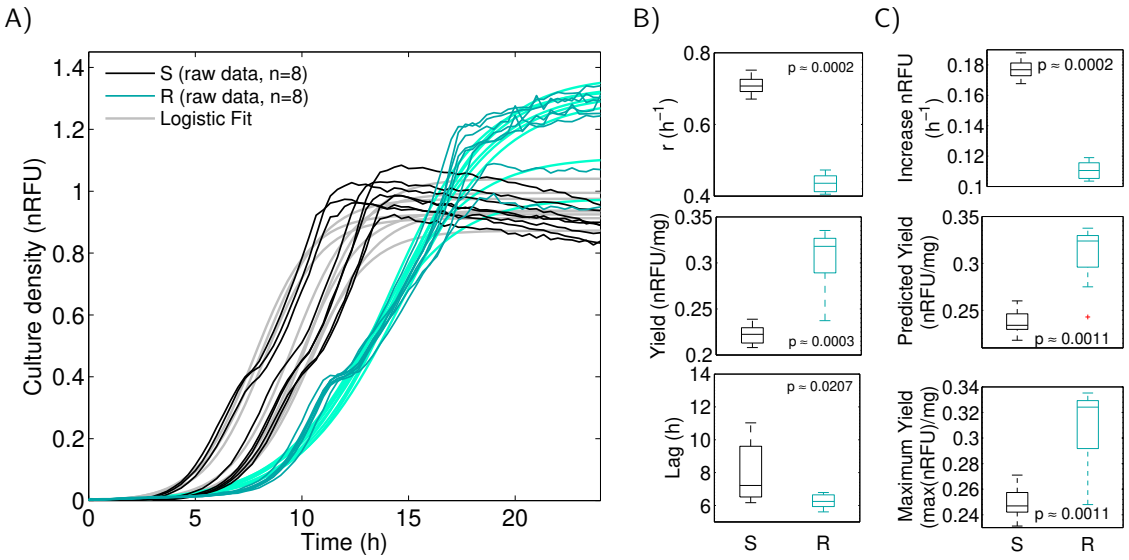

Figure 1. Asymmetric carriage costs of pGW155B. A) Overlapped growth curves of constructs S (black) and R (cyan) in the absence of tetracycline over 24h. I estimated the growth rate *per capita* (r), population size in the equilibrium (K), biomass yield, and lag duration from a 4-parameter logistic model fitted to growth data (see methods). Data fits for constructs S and R are shown in grey and light cyan, respectively. **B)** Box plots showing the median (centre of the box), 25th and 75th percentile for each of the aforementioned parameters. The whiskers extend to the most extreme data points that are not outliers, and these are individually represented. The p value shown refers to a Mann-Whitney U-test (8 replicates) that I used to test differences in the parameters between both constructs. **C)** Alternative metrics for growth rate and biomass yield: Forward Euler approximation (top), data fit predicted yield (middle), and maximal yield across all time points (bottom). The p values correspond to Mann-Whitney U-tests (8 replicates).

Despite their lower growth rate, the construct R, harbouring pGW155B, attained larger population sizes during the stationary phase compared to S. Optical density data reports similar dynamics (Figure S1), so the larger population size is likely caused by an increase in cell number as Figure S2 illustrates. Thus, I used this parameter—population size in the equilibrium, K—to estimate the biomass yield (y) of both strains, a proxy for metabolic efficiency defined as $y = K/glc$ [27, 35], where glc is the supply of glucose. Note that changes in cell size may confound optical density and fluorescence readings. This phenomenon, however, leaves a signature [36] in growth curve data that I did not find in my data (Figures S1, S3 and S4). The above metric for yield suggests the construct R is more efficient than S, despite their slower growth rate ($y_{\mathsf{S}} = 0.222 \pm 0.004$ normalised relative fluorescence units (nRFU) per mg of glucose in construct S, for $y_{\mathsf{R}} = 0.305 \pm 0.013$ nRFU per mg of glucose in construct R; mean ± standard error with 8 replicates; Mann-

Whitney U-test $p = 3.108 \times 10^{-4}$, ranksum = 37; Figure 1B).

Another parameter that I found sensitive to pGW155B was the duration of the lag phase—time preceding active growth. Using normalised fluorescence data, I found the lag phase to be ∼2h shorter in the construct R ($\text{lag}_\text{S} = 7.984 \pm 0.702$ hours *versus* $\text{lag}_\text{R} = 6.257 \pm 0.164$ hours, mean ± standard error with 8 replicates; Mann-Whitney U-test $p = 0.021$ with ranksum= 90; Figure 1B). However optical density data suggested the opposite, with the lag for R being nearly 4h longer than S's ($\text{lag}_\text{S} = 7.041 \pm 0.287$ hours *versus* $\text{lag}_\text{R} = 10.908 \pm 0.586$ hours; Mann-Whitney U-test $p = 1.554 \times 10^{-4}$ with ranksum=36; Figures S1B). The lag for the construct S did not change substantially in both normalised fluorescence and optical density datasets. The ratio between normalised fluorescence per optical density units remained constant in R despite this asynchrony in lag reported by both datasets (Figure S5). Importantly, the variations in growth rate and yield between both constructs were robust to changes in the metric used as Figures 1C and S1C illustrate: Growth rate was significantly lower in R, compared to S's, whereas yield was significantly higher.

Selection on yield allows co-maintenance of S and R. Next I asked whether selecting for yield instead of growth rate could help R avoid extinction and, more importantly, preserve its non-transmissible plasmid. I mixed equal proportions of two pure cultures of S and R, grown overnight, in media containing no antibiotic (see methods). I incubated this mixed culture at 30^oC until *both* constructs reached the stationary phase (∼ 24h as per data in Figure 1A), and then I propagated the mixed culture into a new plate containing fresh media. The pairwise competition assay lasted seven days in total. Figure 2A shows that both S and R can be co-maintained. The construct R was more abundant overall, albeit this dominance changed over time: By day seven, S was dominant in some replicates, whereas R was in the others. The change in error bars is indicative of ongoing adaptation to the mixed culture conditions but, interestingly, I found no significant change in the number of pGW155B borne by R-cells during the competition (Figure 2B, Mann-Whitney U-test $p = 0.1$ and ranksum= 15 for 3 replicates).

Now, the cost of plasmid carriage is commonly measured using *fitness* [17, 19, 21, 23, 37] and this metric relies on growth rate [14, 15]. How well does fitness predict the outcome of this competition? Fitness is measured in pairwise competition assays between two microbial types, here S and R, and it is given by [14, 15]

$$W_{ij} = m_i/m_j \tag{1}$$

where W_{ij} is the fitness of species i with respect to species j, and m is the growth rate of each species during the competition measured as Malthusian growth rate (m_i). Crucially, Malthusian growth implies pure exponential, density-independent, growth rate. It is estimated by measuring the relative abundance of each species over time. For species i, for instance, the Malthusian growth rate is given by [14]

$$N_i(t) = N_i(0)e^{m_i t} \longrightarrow m_i = ln[N_i(t)/N_i(0)]/t \tag{2}$$

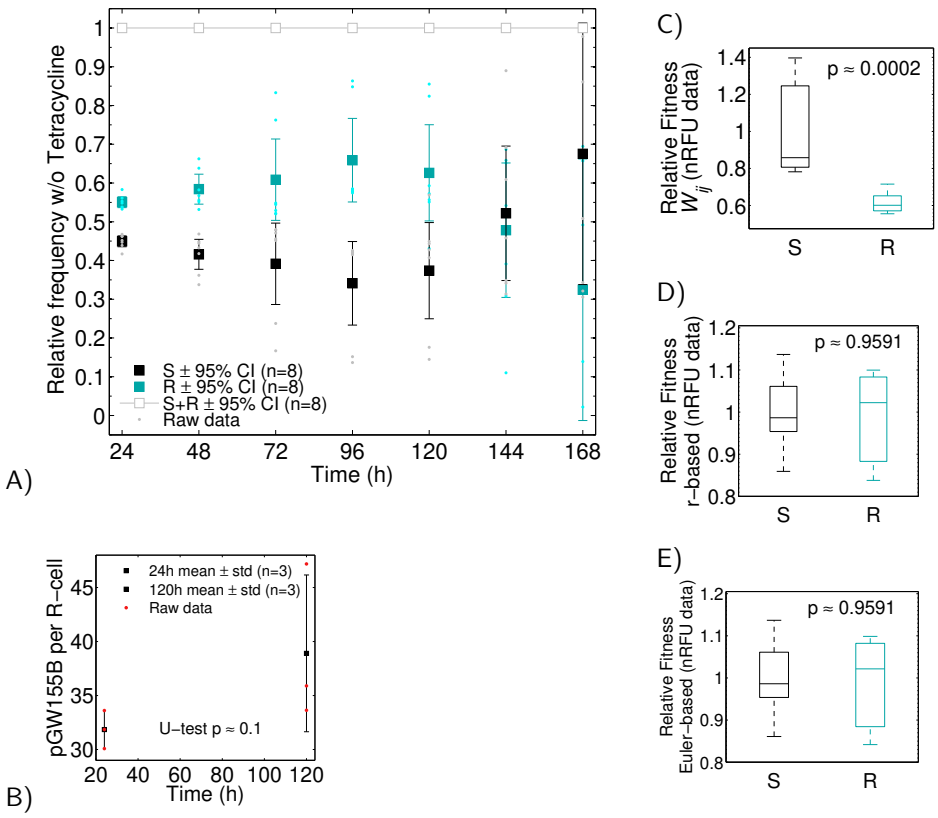

Figure 2. Co-maintenance of S and R without tetracycline not captured by classic fitness metric W_{ij}. A) Relative frequency of constructs S (black) and R (cyan) in mixed culture conditions without tetracycline. Error bars represent mean ± 95% confidence intervals, with 8 replicates. Raw data shown as dots. **B)** pGW155B copy number per R-cell from three representative replicates, measured using quantitative PCR (see methods), after 24 and 120h of co-maintenance. Error bars represent mean ± standard deviation, and raw data is shown as red dots. The p value shown corresponds to a Mann-Whitney U-test used to measure significant differences in plasmid copy number. **C-E)** Box plot showing the median (centre of the box), 25th and 75th percentile for relative fitness using Malthusian growth rate (W_{ij}, C), growth rate *per capita* estimated from a 4-parameter logistic model (D), and forward Euler approximation (D, see main text). The whiskers extend to the most extreme data points that are not outliers. The p value shown corresponds to Mann-Whitney U-tests (8 replicates) to measure significant differences between the fitness in each construct.

where $N_i(0)$ is the initial abundance of species i, $N_i(t)$ the abundance of species i at time t. Note that $N_i(0)$ must be positive and that $N_i(t) = N_i(0)$ when $t = 0$.

Initially, I calculated W_{RS} using data from the first day of competition between S and R. After applying equations 2 and 1 to growth data, I found that S was around 35% fitter than R (Mann-Whitney U-test $p = 1.554 \times 10^{-4}$ with ranksum= 100, 8 replicates; Figure 2C). With this metric, the expectation would be that S should obliterate R during a prolonged competition assay. But this is not what I found.

Fitness relies on density-independent growth, as defined above by equation 2 [14]. But Figures 1A and S1A show the growth of both constructs is *density-dependent*—that is,

logistic, not exponential. I recalculated W_{RS} using the growth rate estimates from a 4-parameter logistic model, that I fitted to growth data of each construct in competition (see methods), and the fitness of S and R was virtually the same (Figure 2D). Now, it can be argued that, for a subset of the data containing only the growth phase, density-independent growth can be a good approximation for growth rate. Indeed, growth rate is also calculated using linear regressions on logarithmic growth data, where Malthusian growth rate is given by the slope of the line fitted [9, 10, 37]. To eliminate the underlying conflict between the assumptions of density-dependent and density-independent growth rates, I used a neutral metric: The forward Euler approximation (r_e). Applied to growth data of both constructs on competition, I defined r_e as

$$r_e = \max_{0 \leq t \leq 24h} \left[\frac{1}{D_i(t)} \cdot \frac{D_i(t + \Delta t) - D_i(t)}{\Delta t} \right] \tag{3}$$

where $D_i(t)$ is the growth data of species i—S or R—at time t, Δt the interval between reads in hours (0.3h), and $D_i(t + \Delta t)$ the growth data of species i at time $t + \Delta t$. Note that r_e has dimensions of t^{-1}. As defined, r_e shows how fast growth data changes over time and, in pure cultures, it was fastest in S-cells ($r_e = 0.177 \pm 0.002$ h^{-1} for construct S and $r_e = 0.111 \pm 0.002$ h^{-1} for construct R, mean $\pm$ standard error with 8 replicates; Mann-Whitney U-test $p = 1.554 \times 10^{-4}$ with ranksum = 100; Figure 1C). The results is, qualitatively, consistent with the growth rate estimate of the 4-parameter logistic model that I used. Finally, I recalculated W_{RS} using the forward Euler approximation estimate and the fitness of both S and R was virtually identical as Figure 2D shows.

Note that identical fitness for both R and S contradict data in Figure 1. This figure shows the growth dynamics of both constructs growing as pure cultures, but fitness is estimated when both constructs grow alongside in mixed cultures. As Figure S6 illustrates, the growth rate of plasmid-bearing construct R is sensitive this change in culture conditions. R grows substantially faster in mixed culture (Mann-Whitney U-test $p = 3.108 \times 10^{-4}$, ranksum = 84; with 8 replicates), whereas the growth rate of S remained unchanged (Mann-Whitney U-test $p = 0.152$, ranksum = 43; with 8 replicates). The result is that both constructs have identical growth rate in mixed culture conditions (Mann-Whitney U-test $p \approx 1$, ranksum = 52; with 8 replicates), whence the same fitness.

'Mutant Selection Window' (MSW) hypothesis sensitive to asymmetries in carriage costs. Deciding whether pGW155B is costly can be problematic, given my results. This uncertainty has practical implications, particularly for clinicians and evolutionary biologists, as I will now demonstrate. The notion of 'selection window' is fundamental for clinical therapies designed to minimise the emergence of resistant mutants. The 'mutant selection window' (MSW) hypothesis states [38–40] that resistant mutants emerge within a specific range of drug concentrations: between the minimal selective concentration (MSC) [40], sub-inhibitory, and the mutant prevention concentration (MPC) [41], supra-inhibitory. By definition [38], no resistant mutant emerges above or below these concentrations. But there is an inconsistency in how MSC and MPC are estimated.

The MPC is based on whether microbes form colonies on Petri dishes containing a range of drug concentrations. Its interpretation is binary, either the colonies form or they do not [39]. The MSC, on the other hand, relies on growth rate data [40, 42, 43]. And here is where the asymmetry of carriage costs is relevant. The MSC assumes that resistant microbes, either by chromosomal mutations or plasmid carriage, divide at slower rates than their sensitive counterparts [40, 43, 44]. Thus, when resistant and sensitive microbes are exposed to the same range of drug concentrations, there is a concentration at which both microbes have identical growth rate, given their different sensitivities: The MSC. The resistant type will therefore divide faster at concentrations above the MSC, whereas below it the sensitive type divides faster. In other words, drug-sensitive microbes outgrow resistant mutants below the MSC and *vice versa*. Given the similarity of this rationale to that for plasmid persistence, I asked whether MSCs can reliably predict the selection of mutants harbouring plasmids with antibiotic resistance genes. As Figure 2A suggests, it does not.

To estimate the MSC I first needed to expose the constructs S and R to tetracycline, calculate the growth rate for each construct grown in each concentration (see methods), and determine their dose-response profiles (Figure 3A). Note that MSCs are calculated by overlapping the dose-response of each construct grown separately as pure cultures [40]. Consistently with the assumption behind the MSC, the construct R grew 0.27 ± 0.015 h^1 slower than S in the absence of tetracycline (mean ± standard error with 8 replicates; Mann-Whitney U-test $p = 1.554 \times 10^{-4}$, ranksum = 100). This means there must be a concentration at which both constructs have identical growth rates and the dose-response profiles cross: 0.066 ± 0.004 µg/mL of tetracycline.

To answer whether MSCs predict the selection of drug-resistant mutants harbouring plasmids, I mixed equal proportions of two pure cultures of each construct—grown overnight—and exposed them to increasing tetracycline concentrations. These concentrations are constrained by S, the most sensitive strain, as using concentrations above its IC_{99} (concentration inhibiting 99% its growth rate) are equivalent to pure cultures of R as Figure 3A illustrates. As above, I propagated the mixed culture into a new plate containing fresh media and antibiotic after 24h of growth, and repeated the transfers for seven days. I tracked daily changes in MSC, but they were not significant (Kruskal-Wallis H-test $p = 0.446$ and χ^2-statistic $= 5.795$ with 8 replicates, Figure 3B). It is therefore reasonable to expect that construct R would grow faster above the MSC, and construct S below the MSC—consistently with [40]. However, given the yield benefits of pGW155B, R-cells outnumbered S even below the minimal selective concentration (Figure S7).

I calculated the selection coefficient [15], s, for the construct R as

$$s = ln[r_i(t)/r_i(0)]/t \tag{4}$$

where $r_i(0)$ is the initial ratio of species i—here the construct R—and $r_i(t)$ the ratio of i at time t. Selection for species i is denoted by coefficients above zero ($0 < s$), whereas coefficients below zero ($0 > s$) imply selection against i. Consistently with my previous

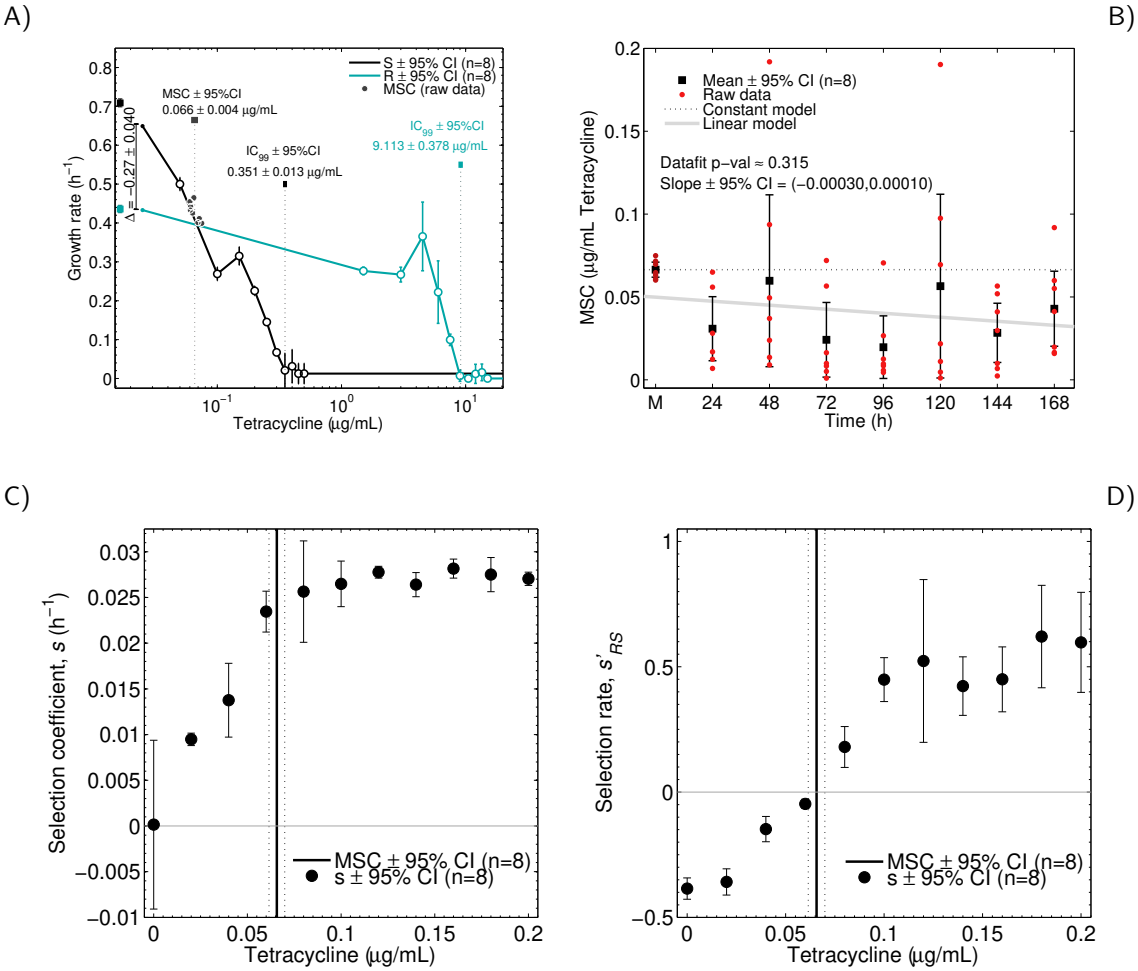

Figure 3. Selection of tetracycline-resistant construct R below the minimal selective concentration (MSC). A) Dose-response profile for each construct showing the change in growth rate, from fluorescence data, with increasing tetracycline concentrations. The relative difference in growth rate is noted by Δ (mean $\pm$ 95% confidence, $n = 8$). The minimal selective concentration (MSC) sat at 0.066 $\pm$ 0.004 µg/mL of tetracycline. **B)** Variation in MSC over time, with respect to that measured in monoculture (M). Error bars show mean and 95% confidence interval (8 replicates), with the raw data shown as red dots. A horizontal, dotted line represents the null hypothesis that MSC does not change through time. The grey line shows the fit of the linear model $a = bx + c$ to data where a is the MSC, b the slope with respect tetracycline concentration (x), and c the intersection with the y-axis when $x = 0$. The p value shown corresponds to an F-test on the regression model (F-statistic$= 1.03$) to test whether the linear model fits significantly better than a generate model consisting of only a constant term (horizontal dotted line). **C)** Selection coefficient (s) for the tetracycline-resistant construct R at different drug concentrations. The vertical black lines represent the mean (thick and black) and 95% confidence (dotted) of the MSC in A). Error bars show the mean $\pm$ 95% confidence of s at each drug concentration (8 replicates). This coefficient is measured as the ratio of construct R at the beginning ($t = 0h$) and end ($t = 168h$) of the experiment as given by the expression $s = ln[r_i(t)/r_i(0)]/t$ (see main text). **D)** Selection rate of tetracycline-resistant construct R with respect to its tetracycline-sensitive counterpart S (s_{RS}). Error bars show the selection rate for R at each tetracycline concentration (mean $\pm$ 95% confidence, 8 replicates). The vertical black lines represent the mean (thick and black) and 95% confidence (dotted) of the MSC in A). This metric is calculated using the fitness (W_{ij}) in Figure 2C, as given by the formula $s_{ij} = W_{ij} - 1$ (see main text).

finding, $s \approx 0$ for R when the mixed culture was not exposed to tetracycline (Figure 3C). But it was significantly positive at any drug concentration used: For 0.02 µg/mL of tetracycline, the lowest concentration I used, $s = 0.0095 \pm 0.003$ h^{-1} (Mann-Whitney U-test against 0 µg/mL $p = 0.007$, ranksum= 32 with 8 replicates). This concentration is well below its IC_{99} (9.113 ± 0.378 µg/mL of tetracycline, mean ± 95% confidence with 8 replicates, Figure 3A) and, most importantly, the minimal selective concentration (MSC). It can be argued that equation 4 above relies on cell number only. A similar metric exists [14, 45] for Malthusian growth rates, where the selection coefficient of species i with respect to species j is given by

$$s'_{ij} = \frac{m_i - m_j}{m_j} = W_{ij} - 1 \tag{5}$$

where m_i and m_j is the Malthusian growth rate of species i and j, respectively, and W_{ij} the fitness of species i with respect to j (see equation 1). Note that this expression is independent of time. Figure 3D illustrates s'_{RS} in all tetracycline concentrations used. The prediction is, indeed, compatible with the rationale behind the minimal selective concentration. But it fails to explain the persistence of construct R below the MSC, despite its negative selection coefficient.

The difference in errorbar size in Figures 3C, 3D and S7 suggests the likely emergence of mutants in either construct. Given the identical genetic background of both constructs (*Escherichia coli* MC4100, where pGW155B and fluorescence markers are the only differences), I used quantitative PCR to measure the relative abundance of pGW155B in R-cells (see methods) and found that R-cells exposed to more tetracycline bore more copies of the plasmid. As I describe in Supplementary Text I, pGW155B lacks partition mechanisms and its persistence relies on stochastic segregation during cell division. I developed a mathematical model (see Supplementary Text I) supporting that the increase in pGW155B that I observed is compatible with imperfect segregation events.

III. Discussion

Density-independent (Malthusian) growth rate constitutes a central tenet in classical population genetics [29, 45, 46]: advantageous genotypes have faster growth rates and sweep to fixation whereas those with lower growth rates are lost. This rationale is embraced by evolutionary and, indeed, plasmid biology [15, 17, 19, 37, 47, 48] where plasmid carriage cost is measured exclusively in terms of growth rate. However, theory is farther along than data in population genetics [49, 50]. For example, exclusive selection on growth rate clashes with rK selection theory [51].

This theory assumes that natural selection acts differently on populations in a manner contingent on their size [52]. When population size (K) is large, resources are scarce and growth slow, thus adaptations that increase K are selected. Conversely, if a population is small resources are likely more abundant. Here, natural selection is expected to favour faster growth rates (r) [53, 54]. In other words, plasmids that reduce growth rate can

still be maintained if selection acts on population size. Growth data is scarce in plasmid biology literature, however there is evidence that plasmid carriage can increase population size [8, 24, 55].

Applying rK selection theory to plasmid biology is unorthodox, but the core mechanism for plasmid persistence—horizontal gene transfer—fails to explain the abundance of non-transmissible plasmids [19]. When plasmids incur asymmetric costs, where a reduction in growth rate is followed by an increase population size, my work shows that selecting on this latter trait suffices to preserve non-transmissible plasmids consistently with rK selection theory. Along with periodic selection on plasmid-borne genes (i.e. exposure to tetracycline) as suggested elsewhere [19], the asymmetry in costs can explain the abundance of non-transmissible plasmids.

My data also question the rationale behind 'mutant selection windows' (MSW). First, the parameter defining where these windows begin, the minimum selective concentration (MSC), is not robust. While I found MSCs between R and S with growth rate data and, indeed, the MSCs did not change over time, I failed to find them in other traits—cell density data (Figure S8). Thus, whether selection windows exist would depend on the trait measured. Second, the selection coefficients that rely on growth rate data (s'_{RS}) are consistent with the notion that MSCs define the start of selection windows. s'_{RS} predicts the selection against the tetracycline-resistant construct R below the MSC, whereas above this concentration R would be favoured. However, my data show co-maintenance of both constructs below the MSC. It is therefore prudent to assume that selection for resistance begins with antibiotic exposure, regardless of the dose. My data suggests that such selection requires little exposure: The lowest drug concentration where R showed a significantly positive selection coefficient was 0.02 µg/mL of tetracycline—around 0.2% the minimum inhibitory concentration for this construct.

It is noteworthy to mention how these relatively low tetracycline concentrations can trigger the emergence of R-mutants harbouring more copies of pGW155B (see Supplementary Text I). Like pGW155B, many natural plasmids lack partition systems that ensure the even distribution of plasmids across both daughter cells [56, 57] and, therefore, rely on stochastic segregation to be maintained. While this mechanism drives plasmid loss [19], it is unusual to find experimental data showing that it can also help cells increase the number of plasmids they host. This is particularly relevant for plasmids that carry antimicrobial-resistance genes: A balance between plasmid gain and plasmid loss could further the persistence of plasmids among microbial populations between selection events. Now, many antimicrobial drugs inhibit microbial growth by reducing growth rate [40], and growth rate can influence plasmid copy number control mechanisms [58]. pGW155B and many natural plasmids lack these controls systems that are sensitive to growth rate [56, 57]. However, consistently with [58], R-mutants with more copies of pGW155B—exposed to more tetracycline—also divided at slower rates.

My work demonstrate that microbes pay plasmid carriage costs in all phases of growth, and not just in growth rate. pGW155B demonstrated that carriage costs can be asym-

metric: while this plasmid imposed a reduction in growth rate, it benefited biomass yield, helping *E. coli* reach larger population sizes. Thus, when selection favours yield over growth rate, plasmids that would otherwise be lost can persist within microbial populations. Importantly, common metrics used in the literature focus exclusively on growth rate, failing to predict the maintenance of plasmids that impose a cost in growth rate. The mechanism controlling the copy number of pGW155B is likely based on stochastic segregation, and I found this mechanisms to be particularly sensitive to tetracycline exposure favouring the emergence of daughter cells with additional plasmid copies.

IV. Methods

Media and Strains. I used the strains of *Escherichia coli* GB(c) and Wyl [59] (a gift from Remy Chait and Roy Kishony), and M9 minimal media supplemented with 0.4% glucose and 0.1% casamino acids (w/v). I made tetracycline stock solutions from powder stock (Duchefa #0150.0025) at 5mg/mL in 50% ethanol, filter sterilised, and stored at -20^oC. Subsequent dilutions were made from this stock in supplemented M9 minimal media and kept at 4^oC.

Batch transfer protocol. I inoculated a 96-well microtitre plate containing 150µg/mL of supplemented M9 media with a mixture of two overnight cultures, one of *E. coli* GB(c) and another of *E. coli* Wyl (Figure S2). The overnight culture for GB(c) was supplemented with 100ng/mL of tetracycline to preserve the plasmid pGW155B carrying *tet(36)* [59], centrifuged and removed prior adding to the microtitre plate. I incubated the plate at 30^oC in a commercial spectrophotometer and measured the optical density of each well at 600nm (OD_{600}), yellow florescence for the S strain (YFP excitation at 505nm, emission at 540nm), and cyan fluorescence for the R strain (CFP at 430nm/480nm) every 20min for 24h. After each day I transferred 1.5µL of each well, using a 96-well pin replicator, into a new microtitre plate containing fresh growth medium and tetracycline.

Growth parameter estimation. Yellow and cyan fluorescence protein genes were constitutively expressed given the constant ratio between fluorescence and optical density (Figure S5). This enabled me to use fluorescence data as a proxy for cell density in mixed culture conditions. I normalised fluorescence readings using a conversion factor, n_f, calculated by growing each construct in a range of glucose concentrations, and regressing the linear model $RFU = n_f \cdot OD + c$ where RFU is relative fluorescence units data, OD optical density data, n_f the conversion factor between fluorescence and optical density, and c the crossing point with the y–axis when $OD = 0$.

I imported the resulting OD time series data set (Figures S3 and S4) into `MATLAB R2014b` to subtract background and calculate growth rate *per capita* (r) using the following algorithm. First, I fitted three mathematical models to data: 1) linear model $g(t) = b + r \cdot t$, 2) exponential model $g(t) = b + C \cdot \exp(r \cdot t)$ and 3) logistic model $g(t) = b + K/(1 +$

$C \cdot \exp(-r \cdot [t - l]))$. The terms $g(t)$ denote culture growth through time (in OD, YFP, or CFP units), b the inoculum size used to subtract the background, C is a parameter, K the maximal population size attained and l the duration of the lag phase. I used the growth rate reported by the model with the lowest corrected Akaike Information Criterion (AICc). To estimate the biomass yield I divided OD data in stationary phase by the glucose supplied [35]. I also used the highest density across all time points and the density reported by the data fit, both divided by glucose supply, as alternative metrics for biomass yield.

Drug sensitivity parameter estimation. I defined the minimum inhibitory concentration (MIC or IC_{99}) for each trait as the tetracycline required to reduce the trait of the bacterium by a factor of 99%, compared to the tetracycline-free control. The MICs were 0.364 ± 0.012 (mean ± 95% confidence, 8 replicates), and 0.351 ± 0.013 of tetracycline for the strain S using culture density and growth rate, respectively. For the strain R they were 11.121 ± 1.735, and 9.113 ± 0.378 µg/mL.

DNA material extraction. For each concentration, I sampled three representative 150µg/mL replicates that I divided into two groups for chromosome and plasmid DNA extraction. I used 'GeneJet DNA' (ThermoScientific #K0729) and 'GeneJet Plasmid' (ThermoScientific #K0502) extraction kits to extract chromosomal and plasmid DNA (pDNA) from the samples, respectively, and used Qubit to quantify DNA and pDNA yields. Both extracts were diluted accordingly in extraction buffer, as per in manufacturer instructions, to normalise DNA across samples.

Quantitative PCR and plasmid copy number estimation. I used `primer3` to design two pairs of primers with melting temperature (T_m) of 60^oC and non-overlapping probes with T_m of 70^oC. The amplicon ranges between 100 to 141bp depending on the locus (Table 1). Two reaction mixes were prepared using the kit 'Luminaris Color Probe Low ROX' (ThermoScientific #K0342), adding 0.3µM of each primer and 0.2µM of the probe as per manufacturer specifications. Following a calibration curve for each reaction (Figure S9) I added 0.01ng of chromosomal or plasmid DNA material to each of the reaction mixes.

To estimate the relative copies of pGW155B per R cell, I calculated the corresponding proportion of chromosomal DNA corresponding to the R-type from data in Figure 3D and used the formula [19]

$$cn = \frac{(1 + E_c)^{Ctc}}{(1 + E_p)^{Ctp}} \times \frac{S_c}{S_p},$$

where cn is the number of plasmid copies per chromosome, S_c and S_p are the size of the chromosome and pGW155B amplicon in bp, E_c and E_p the efficiency of the qPCR taken from data in Figure S9, and Ctc and Ctp are the cycles at which I first detected product amplification (C_t).

Table 1. Primers and probes designed using `Primer3`. Amplicon ranging from 100 to 141bp. T_m indicates the estimated melting temperature.

Target gen	**Sequence** ($5' \rightarrow 3'$)	$\mathbf{T}_m$ (^{o}**C**)	**Feature**
tatB	CGATGAAGCGTTCCTACGTT	60.27	Forward
	TCATGCGCAGCTTCATTATC	59.94	Reverse
	AAGGCGAGCGATGAAGCGCA	70.70	Probe
tet(36)	ATTGGGCATCTATTGGCTTG	59.22	Forward
	CCGATTCACAGGCTTTCTTG	60.76	Reverse
	AGCCTTTGCCAATTGGGGCG	70.37	Probe

Competing interests

The author declares no competing interests.

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I. Supplementary Text

Tetracycline-resistant mutants with additional copies of pGW155B can be selected below the minimal selective concentration (MSC). The drug concentrations that I used suppose 0.2% to 2% the minimum inhibitory concentration for the construct R (see Figure 3A in main text). Despite the relatively low tetracycline used, Figure S10A reveals substantial changes in the growth rate of R between the first and last day of the experiment (linear regression $p \approx 1.45 \times 10^{-4}$, F-statistic= 16.7, adjusted coefficient of determination $R^2 = 0.222$; slope parameter $1.876, p \approx 1.99 \times 10^{-4}$, t-statistic= 3.9915; 8 replicates). It is reasonable to expect an increase in growth rate as R adapts to the growth conditions—the same for seven consecutive days. However, I found a concomitant change in yield and lag—redolent of the asymmetric costs of pGW155B shown in the main text, in Figure 1 (Figures S10B and C). Given the experimental setup, it is difficult to expose chromosomal mutations in both, R and S, and underpin those that ease pGW155B carriage (see main text). Thus, I asked whether the number of plasmids borne by R cells changed over time with different tetracycline concentrations—it did.

To quantify the relative abundance of pGW155B within R cells, I sampled representative cultures on days one and five, calculated the proportion of chromosomal DNA corresponding to the construct R, and used quantitative polymerase chain reaction (qPRC) to measure the number of plasmids borne per cell (see methods). The initial pool of cells from this strain, grown overnight in pure culture and used to inoculate the mixed cultures, contained 30.21 ± 6.72 copies of pGW155B per cell (mean ± 95% confidence, $n = 3$). Without tetracycline, the number of pGW155B did not change significantly after one and five days of competition against S (Mann-Whitney U-test $p = 0.1$ and ranksum = 15 with 3 replicates, Figure S10D). However, the relative abundance of pGW155B changed rapidly with increasing drug concentrations: I observed a 2-fold increase in the number of plasmids hosted by R within 24h, and 6- to 10-fold after five days of depending on tetracycline concentration (Figures S10E and F).

To understand the variation in plasmid copy number with respect to drug concentration, I fitted two mathematical models to qPCR data. First, the linear model $p_c = p_0 + d\kappa$ and the constant model $p_c = \kappa$, where κ denotes the slope or proportionality constant, p_0 the initial number of copies borne by each R cell and d the antibiotic supplied. The constant model assumes no change in the number of plasmids borne, and it was extremely unlikely (relative likelihood with respect to the linear model ≈ 0.034, Figure S10E). Instead, the linear model suggests that plasmid copy number correlates with drug concentration, where the constant of proportionality $\kappa = 161.87 \pm 110.37$ plasmids per mL per microgram of drug per cell (t-statistic = 2.874, $p = 0.009$ and 95% confidence interval (45.08, 278.662)). Albeit significant, with an adjusted coefficient of determination (R^2) of 0.246, the linear model does not entirely capture the changes observed in qPCR data. A switch-like, non-linear model, say, the logistic model (see methods), explained better the variation in the number of pGW155B that I observed (adjusted R^2 of 0.472). After

five days of exposure to tetracycline, the constant κ increased from 161.87 ± 110.37 to 880.19 ± 705.71 plasmids per mL per microgram of drug per cell (Figure S10F, t-statistic $= 2.4446$, $p = 0.0229$, and 95% confidence interval $(133.5, 1626.9)$). The predictive power declined for the logistic model, albeit it was still better than that for the linear model (adjusted $R^2 = 0.411$ *versus* 0.261).

Tetracycline influences the random segregation of pGW155B during cell division. Now the question is this: how did R cells gain additional copies of pGW155B? The plasmid lacks a *rep* gene to control tightly the partition of plasmids after cell division [1–3] (addgene vector database accession #2853). While pGW155B is a synthetic plasmid, many natural plasmids also lack partition systems [3, 4] so I hypothesised the following: That random segregation, the mechanism underlying plasmids loss in the absence of selection [5], can also facilitate their accumulation in bacteria. Exposure to tetracycline would, therefore, skew the segregation of plasmids to favour daughter cells with additional copies of pGW155B.

Suppose a microbe that harbours a non-transmissible plasmid, and neither the microbe's chromosome nor the plasmid can mutate. The plasmid imposes a cost $0 < \Gamma < 1$ on the microbe's growth rate, and the cost is proportional to the number of plasmids hosted as we may expect. Now, if the microbe bears n copies of the plasmid and it replicates alongside the host's chromosome, the only means to gain or lose plasmids would be through the imperfect segregation of plasmids during cell division: One daughter cell would carry $n + 1$ plasmids whereas the other is left with $n - 1$. This process is how pGW155B, I hypothesise, changed its relative abundance in *E. coli*.

Let me assume these imperfect segregations are a Markov process, where they occur with a probability σ and are independent of previous imperfect segregation events. For simplicity, I assume that only one plasmid can be gained or lost during each imperfect segregation. Note that, regardless of the initial number of plasmids present in the inoculum, j subpopulations will emerge—containing from 0 to $j - 1$ copies—derived from this rationale as I show below. Thus, the population of the aforementioned microbe is given by the time-series vector $\mathbf{B}(t, A, S) = (b_1(t, S, A), b_2(t, S, A), \ldots, b_j(t, S, A))$. This vector contains the density of j different subpopulations of the same genotype, but hosting different number of plasmids, that sum together to form B at time t given a carbon source S and an antibiotic A. To make the model compatible with the batch transfer protocol used in this study, I further assume that $\mathbf{B}$ is transferred to an environment with replenished S and A at fixed time intervals, k, usually at 24h, satisfying the expression

$$\mathbf{B}_k(t_0, S_0, A_0) = \eta \cdot \mathbf{B}_{k-1}(t_{k-1}, S_{k-1}, A_{k-1})$$

where $0 < \eta \ll 1$ denotes the fraction transferred (typically $\eta = 0.01$). Given the above assumptions, the resulting probability matrix for the Markov process (M) is

$$M = \begin{pmatrix} 1 & \sigma & 0 & & \cdots & 0 \\ 0 & 1-2\sigma & \sigma & 0 & & \vdots \\ 0 & \sigma & 1-2\sigma & \sigma & \ddots & \\ & 0 & \sigma & 1-2\sigma & \ddots & 0 \\ \vdots & & \ddots & \ddots & \ddots & \sigma \\ 0 & \cdots & & 0 & 0 & 1-\sigma \end{pmatrix}.$$

Although only one plasmid can be gained or lost in each imperfect segregation event, cells that lose all their plasmids cannot recover them (i.e. cells that only host one plasmid) and these cells become sensitive to the antibiotic A. Now, the column vector $\mathbf{U}(S) = (U(b_1, S), U(b_2, S), \ldots, U(b_j, S))$ of uptake rates of all subpopulations is determined by the Monod function

$$U(b, S) = V^{max} \frac{bS}{k_m(b) + S}.$$

The maximum uptake rate is given by V^{max}, and $k_m(b_n)$ is the associated half-saturation constant for cells of the n-th subpopulation where $k_m(b_n)$ is a smooth function. Let me now define c_n as the biomass yield of cells from the n-th subpopulation, where c_n can be derived from the maximal uptake rate of cells in subpopulation n. This can be written as $c_n = c(b_n)$, where $c(\cdot)$ is a smooth function. The growth rate of each subpopulation is given by the vector $\mathbf{G}(S, A) = (G(b_1, S, A), G(b_2, S, A), \ldots, G(b_j, S, A))$ at carbon concentration S, where

$$G(b, S, A) = c(b) \cdot U(b, S) \cdot (1 - \Gamma) \cdot \frac{1}{1 + \kappa(b)A^2}.$$

Note that $\mathbf{G}(S, A)$ is modulated by the costs imposed by plasmid carriage Γ, and the antibiotic A. The term $\frac{1}{1+\kappa(b)A^2}$ describes the inhibition exerted by the drug on the n-th subpopulation of B (b_n), where $\kappa(b_n)$ is the affinity of the antibiotic for its substrate and 2 is the Hill coefficient. The following system of ordinary differential equations describe the change in $\mathbf{B}$ over time, given S and A

$$\frac{d\mathbf{B}}{dt} = M\Big((1 - \Gamma) \cdot \mathbf{G}(S, A_0) \circ \mathbf{B}\Big), \tag{6a}$$

$$\frac{dA_i}{dt} = -d \cdot A_i + \phi \cdot (A_e - A_i) \cdot \sum_{n=1}^{j} b_n, \tag{6b}$$

$$\frac{dA_e}{dt} = -d \cdot A_e - \phi \cdot (A_e - A_i) \cdot \sum_{n=1}^{j} b_n, \tag{6c}$$

$$\frac{dS}{dt} = -\langle \mathbf{U}(S), \mathbf{B} \rangle, \tag{6d}$$

where $\langle \mathbf{U}(S), \mathbf{B} \rangle$ represents the Euclidean inner product of two vectors of the same dimension, $\langle \boldsymbol{u}, \boldsymbol{v} \rangle = \sum_{n=1}^{j} u_n v_n$. Finally, the carbon uptake follows Michaelis-Menten kinetics

(see above), but the antibiotic A diffuses from the environment (A_e) into the cells (A_i) with a rate ϕ.

Figures S11A and B illustrate the qualitative change in population structure resulting from the exposure of B to the antibiotic A. When the microbe grows in the absence of drug, the subpopulation with fewer copies of the plasmid is favoured given their lower relative costs. In this condition, cells harbouring more copies are rare. However, the distribution changes when the microbe is exposed to A: More copies of the plasmid are needed to overcome the antibiotic challenge. Crucially, with more antibiotics, subpopulations hosting higher number of copies are favoured—despite the costs of plasmid carriage.

The experimental data set is consistent with this prediction (Figures S11C, D and E). If the initial pool of *E. coli* cells contained 30.21 ± 6.72 copies of the plasmid, after 24h of antibiotic challenge cells containing more copies rapidly emerged. To find the distribution that best fits the data, I tried all distributions available in `MATLAB 2014a` using the built-in routine `fitdist`. Then, I used the negative log-likelihood (NLogL) function and corrected Akaike Information Criterion (AICc) as metrics for the goodness of fit, and sorted the distributions accordingly. I considered the best distribution to be that with the lowest NLogL and AICc. After 24h of exposure to tetracycline, the data followed a Nakagami distribution (corrected Akaike Information Criterion AICc = 198.14, Negative Log-Likelihood $NLogL = 96.79$) with parameters for shape $\mu = 2.18$ and scale $\omega = 1.79 \times 10^3$ (95% confidence intervals are $\mu = (1.29, 3.71)$ and $\omega = (1367.1, 2348.9)$). The mean copies of plasmids borne per cell after this period were 39.95 ± 10.58 (mean $\pm$ 95% confidence).

These parameters changed when I prolonged the antibiotic challenge. After 120h of exposure to tetracycline the mean copies per cell increased to 169.58 ± 90.14 and the data now followed a skewed BirnbaumSaunders distribution (AICc = 290.19, NLogL = 142.81 *versus* AICc = 293.66 and NLogL = 144.54 for the Nakagami distribution) with parameters for shape $\gamma = 0.73$ and scale $\beta = 133.52$ (95% confidence $\gamma = (97.07, 169.98)$ and $\beta = (0.52, 0.94)$). Thus, the antibiotic challenge increased the frequency of cells bearing more copies of pGW155B consistently with this theory (Figure S11E) where the imperfect segregation of plasmid during cell division would be the underlying mechanism.

Supplementary References

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II. Supplementary Figures

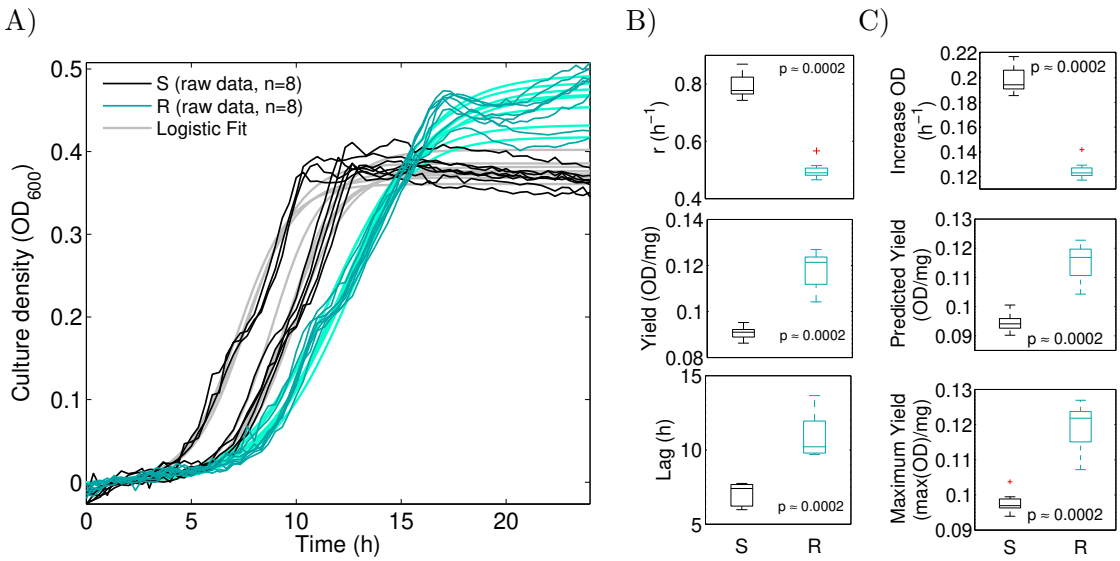

Figure S1. Asymmetric carriage costs of pGW155B (optical density data). A) Overlapped growth curves of constructs S (black) and R (cyan) in the absence of tetracycline over 24h. I estimated the growth rate *per capita* (r), population size in the equilibrium (K), biomass yield, and lag duration from a 4-parameter logistic model fitted to growth data (see methods). Data fits for constructs S and R are shown in grey and light cyan, respectively. **B)** Box plots showing the median (centre of the box), 25th and 75th percentile for each of the aforementioned parameters. The whiskers extend to the most extreme data points that are not outliers, and these are individually represented. The p value shown refers to a Mann-Whitney U-test (8 replicates) that I used to test differences in the parameters between both constructs. **C)** Alternative metrics for growth rate and biomass yield: Forward Euler approximation (top), data fit predicted yield (middle), and maximal yield across all time points (bottom). The p values correspond to Mann-Whitney U-tests (8 replicates).

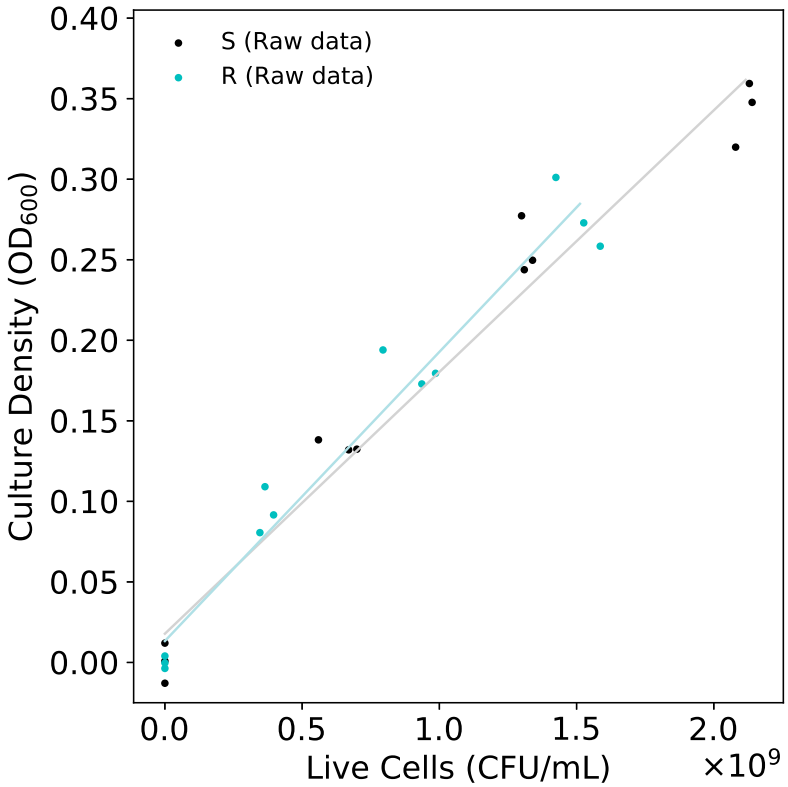

Figure S2. Calibration curve to translate optical density data to number of *Escherichia coli* cells. I fitted the linear model $a = bx + c$ to optical density and colony counting data (dots) to calculate the number of optical density units (OD_{600}) per cell. a denotes the optical density readings measured at 600nm, c the crossing point with the $y-$axis when $x = 0$, and b the conversion factor between optical density and number of cells (x). I interpolating optical density readings to calculate the number of cells within a culture as $x = (a - c)/b$. For the strain S, $b = 1.62 \times 10^{-10}\ OD \cdot mL \cdot CFU^{-1}$ and $c = 1.78 \times 10^{-2}\ OD$, whereas for R $b = 1.79 \times 10^{-10}\ OD \cdot mL \cdot CFU^{-1}$ and $c = 1.33 \times 10^{-2}\ OD$.

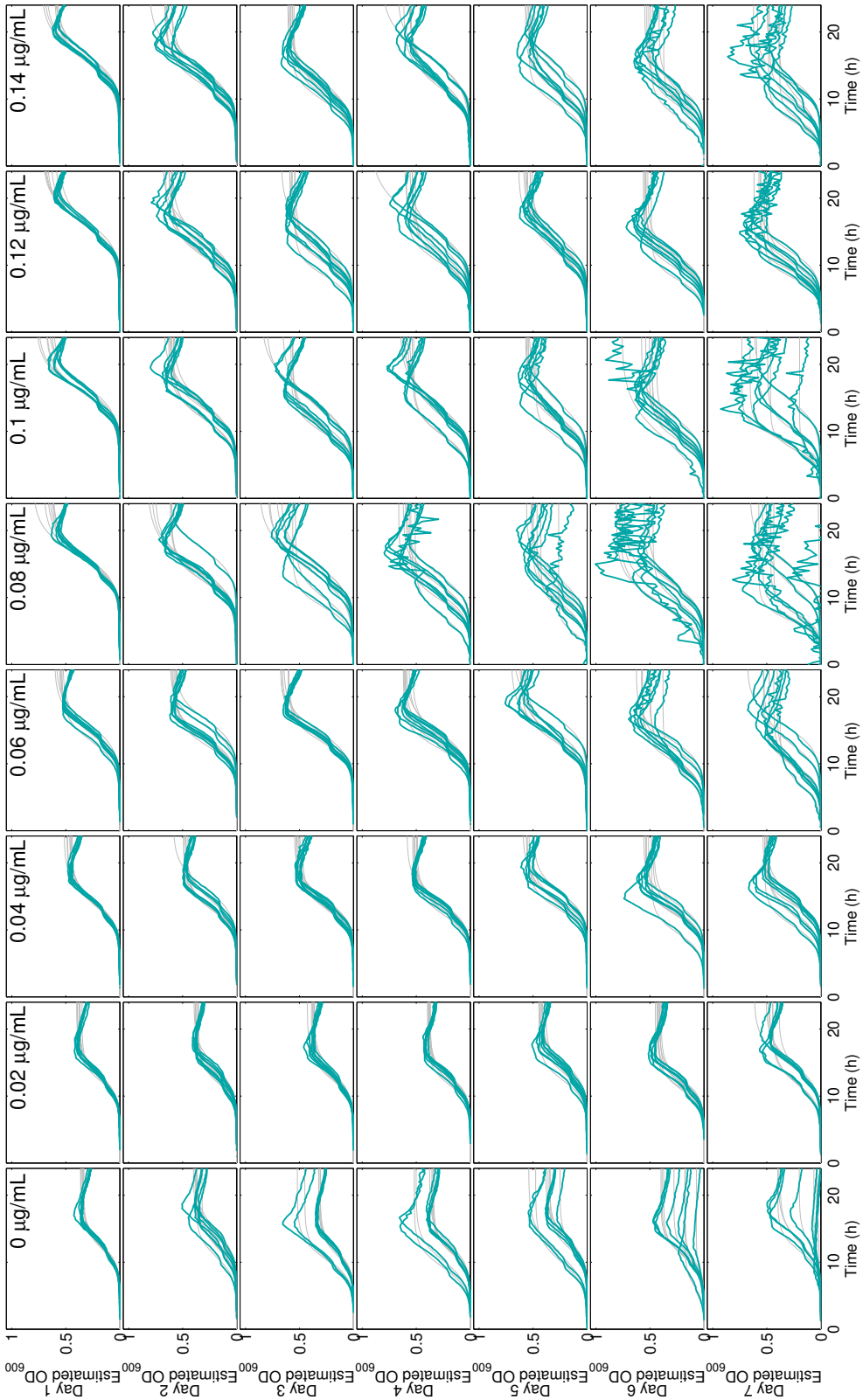

Figure S3. Raw data and model fit for resistant R strain in mixed culture conditions. Raw optical Density data for GB(c) measured every 20min for seven days (blue). The best fit to data (see methods in main text) used to calculate bacterial fitness is shown in grey. Each column represents one day worth of data for a given condition, and each row the data set for one tetracycline concentration.

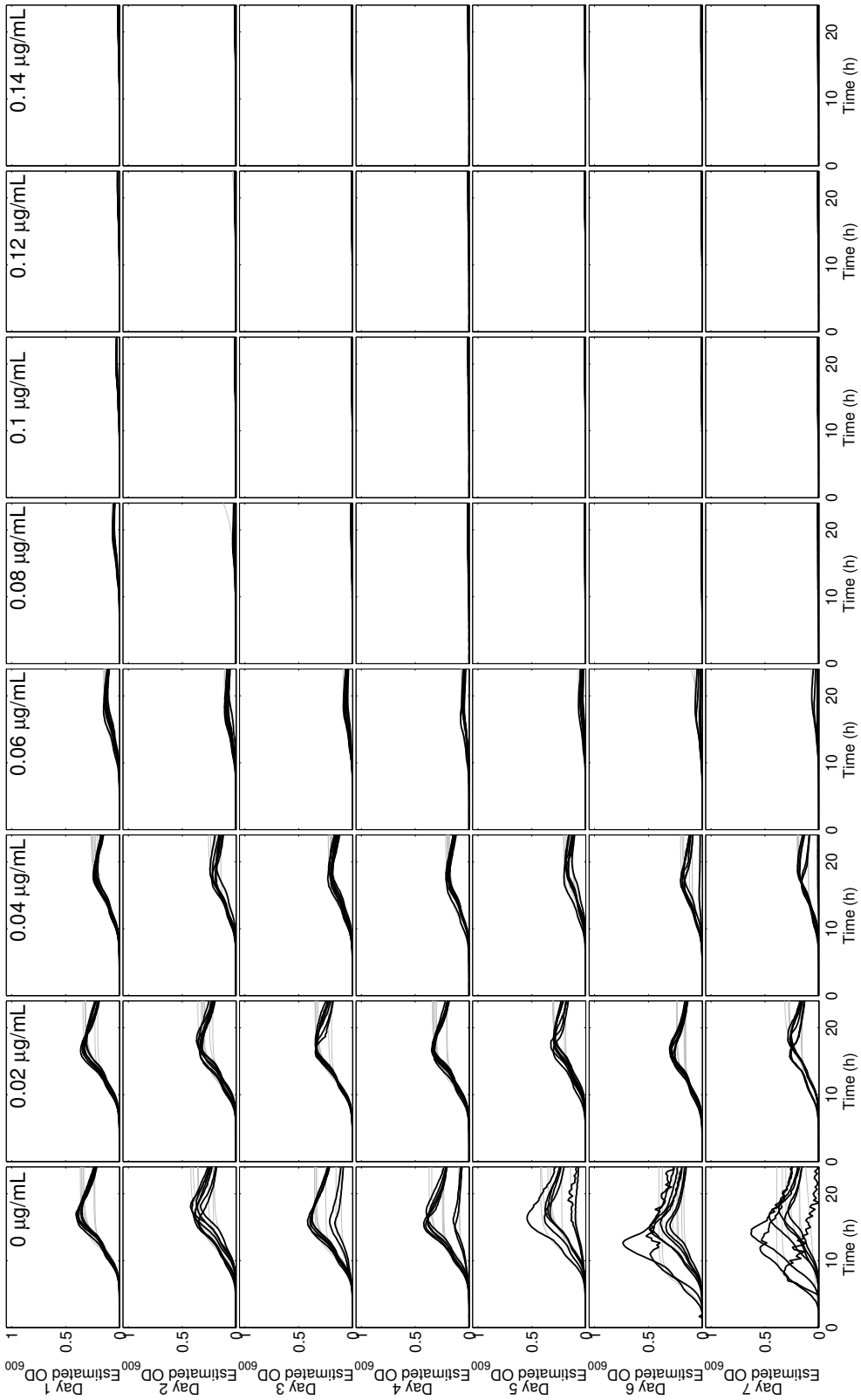

Figure S4. Raw data and model fit for sensitive S strain in mixed culture conditions. Raw optical Density data for Wyl measured every 20min for seven days (black). The best fit to data (see methods in main text) used to calculate bacterial fitness is shown in grey. Each column represents one day worth of and each row the data set for one tetracycline concentration.

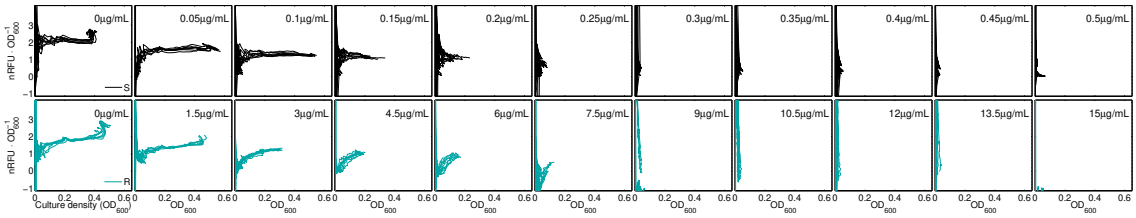

Figure S5. Changes in relative fluorescence over time in R and S strains in pure culture conditions. Raw change in florescence, per optical density units, measured every 20min for 24h for the S- (black) and R-type. Each column represents the data set for each tetracycline concentration used.

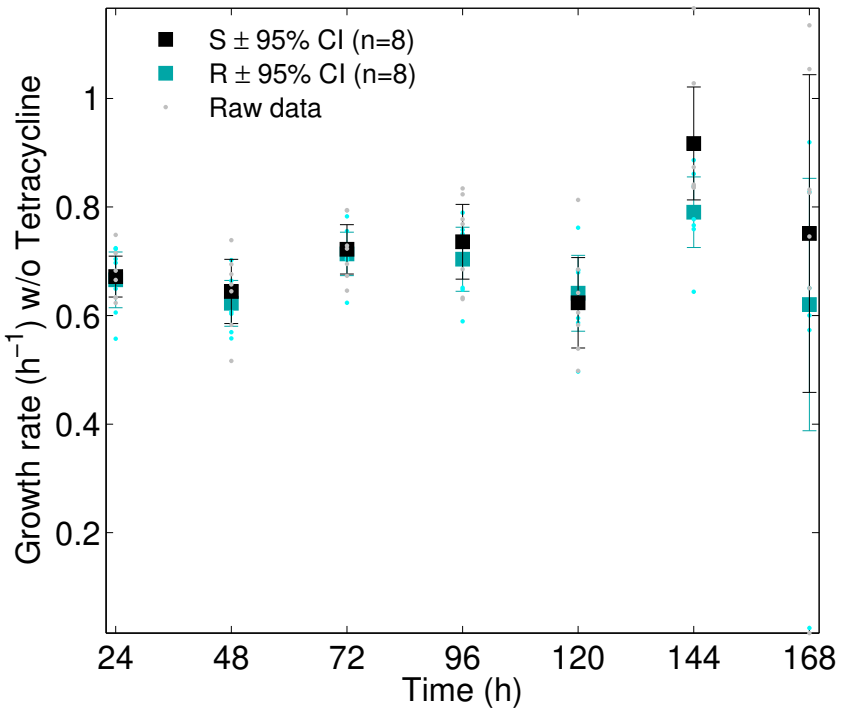

Figure S6. Change in growth rate over time without tetracycline. Growth rate of constructs S (black) and R (cyan) in mixed culture conditions without tetracycline. Error bars represent mean ś 95% confidence intervals, with 8 replicates. Raw data shown as dots.

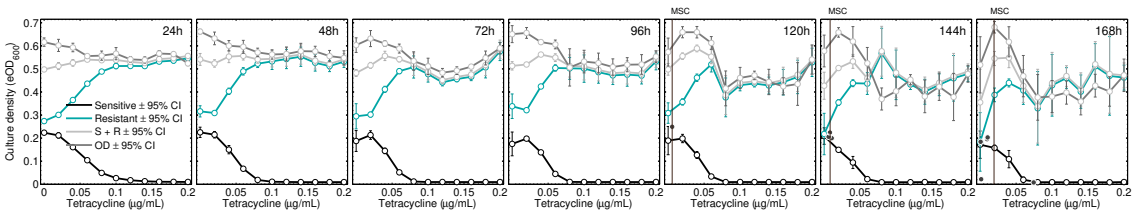

Figure S7. Dose-response profile for tetracycline in mixed culture conditions. Dose-response profiles for each strain (S in black, R in cyan) showing the change in culture density with increasing tetracycline concentrations over time. Error bars represent mean ± 95% confidence, with 8 replicates. Data in light grey is the optical density estimated by adding both normalised fluorescence datasets, whereas the optical density measured at 600nm is shown in dark grey. Where detectable, the minimal selective concentration is shown individually, for each replicate, as a black dot.

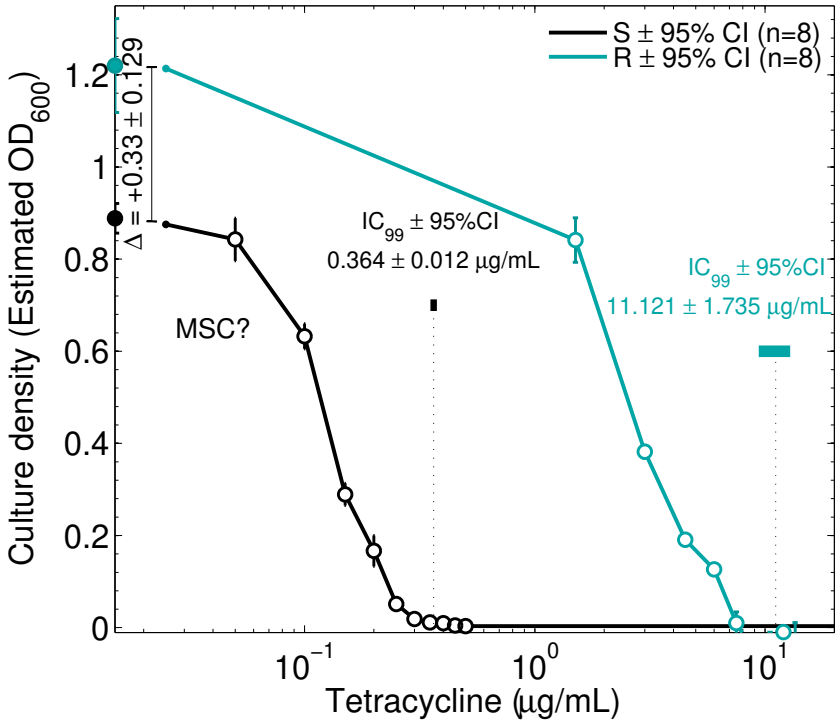

Figure S8. Dose-response profile for tetracycline (optical density data). Change in culture density for both constructs (S in black, R in cyan) in pure culture conditions, with increasing tetracycline concentrations. Error bars show mean ± 95% confidence with 8 replicates. A vertical, dashed line highlights the inhibitory concentration 99 (IC_{99}, concentration with 99% reduction in optical density with respect to the drug-free control), where the 95% confidence is noted by a horizontal line atop the IC_{99}. Note the profiles do not cross, and therefore I could not compute the minimal selective concentration or MSC. Δ shows the difference in optical density between both constructs in the absence of tetracycline (mean ± 95% confidence).

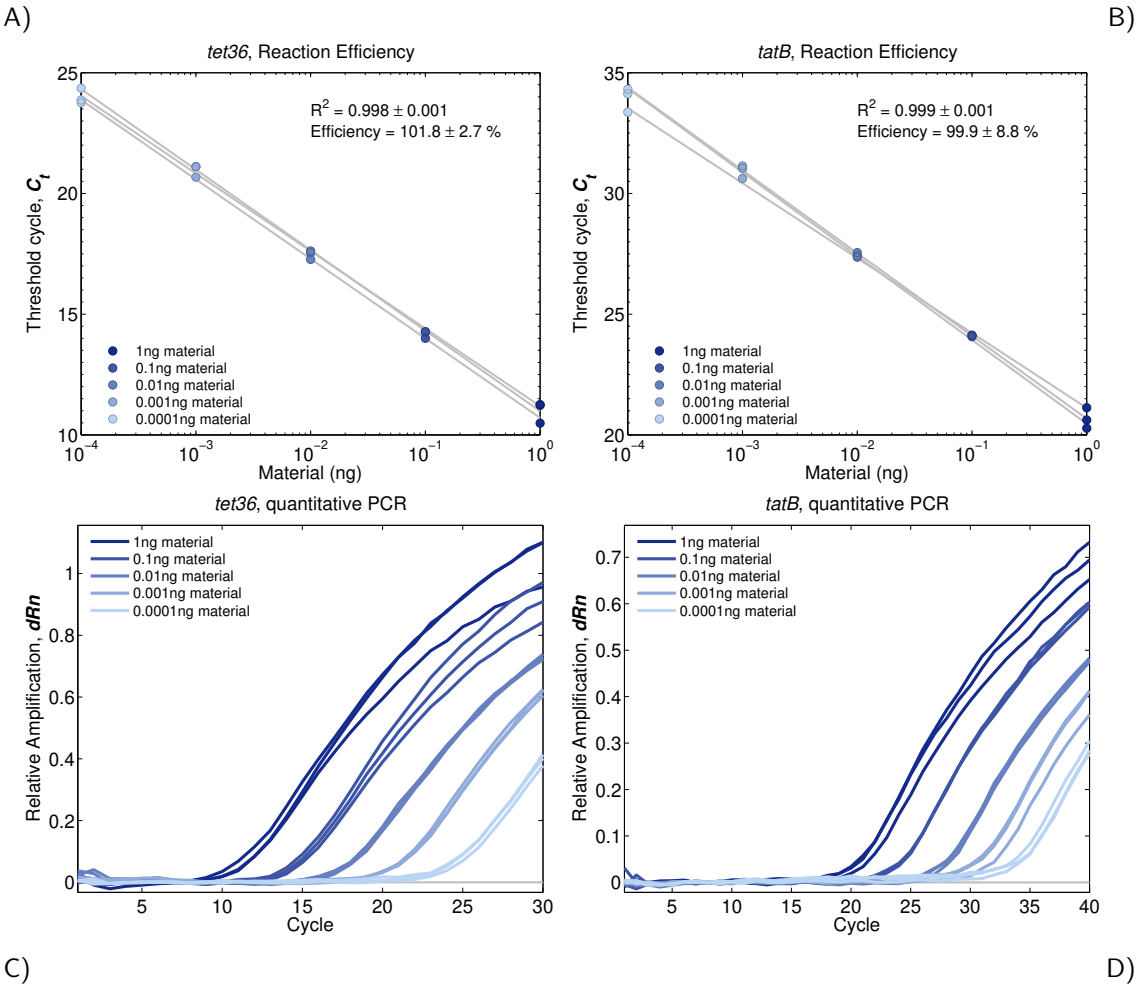

Figure S9. Quantitative PCR calibration curves for *tet(36)* and *tatB*. Reaction efficiency for the set of primers and probes, listed in Table 1 the main methods section, for *tet(36)* (A) and *tatB*. The efficiency was calculated as $E_f = 10^{-1/Slope} - 1$, and the slope term calculated by fitting a linear model to qPCR threshold cycle (C_t) data. The mean $\pm$ standard deviation for the adjusted coefficient of determination R^2 and efficiency are shown in the figures. The amplification curves for each reaction, using 3 replicates, are shown in C) and D), respectively.

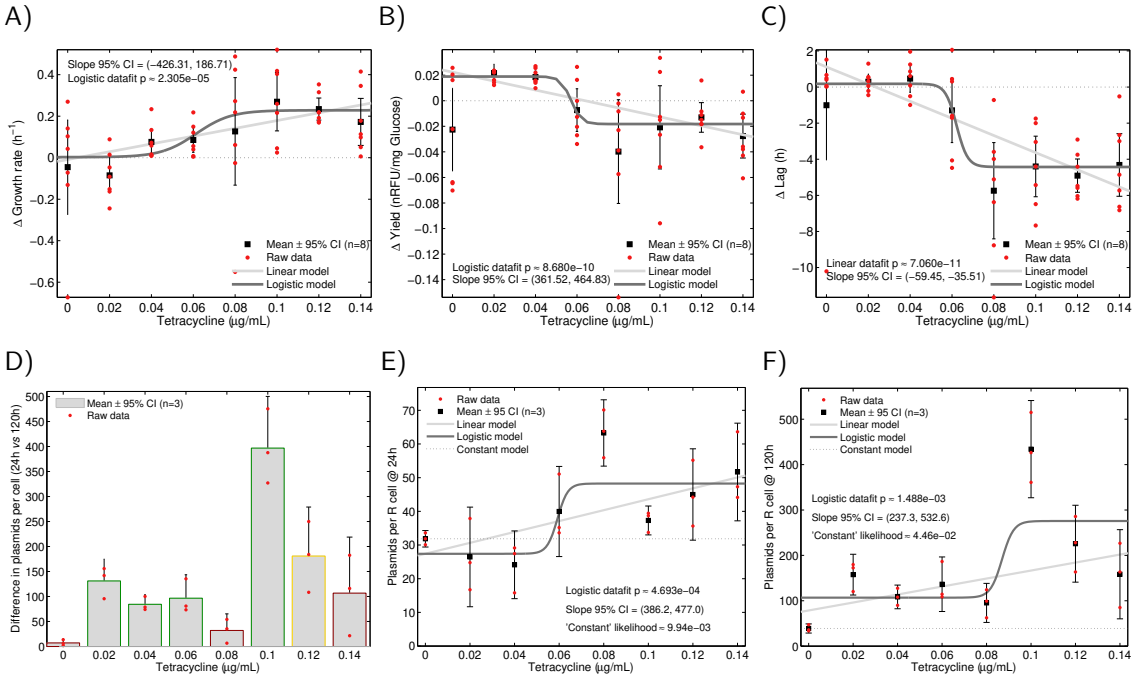

Figure S10. Variation in pGW155B copy number induces phenotypic changes in traits sensitive to plasmid carriage. A-C) Relative change in growth rate (A), biomass yield (B), and lag (C) after 168h exposure to different tetracycline concentrations. Error bars show the mean and 95% confidence interval of each trait at different drug concentrations, and raw data is shown as red dots. The horizontal, dotted black line represents the null hypothesis that no change occurs after 168h of exposure. The light grey line represents the linear model $a = bx + c$, where a notes the relative phenotypic difference, b the slope with respect tetracycline concentration (x), and c the intersect with the y-axis when $x = 0$. The dark grey line represents a 4-parameter logistic model (see main methods). The statistics shown in A-C are those for the model with the highest adjusted coefficient of determination (R^2), where the p value shown corresponds to an F-test on the regression model to test whether it fits data significantly better than a generate model consisting of only a constant term (horizontal dotted line). **D)** Variation in pGW155B copy number after five days (120h) of exposure to tetracycline. Bars denote the mean and the errorbar 95% confidence of the mean; those with a green edge have a significant increase in copy number ($p < 0.05$) according to Welch's *t*-test, red if it was not ($p > 0.05$), and yellow if the test was inconclusive ($p \approx 0.05$). Raw difference in qPCR data is shown as red dots. **E-F)** Copies of pGW155B per R-cell exposed to increasing tetracycline concentrations after 24h (E) and 120h (F). Error bars note the mean and 95% confidence interval of qPCR data, and red dots raw qPCR data. The black dotted line represents the null hypothesis—no change in plasmid copy number. The predictions from linear and 4-parameter logistic models analog to those above are shown in light grey and dark grey, respectively. The statistics shown in A-C are those for the model with the highest adjusted coefficient of determination (R^2), where the p value shown corresponds to an F-test on the regression model to test whether it fits data significantly better than a generate model consisting of only a constant term (horizontal dotted line). Also shown is the relative likelihood of the null hypothesis with respect to the model (logistic or linear) with the highest adjusted coefficient of determination (R^2).

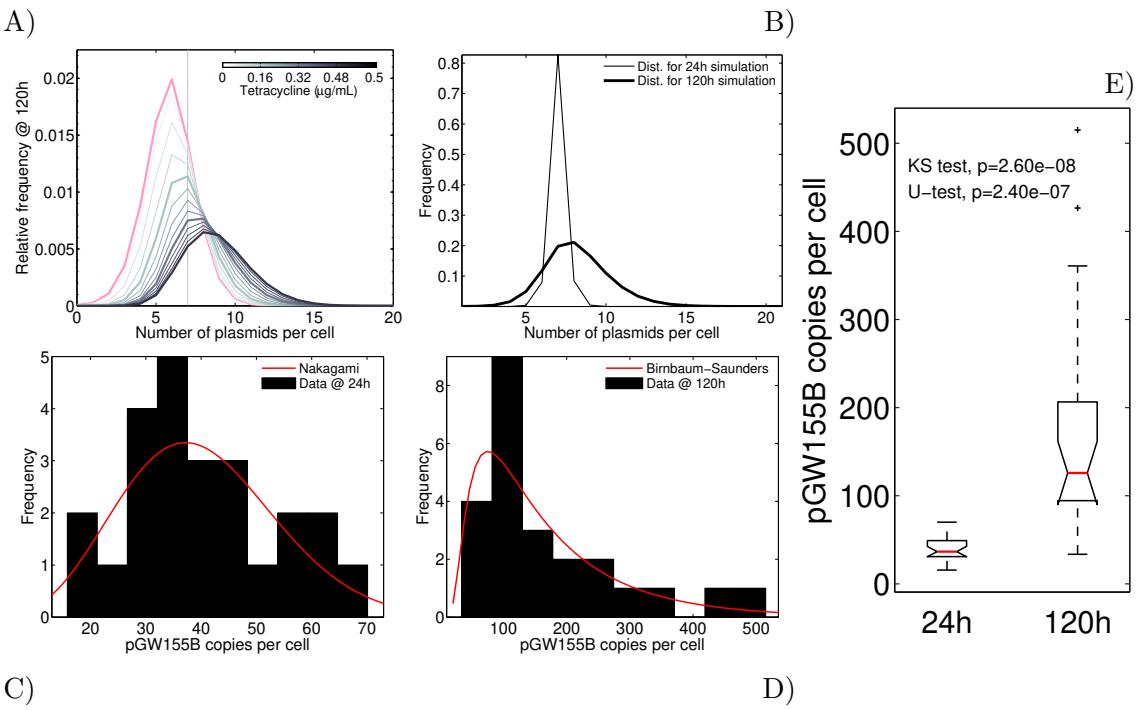

Figure S11. Influence of tetracycline concentration on the random segregation of pGW155B during cell division. A) Theoretical distributions of plasmid copy number as a function of antibiotic concentration. The thin, vertical line illustrates the initial distribution of plasmids—analog to the inoculum in the experimental setup. The distribution of plasmids for each drug concentration is shown in different colours from light green (low drug) to black (high drug), with thicker lines denoting higher drug concentrations. The distribution in the absence of antibiotic is shown in pink. **B)** Pooled frequency of plasmids after 24h and 120h of exposure to the drug using simulated data. **C-D)** Distribution of pGW155B copies in R-cells after 24h (C) and 120h (D) of exposure to tetracycline based on qPCR data. In red I represent the continuous distributions that best fit the data (red lines): 'Nakagami' for the 24h dataset and 'BirnbaumSaunders' for the 120h dataset. **E)** Two-sample Kolmogorov-Smirnov (KS) and MannWhitney U-tests to test whether both datasets come from different distributions. The box plot shows the median (red), 75th and 25th percentile of the data set, and the whiskers extend to the most extreme data points not considered outliers. The outliers are plotted individually.