Persistence under pressure: exploring the impact of conjugation rate evolution on the stability of plasmids

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Abstract

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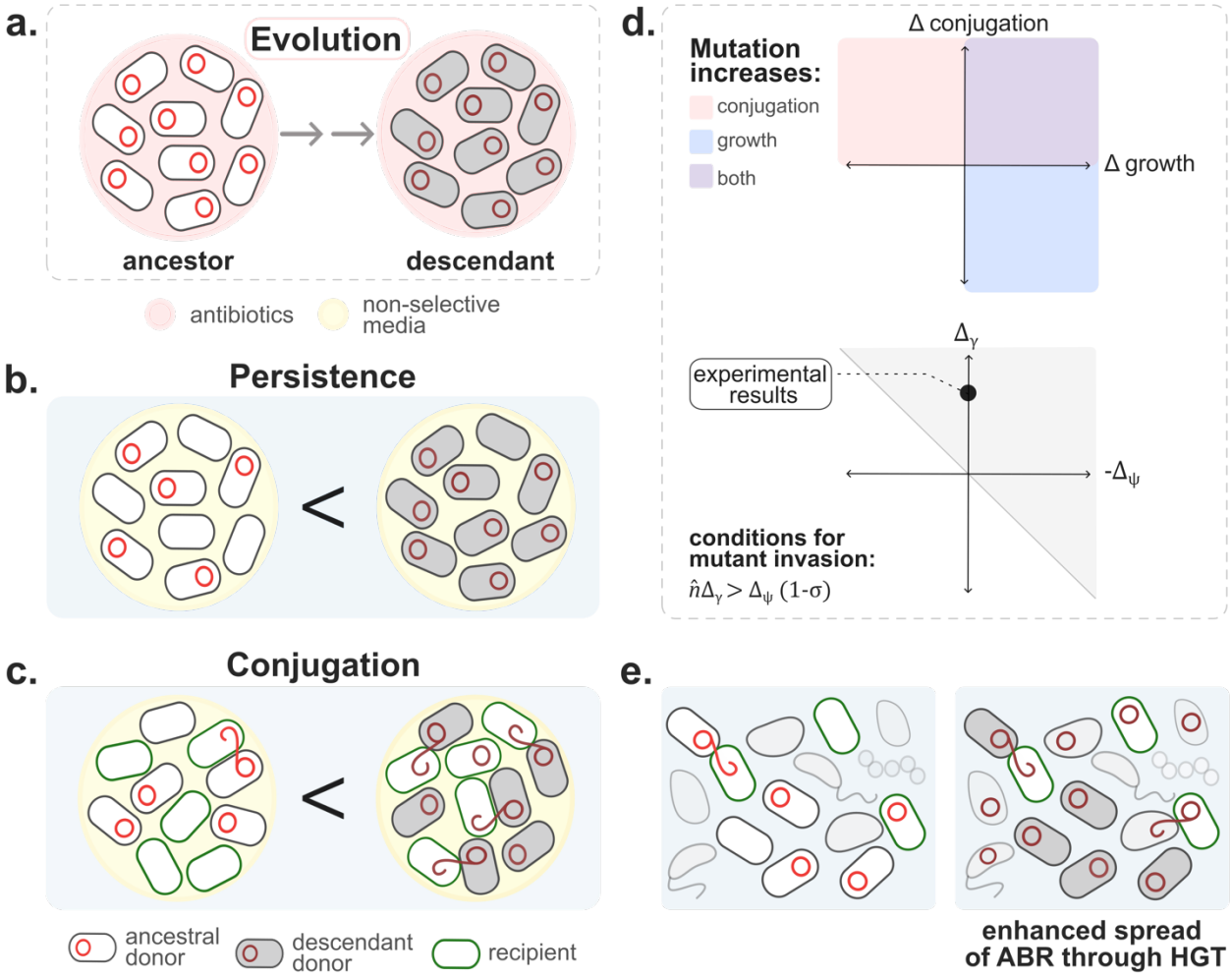
Plasmids are small, extrachromosomal DNA elements commonly found in bacteria, often carrying accessory genes such as antibiotic resistance genes. They play a pivotal role in disseminating antibiotic resistance within bacterial populations through the process of conjugation, enabling transfer between different bacterial strains or species horizontally, rather than vertically through cellular division. In the absence of selection for the plasmid, its presence in the population tends to decrease due to associated fitness costs. However, coevolution between hosts and plasmids can lead to enhanced plasmid persistence, allowing them to persist even after the selective pressure is removed. There are a variety of ways that plasmids become more persistent, including acquiring compensatory mutations to reduce the cost of carriage, minimizing segregational loss, and increasing conjugation rates. In this study, we specifically investigated the impact of increased conjugation rates, a less explored yet significant factor contributing to plasmid persistence. We employed the Luria-Delbrück Method (LDM) to estimate the conjugation rate of an ancestral and descendant plasmid-host pair consisting of an *Escherichia coli* host

and an IncP-β plasmid that coevolved under antibiotic selection favoring plasmid maintenance. Remarkably, we observed a significant increase in conjugation rates, which suggests that the increase in plasmid persistence in a population can be partially explained by an increase in the transfer rate after plasmid-host coevolution. To understand the drivers behind this increase, we formulated two hypotheses: (i) a pleiotropic effect of cost reduction and (ii) direct selection for heightened conjugation rates. While the pleiotropy hypothesis is attractive, our findings lacked robust evidence for it, as there was no significant change in growth rate that would indicate a reduction in plasmid cost. Consequently, we explored the direct selection hypothesis. Although we did not find any conjugation-related mutations in the plasmid, our theoretical model suggested that mutations impacting conjugation rates could potentially drive the mutant plasmid's invasion into the population under certain conditions. Our results shed light on the complexities of plasmid persistence and conjugation rates, indicating that selection under antibiotic pressure not only favors retaining antibiotic resistance genes and alleviating associated plasmid costs, but in some cases, may also promote an increase in horizontal transmission of the plasmid. Thus, selective antibiotic conditions may enhance the spread of antibiotic resistance through horizontal gene transfer.



Graphical Abstract. (a) A study by Jordt et al. (2020) conducted an evolution experiment in Escherichia coli (black-outlined rods) with an IncP-β plasmid (red circles) encoding antibiotic resistance, in the presence of an antibiotic (red-shaded medium). Changes occurring over the evolutionary trajectory are shown as darker shading. **(b)** A persistence assay revealed increased persistence in descendants compared to ancestors. **(c)** We probed the impact of conjugation rates on plasmid persistence, uncovering a significant increase. This suggests enhanced transfer post-plasmid-host coevolution contributes to higher persistence. **(d)** Mutations in a region of the phenotype space (top graph, colored shading) could enhance persistence via increases in growth rate (blue), conjugation (red), or both (purple). We considered two hypotheses here. First is the "indirect selection" hypothesis, where direct selection for improved growth has a positive pleiotropic effect on conjugation rate (such mutations would land in the purple region). Second is the "direct selection" hypothesis, where higher rates of conjugation are directly favored (such mutations would land in either the red or purple regions, or along the positive y-axis). We employed a mathematical model to explore the conditions for mutant plasmid invasion. The model indicates that the mutant plasmid can successfully invade the population when the product of the proportion of plasmid-free cells ($\hat{n}$) and the change in conjugation rate (Δ_γ) is larger than the product of the change in the cost of plasmid carriage (Δ_ψ) and the probability of retaining the plasmid (1-σ). This condition establishes a threshold line, with mutation effects falling above it representing mutants that can invade (bottom graph, gray shading). The descendant from our case study is denoted by the black dot for reference.

Its location suggests that the increase in conjugation rate could be explained by the direct selection hypothesis. **(e)** Our results shed light on the complexities of plasmid persistence and its link to antibiotic-driven horizontal transmission, suggesting that antibiotic selection could potentially encourage not only the preservation of antibiotic resistance genes but also facilitate an increase in horizontal gene transfer.

Acknowledgements

The completion of this thesis owes much to the invaluable contributions and support of my co-authors: Benjamin Kerr, Elizabeth Duan, Eva Top, and Olivia Kosterlitz. Their dedication and assistance, particularly Ben and Olivia, who played pivotal roles in guiding my early graduate school training, have been instrumental to the success of this endeavor. Furthermore, I extend my deepest gratitude to my friends and family. Special recognition is due to Richard Moyer for his indispensable editorial guidance throughout the manuscript preparation process and his continuous love and support during my studies. My sincere appreciation also goes to Ada Tirado de Jesús, Melanie Miranda Muñiz, and Melissa Muñiz Tirado for their unwavering affection and encouragement.

Persistence under pressure: exploring the impact of conjugation rate evolution on the stability of plasmids

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Introduction

Horizontal gene transfer (HGT) is an important process in bacterial evolution, exerting a pivotal influence on microbial adaptation and ultimate survival. Unlike vertical gene transfer, which occurs through reproduction, HGT is the transfer of genetic material between different, often unrelated, organisms. This genetic exchange greatly enriches the genetic diversity of bacteria, enabling the acquisition of new traits such as antimicrobial resistance (AMR), pathogenicity, and the biodegradation of toxic compounds (de la Cruz & Davies, 2000; Thomas & Nielsen, 2005). Understanding the mechanisms and consequences of HGT is essential for preventing the spread of AMR, combating the emergence of infectious diseases, and gaining insights into the evolution of bacterial communities.

In bacteria, important vehicles of HGT are plasmids. Plasmids are extrachromosomal DNA molecules that replicate separately from the host chromosome (Frost et al., 2005; Norman et al., 2009). Bacteria carrying certain plasmids can go through a process called conjugation which enables the transfer of genetic material between cells through a contact-dependent mechanism. Conjugative plasmids are widely distributed in the prokaryotic world, and understanding how they disseminate within microbial systems is a crucial component to understanding the ecology, evolution, and epidemiology of bacterial communities (Kottara et al., 2016; Norman et al., 2009; Ochman et al., 2000; Olesen et al., 2022). Importantly, conjugative plasmids play a significant role in the dissemination of AMR via HGT; e.g., when compared to other mobile and conjugative elements, plasmids exhibit a higher abundance of AMR genes (Baker et al., 2015; Botelho & Schulenburg, 2021).

In the absence of selective conditions favoring plasmid-encoded products, plasmids can be costly to their hosts (Bergstrom et al. 2000; Dionisio et al., 2005; Stewart & Levin, 1977). In such a case, segregational loss of the plasmid during cell division and subsequent selection against plasmid-bearing cells can lead to plasmid loss at the level of a bacterial population The faster plasmid-free cells displace plasmid-bearing cells, the less “persistent” the plasmid (Bergstrom et al. 2000, Jordt et al. 2020). However, recent studies have described multiple ecological and evolutionary factors affecting plasmid persistence. To start, the fitness burden of plasmid carriage is highly dependent on the identity of the host (De Gelder et al., 2008). Additionally, interactions between coinfecting plasmids within a host cell can influence plasmid persistence (San Millan et al., 2014). Evolutionarily, compensatory mutations that ameliorate fitness costs can occur in the plasmid, host chromosome, or both (Brockhurst & Harrison, 2022). High conjugation rates promoting infectious transmission have also been proposed as a mechanism promoting plasmid persistence (Brockhurst & Harrison, 2022; De Gelder et al., 2008; Kottara et al., 2016; Stewart & Levin, 1977). A study on *Escherichia coli* revealed that common conjugative plasmids can be transferred at rates significant enough to support plasmid maintenance, even in the presence of associated fitness costs (Lopatkin et al., 2017). Furthermore, plasmid mutations that affect transfer rate have been characterized (Cheah & Skurray, 1986; Kohler et al., 2018; Poidevin et al., 2018; Virolle et al., 2020; Yoshioka et al., 1987), opening the door to the evolution of conjugation rate itself. Thus, understanding how these genetic elements persist in complex bacterial communities also requires an examination of plasmid conjugation rates and their potential evolution (Kosterlitz et al. 2022).

Jordt et al. (2020) conducted a study providing valuable insights into the interplay of host-plasmid coevolution, plasmid stability, and persistence. Through antibiotic selection, they demonstrated that coevolution between hosts and plasmids led to enhanced persistence of the plasmid under conditions in which the antibiotic was absent. The authors attributed the increased persistence to compensatory evolution, in which the cost of plasmid carriage was reduced. In addition to the amelioration of fitness costs, another possible explanation for the increased persistence is a higher conjugation rate. Initial examinations suggested that these strains not only acquired compensatory mutations but also exhibited higher

conjugation frequencies. However, it is important to note that the methods to measure conjugation available at the time of the study were biased due to constraints imposed by violating model assumptions (Kosterlitz et al., 2022; Kosterlitz & Huisman, 2023).

To investigate the interplay between persistence and conjugation rates, we employed the Luria-Delbrück Method (LDM) to estimate rates of plasmid transfer (Kosterlitz et al., 2022). This approach overcomes traditional method obstacles, avoiding restrictive assumptions about growth and transfer rates for each population in the assay. In this context, we estimated the conjugation rates of an ancestral and evolved plasmid-host pair from the study of Jordt et al. (2020). Our results indicate that the improved persistence of the plasmid can be attributed to an increased transfer rate arising from plasmid-host coevolution. Below, we discuss conditions contributing to the evolution of an increased conjugation rate. We describe hypotheses for the indirect and direct selection of conjugation rate and build a preliminary mathematical model to explore the plausibility of one version of direct selection for plasmid transfer. These findings provide valuable insights into factors influencing plasmid conjugation, underlining the significance of considering changes in transfer rates when examining the long-term stability and spread of plasmids in bacterial populations.

Methods

Bacterial Strains

We obtained an *E. coli* MG1655_SR strain from Jordt et al. (2020). This strain is a streptomycin- and rifampicin-resistant derivative of MG1655_SR that was used in an evolution experiment (outlined below) in which an ancestor was connected to its descendant through a few hundred generations under batch culture conditions. It contains the self-transmissible plasmid pALTS29 from the plasmid group IncP-1β, carrying genes conferring resistance to chloramphenicol (*cmlA1*), macrolides (*mph(E)* and *msr(E)*), sulfonamide (*sul1*), and a gene encoding a class-D beta-lactamase (*blaOXA*). A more detailed description of the ancestral plasmid can be found in Law et al. (2021).

For simplicity, we will refer to the starting strain, or ancestor, as E(p) and the descendant as E'(p'). In this notation, E represents the bacterial host, p the plasmid, and the apostrophes denote that evolution

has taken place (and changes have occurred in both the host, E', and the plasmid, p'). These plasmid-containing strains, E(p) and E'(p'), will be used as the plasmid donors in the conjugation assays. In addition to the strains obtained from the previous study, we derived a recipient strain from the same isogenic strain as E(p), labeled as E(Ø) in this study. E(Ø) is a plasmid-free *E. coli* MG1655 that was isolated by selective plating in the presence of nalidixic acid, providing a unique selectable marker to distinguish the donor and recipient hosts during the conjugation assay.

Evolution Experiment

In the study conducted by Jordt et al. (2020), replicate populations of E(p) were evolved for 68 culture transfers (~ 400 generations) in Lysogeny broth (LB) containing 25 μg ml^{-1} chloramphenicol (Chl) to select for maintenance of the plasmid (Figure 1a). At the last transfer for each replicate population, an 'evolved isolate' was selected as the descendant, E'(p'). One of these replicate evolved isolates, clone 3, was selected for this study.

At the end of the experiment, the clone 3 descendant's plasmid had a deletion of a large composite transposon containing *mph(E)* and *msr(E)* resistance genes (Figure 1b). Its chromosome had a missense substitution in the *acrR* gene (W63R), a deletion in the *tolA* gene removing a segment of DNA that is 371 base pairs in length, an insertion in the *uspC* gene mediated by the IS51 element, a deletion encompassing 15 genes between *yhiM* and *yhiS*, two mutations in the intergenic region between the *fimE* and *fimA* genes, and a substitution and an inversion of a segment of DNA in an intergenic region. A more detailed description of the mutations accumulated by the descendant can be found in Jordt et al. (2020).

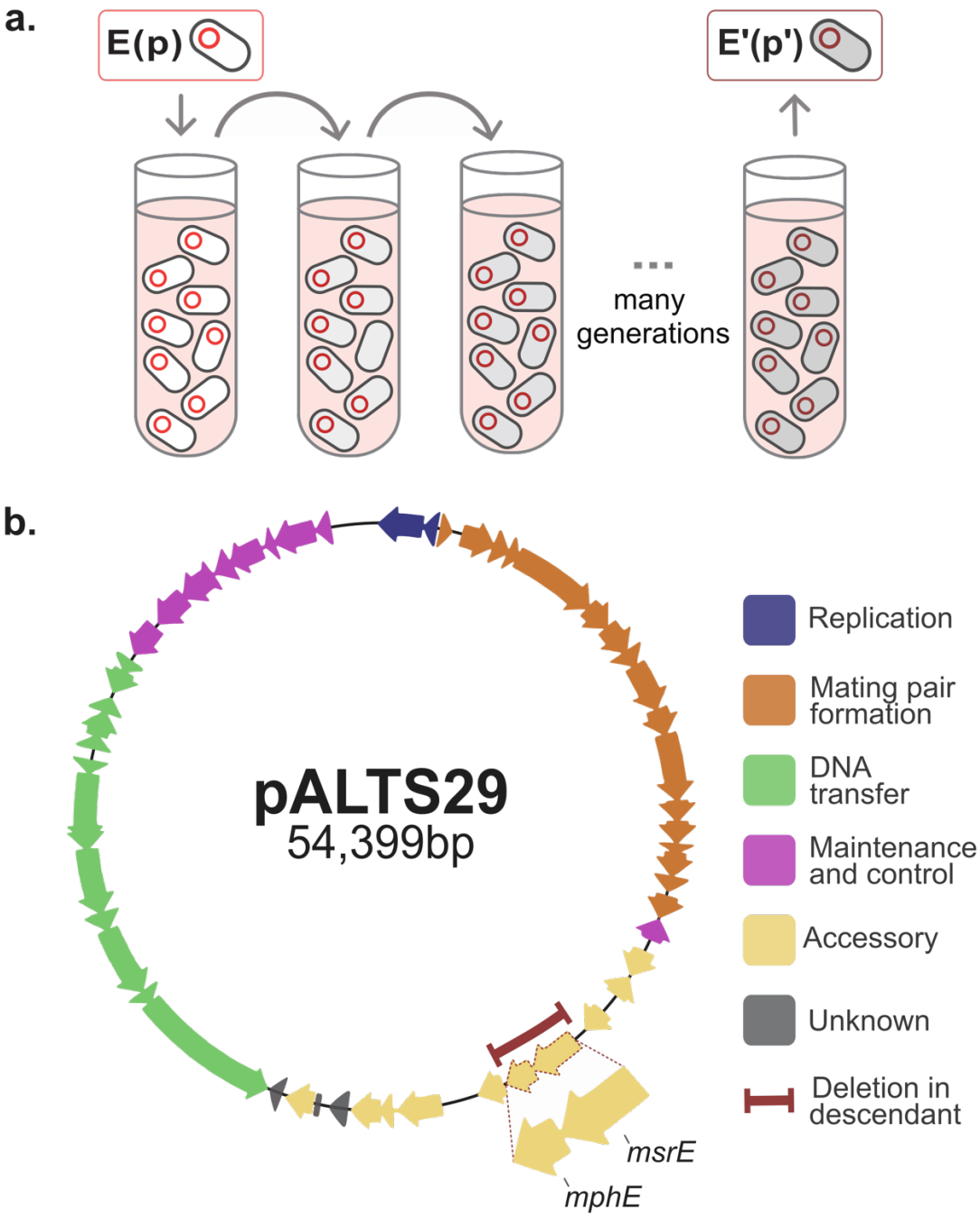

Figure 1. Schematic overview of the evolution experiment and resulting mutations on the plasmid. **(a)** A population of E(p) (*E. coli* MG1655_SR with plasmid pALTS29) was evolved for many generations in the presence of chloramphenicol (red-shaded medium), an antibiotic that selected for the maintenance of the plasmid (red circles). Evolutionary changes are represented by progressively darker shading of the bacteria and plasmids over the sequence. This figure was adapted from Jordt et al. (2020) for clarity. **(b)** At the end of the experiment, the descendant plasmid (clone 3) had a deletion in the large composite transposon IS26 containing *mph(E)* and *msr(E)* resistance genes (red line in genomic map). This deletion removes a segment of DNA that is 2,835 base pairs long. Genes are color-coded by functional/regional categorization. This figure was adapted from Law et al. (2021) for clarity.

Persistence assay

After the evolution experiment, Jordt et al. (2020) propagated the ancestral and descendant strains daily for nine transfers under batch culture conditions without antibiotic selection. The proportion of plasmid-containing colonies (and, by proxy, plasmid-bearing cells in the batch cultures) was determined via streaking on selective and non-selective agar. The rate of decay of the proportion of plasmid-bearing cells (inferred from the streaking results) is inversely related to the "persistence" of the plasmid. As shown in Figure 2b, the persistence profiles between the ancestral E(p) and descendant E'(p') differ. Their results demonstrate a decrease in the proportion of cells containing the plasmid in the ancestral E(p) population when the relevant antibiotic, chloramphenicol, was absent. However, plasmid-host coevolution in the presence of chloramphenicol led to greater plasmid persistence in the absence of this antibiotic for the E'(p') population.

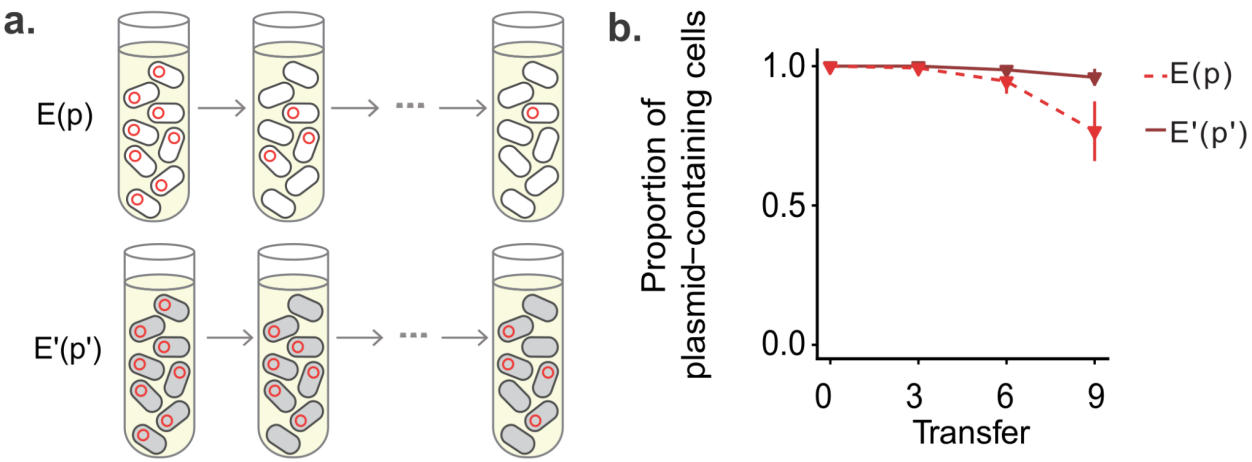

Figure 2. Persistence assay overview and results. (a) Isolates of E(p) and E'(p') were propagated in non-selective media (yellow-shaded medium) over a small number of transfers to establish the proportion of plasmid-containing colonies. **(b)** Persistence profile of the ancestor and descendant strains. For the ancestral isolate (E(p)), the proportion of cells containing the ancestral plasmid decreased in the absence of chloramphenicol. However, the coevolution of this host and plasmid with chloramphenicol (as in Figure 1) led to greater plasmid persistence in the absence of antibiotics (i.e., the plasmid-bearing proportion of the population for the E'(p') isolate decays more slowly than the E(p) isolate). Every point indicates the mean of three replicate persistence assays. Bars indicate the standard error of the mean of replicate cultures. This figure was modified from Jordt et al. (2020), where only the persistence profiles of the isolates relevant to this study are included.

Conjugation assay

Before the conjugation assays, the minimum inhibitory concentration (MIC) of the ancestral E(p) and descendant E'(p') strains was determined in both agar and liquid LB media. These concentrations were then used for all future assays. To select for plasmid-containing cells, LB containing the antibiotic chloramphenicol was used at 25 μg ml^{-1} for liquid media and 12.5 μg ml^{-1} for agar selective plates. To isolate E(Ø) and any transconjugants resulting from conjugation events, we used liquid media containing LB supplemented with nalidixic acid at a concentration of 30 μg ml^{-1}, while the agar selective plates contained LB with nalidixic acid at a concentration of 15 μg ml^{-1}. To prevent low estimations during the conjugation assay due to the transconjugants failing to establish a lineage under the concentrations of antibiotics used, we employed a method developed by Alexander & MacLean (2020), with the suggested adjustments by Kosterlitz et al. (2022), to measure the extinction probability of the transconjugants under these concentrations. Based on the analysis conducted, we determined that there is an undetectable probability of extinction for all strains and transconjugants at the above antibiotic concentrations.

The conjugation assays were performed in accordance with the Luria–Delbrück Method (LDM) developed by Kosterlitz et al. (2022). The basic approach of this method consists of mixing exponentially growing donor and recipient cultures and inoculating a 96-well deep-well plate. The cocultures are incubated in a non-selective growth medium until a critical time point ($\tilde{t}$) where the first conjugation event between the donors and recipients is expected to occur. A separate assay conducted before the LDM determines this critical time point and the initial culture densities to use during this assay. At this predetermined time, a transconjugant-selecting medium that selects against donors and recipients is added to determine the presence or absence of transconjugant cells in each coculture. The number of non-turbid wells resulting after prolonged incubation (p_0), along with the initial and final densities of the donors and recipients (D_0, R_0, $D_{\tilde{t}}$, and $R_{\tilde{t}}$, measured in wells reserved for this purpose), and a volume conversion factor (f), were recorded to calculate the conjugation rate. The conjugation rate was estimated using an equation derived by Kosterlitz et al. (2022).

$$\gamma_D = f\left\{\frac{1}{\tilde{t}}[-\ln p_0\,]\frac{\ln D_{\tilde{t}}R_{\tilde{t}} - \ln D_0R_0}{D_{\tilde{t}}R_{\tilde{t}} - D_0R_0}\right\}. \quad [1]$$

We note that the growth rates of donors and recipients (ψ_D and ψ_R) can also be computed from the assay data as follows:

$$\psi_D = \frac{\ln D_{\tilde{t}} - \ln D_0}{\tilde{t}},$$

$$\psi_R = \frac{\ln R_{\tilde{t}} - \ln R_0}{\tilde{t}}.$$

In this study, we conducted two separate LDM assays to measure the conjugation rate of plasmids from either E(p) or E'(p') to the recipient E(Ø) (Figure 3a). To ensure accuracy and consistency, we repeated these measurements on seven replicate populations. To account for any discrepancies caused by variations in the lab environment, every replicate assay for the E(p) isolate was carried out alongside a corresponding replicate assay for the E'(p') isolate. The initial target densities for the ancestral E(p) pair were $5x10^7$ CFU ml^{-1} for the donor and $5x10^5$ CFU ml^{-1} for E(Ø); the culture was incubated for 2.33 hours before adding the transconjugant-selecting medium. For the descendant pair, the initial target densities were $5x10^6$ CFU ml^{-1} for E'(p') and $5x10^4$ CFU ml^{-1} for E(Ø). After incubating for 1.5 hours, the transconjugant-selecting medium was added. In all cases, our coculture volume is 0.1 ml (giving a conversion factor of $f = 10$).

Results and Discussion

An increase in conjugation rate after coevolution can explain plasmid persistence.

Estimating the conjugation rates using the LDM, we found a significant increase in conjugation rate from E'(p') to a plasmid-free recipient compared to its ancestor E(p) (Figure 3b, p = 0.0005828, Mann-Whitney U test, two-tailed). While the increase in conjugation rate might explain the increased persistence of the descendant plasmid (p') compared to its ancestor (p), a question remains as to *why* we find such a lift in transfer rate. We hypothesize that this increase in conjugation might have evolved due to one of two things: (1) A pleiotropic effect of cost reduction, where mutations that lead to the reduction in the cost of plasmid carriage resulted in higher fitness and, as a side effect, lead to the increase of the conjugation rate;

(2) Direct selection for increased plasmid transfer, mediated by mutations in the plasmid, that occurred during the evolution experiment. Below, we discuss these possibilities.

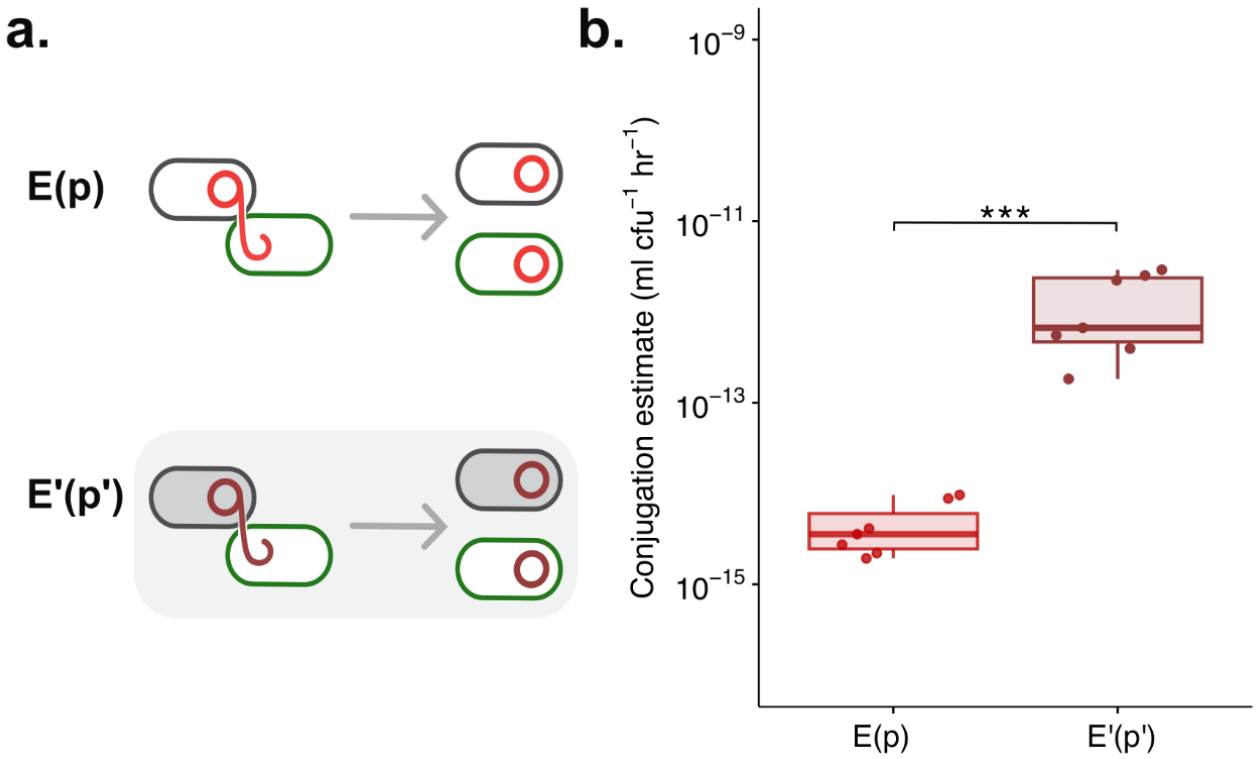

Figure 3. The conjugation rate increased after plasmid-host coevolution. (a) Schematic giving the relevant transfer event for each conjugation assay. Two separate LDM assays were conducted to measure plasmid conjugation from donors (E(p) or E'(p')) to a recipient (E(Ø), green cells). **(b)** Estimates for conjugation rates for E(p) and E'(p'). Each box summarizes 7 replicate estimates, where each data point corresponds to a replicate. The asterisks indicate statistical significance by a two-tailed Mann-Whitney U test ($p = 0.0005828$).

Indirect selection for increased conjugation rate

Previous work has shown that coevolution between host cells and their plasmids under plasmid-selecting conditions can lead to compensatory mutations that lessen the initial costs of plasmid carriage, manifesting as higher growth rates. For instance, Benz and Hall (2023) reported widespread improvements in growth rates associated with increased plasmid persistence after coevolution. Another study by Porse et al. (2016) reported improved persistence of an IncN plasmid through deletions in the transposon IS26, which were associated with an increase in growth rate. Furthermore, Jordt et al. (2020) framed their discussion of improved persistence in terms of increased growth rates due to plasmid cost compensation.

Thus, the selection of increased conjugation rate as a pleiotropic effect of direct selection for improved growth is an attractive explanation.

To investigate this indirect selection hypothesis, we examined the growth rates of the donor cells, E(p) and E'(p'), during their respective conjugation assays. Surprisingly, we found no significant difference in growth rate between E'(p') and its ancestor E(p) (Figure 4a, $p = 0.46$, Mann-Whitney U test, two-tailed). This suggests that the observed increase in conjugation rates cannot be solely attributed to an increase in growth rate. Perhaps compensatory mutations affected other aspects of batch culture growth besides the exponential growth rate. For instance, compensatory mutations that improved growth as the culture was transitioning to a static phase (as nutrients become exhausted) could have been selected. Such mutations, which could pleiotropically raise conjugation rate, would presumably increase the yield (the maximum cell density under our culture conditions), which we also measured in our study. After 24 hours of growth in LB media and plating on agar enriched with chloramphenicol, we observed a significant difference in yield, as measured by the saturation density, between E'(p') and E(p), where the ancestral E(p) strain showed a slightly *higher* yield than the E'(p') descendant (Figure 4b, $p = 0.04723$, Welch two-sample t-test). We emphasize that this difference is opposite of what we would expect if mutations increasing yield were selectively advantageous. While further analysis could be done (e.g., exploring the rate of exit from lag phase), we do not have strong support for the indirect selection hypothesis.

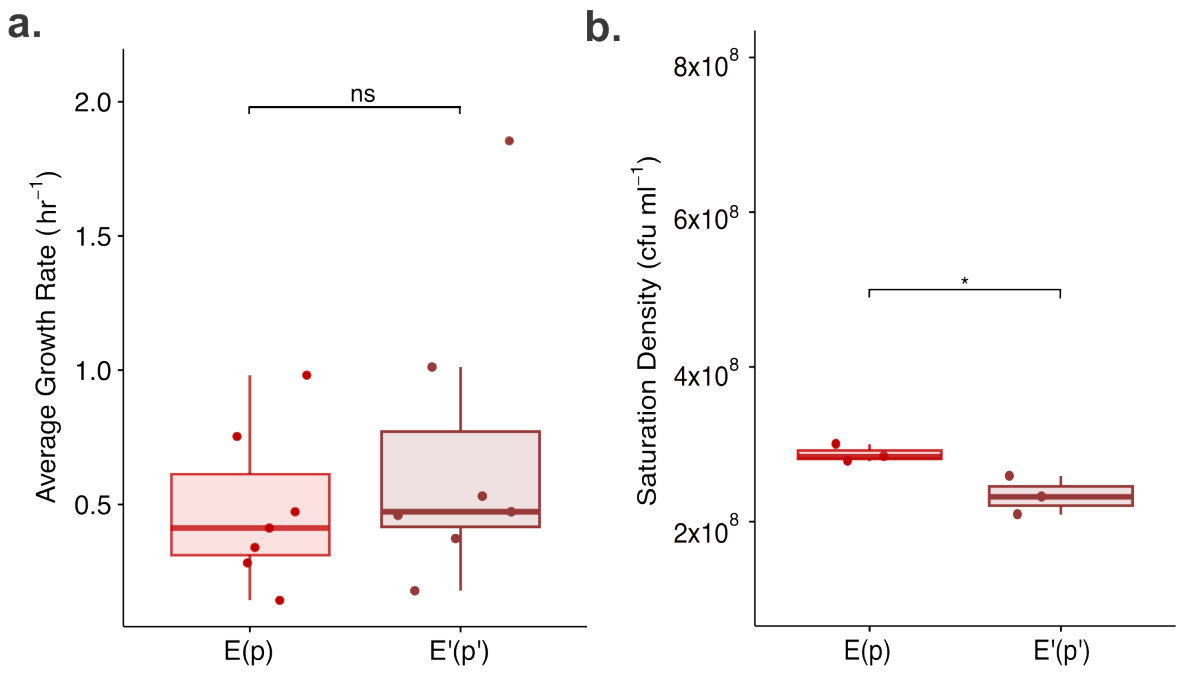

Figure 4. Evaluating factors that might have influenced a fitness increase after coevolution. (a) Growth rate estimates for E(p) and E'(p'). Each box summarizes the average growth rate estimates from seven replicates. Each data point corresponds to a replicate of the LDM procedure. To estimate the average growth rate in each replicate, we calculated the change in density of three wells in that replicate. Based on the outcomes of a two-tailed Mann-Whitney U test (p = 0.46), we determined that there is no statistical significance between the growth rates of E(p) and E'(p'). The letters "ns" denote non-significant results. **(b)** Saturation densities for E(p) and E'(p'). Saturation density, an indicator of yield, was measured after 24 hours of incubation. Each box summarizes the saturation density of each strain, and each data point represents a replicate. A Welch two-sample t-test indicated a statistically significant difference in densities (p = 0.04723) between E(p) and E'(p') after 24hrs of incubation. This is indicated by a single asterisk.

Direct selection for increased conjugation rate

An alternative to our pleiotropy hypothesis is that selection is acting directly to increase the conjugation rate of the plasmid. However, this hypothesis faces a few immediate problems. First, the mutation in the plasmid p' (the deletion between the genes *mph(*E) and *msr*(E) in the composite transposon IS26) did not occur in transfer genes, and such a mutation is not known to directly influence conjugation rate (i.e., it is possible that the causal mutation(s) increasing transfer occurred in the host chromosome). While we currently lack a clear connection between our plasmid mutation and conjugation, we note there has not been much previous exploration of such mutations in light of the transfer rate. Second, and perhaps more concerning, it is not clear why a higher conjugation rate would be selected for in the evolution

experiment of Jordt et al. (2020). This conclusion originates from a common assumption for these systems: *conjugation occurs only from plasmid-bearing to plasmid-free cells*. Due to the presence of antibiotics during the evolution experiment in Jordt et al. (2020) (see Figure 1), plasmid-free cells (generated by segregational plasmid loss) are inhibited. Such evolution experiments, which continuously select for cells carrying the focal plasmid, are typically considered to lack horizontal transmission due to the absence of potential recipients (Dimitriu, 2022). In fact, conjugation rates are expected to decrease under these conditions (Dahlberg & Chao, 2003; Porse et al., 2016) or remain unchanged (Dionisio et al., 2005) due to a reduced "need" for transfer. An influx of recipient cells is assumed to be necessary in order to observe increases in conjugation rates (De Gelder et al., 2008; Dimitriu, 2022; Kottara et al., 2016). However, we feel this hypothesis actually deserves some attention because there might be scenarios where the assumption of a restricted recipient pool in the system is violated.

Given our results, we consider the possibility that, even under selective conditions for plasmid maintenance, there might be a recipient pool available. We note that the antibiotic chloramphenicol is bacteriostatic (preventing growth) rather than bactericidal (causing death), which means that plasmid-free cells could potentially be rescued via horizontal transfer of the plasmid if the process of conjugation could still occur. In a related vein, antibiotic resistance genes encode protein products, which may persist for multiple generations (Kroll et al., 2010) in a cell lineage after the plasmid encoding them has been lost. Such a case of "transient resistance" (Figure 5a) would lead to a population of phenotypically-resistant plasmid-free recipients that could be made genetically-resistant via conjugation. There are few additional ways that segregants could survive, thereby serving as recipients for plasmid transfer. If the level of the antibiotic was low enough, the inhibition by the antibiotic could be imperfect, and some cells without the plasmid might grow (Figure 5b). There is also the possibility of "persister" phenotypes (metabolically inactive states enabling cells to survive stressful conditions, see (Fisher et al., 2017), Figure 5c). Finally, it is important to note that, for some plasmids, conjugation can also occur between two plasmid-containing cells, resulting in superinfection. In this scenario, all cells in the culture become potential recipients, further influencing the conjugation dynamics in the bacterial population. For instance, in a study conducted by Bellanger et al. (2014) on the IncP-β plasmid pB10, the authors observed that the presence of plasmids from the same incompatibility group did not hinder the spread of this plasmid. This study demonstrates that

although superinfection immunity may reduce the transfer efficiency of a plasmid, it does not prevent its transfer entirely.

In all the scenarios illustrated in Figure 5, the working assumption that there are no recipients for plasmid transfer is challenged. Under these scenarios, mutations that increase the rate of plasmid conjugation could be selected directly. If there are plasmid-free recipients available (Figures 5a-c), a mutant plasmid with a higher transfer rate would tend to colonize these recipients first. In the event of superinfection (Figure 5d), there would be an asymmetry in plasmid transfer: the more transmissible mutant plasmid would more often move into cells with the ancestral plasmid than the reverse. Even though the immediate result of such superinfection events is the creation of heteroplasmic cells (where two different plasmids sharing the same nucleotide sequences for all regions involved in the replication and maintenance system coexist within the same cell; Rodríguez-Beltrán et al., 2021) the process of segregational drift (Garoña et al., 2021) would eventually lead to homoplasmic cells, and the asymmetry in transfer should work towards the increase of homoplasmic cells with the mutant plasmid. Of course, we are assuming here that such mutations only affect transfer with no other (pleiotropic) effects.

For a more rigorous exploration of the evolution of conjugation, we built a simple model that allows for pleiotropic effects in the next section. We investigated whether direct selection for higher conjugation rates can facilitate the invasion of a mutant plasmid into a population containing its ancestral variant. To this end, we focused on a concise mathematical model that treats the specific scenario of transient resistance (Figure 5a), providing insights into the conditions under which this scenario could favor an increased plasmid transfer rate.

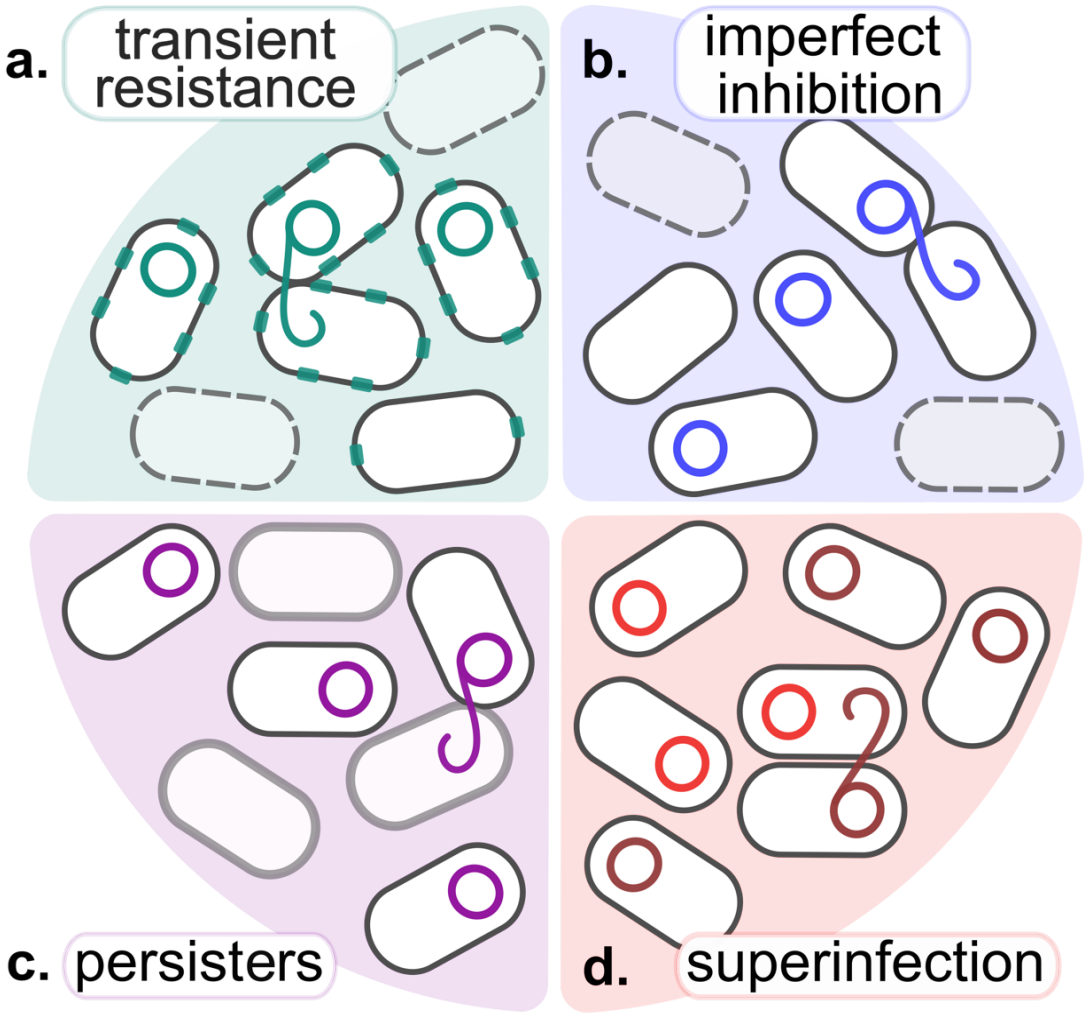

Figure 5. Factors that might increase recipient availability under selection regimes that favor plasmid-containing cells. Higher availability of plasmid-free recipients than expected due to **(a)** transient resistance, where protein products encoded by plasmid genes (green rectangles) may persist in cells even after plasmids are lost for a handful of generations **(b)** antibiotic concentration failing to completely inhibit the plasmid-free cells, or **(c)** the presence of persister cells that are able to survive antibiotic exposure by becoming metabolically inactive. Additionally, conjugation can happen between plasmid-containing cells, leading to **(d)** superinfection. In this last scenario, every cell in the culture becomes a potential recipient.

Model for the direct selection of conjugation under a transient resistance scenario

In this model, we explore the conditions under which a mutant plasmid conferring a greater rate of conjugation could evolutionarily invade a population where cells possessed the ancestral plasmid. To do so, we track the densities of three populations of bacteria. The first population includes cells that possess the ancestral form of the plasmid—the density of such cells is given by the variable A. The second

population includes cells that possess the mutant form of the plasmid—their density is given by M. Finally, the population of cells with no plasmid has its density denoted N. We will use lower-case letters to represent the proportions of each of the three strains in the entire community. Thus, we have:

$$a = \frac{A}{A + M + N},$$

$$m = \frac{M}{A + M + N},$$

$$n = \frac{N}{A + M + N}.$$

For ease, we will describe our three bacterial strains with non-italicized letters A, M, and N, where italicizing each letter will signify strain *density*. Letting X and Y denote arbitrary strains, we can characterize key parameters. We let ψ_{X} and δ_{X} be the growth and death rates of strain X, respectively, where $\mathrm{X} \in \{\mathrm{A}, \mathrm{M}, \mathrm{N}\}$. We let γ_{Y} be the rate that a plasmid transfers from plasmid-bearing cell of strain Y to a plasmid-free cell. Lastly, we let σ_{Y} be the probability that, upon cell division, a mother cell of strain Y generates a daughter that lacks a plasmid. For the last two parameters, $\mathrm{Y} \in \{\mathrm{A}, \mathrm{M}\}$.

We now describe the dynamics within our bacterial community by the following differential equations:

$$\dot{A} = \psi_{\mathrm{A}}(1 - \sigma_{\mathrm{A}})A + \gamma_{\mathrm{A}} aN - \delta_{\mathrm{A}} A,$$

$$\dot{M} = \psi_{\mathrm{M}}(1 - \sigma_{\mathrm{M}})M + \gamma_{\mathrm{M}} mN - \delta_{\mathrm{M}} M,$$

$$\dot{N} = \psi_{\mathrm{N}} N + \psi_{\mathrm{A}}\sigma_{\mathrm{A}} A + \psi_{\mathrm{M}}\sigma_{\mathrm{M}} M - \gamma_{\mathrm{A}} aN - \gamma_{\mathrm{M}} mN - \delta_{\mathrm{N}} N.$$

It is worth emphasizing some of the assumptions of this model here. First, we are assuming growing populations are doing so exponentially (and decaying populations are also doing so exponentially). That is, there is no density-regulated growth in this model. Second, the rate of plasmid transfer is assumed to depend on the *proportion* of the relevant plasmid-bearing cell (variables a and m), as opposed to its *density* (variables A and M). One potential way to justify this assumption is to imagine a laboratory setup in which the population is regularly diluted such that total cell density remains relatively constant (but cell proportions

can change). We can still justify exponential growth in such a case if we consider tracking an ever-expanding population of which the diluted portion is some fractional representation.

We are interested in cases where the mutant has a higher transfer rate. That is, $\gamma_{\mathrm{M}} > \gamma_{\mathrm{A}}$. Thus, we let $\gamma_{\mathrm{M}} = \gamma_{\mathrm{A}} + \Delta_{\gamma}$, where $\Delta_{\gamma} \geq 0$. Generally, this increased transfer may come at a cost. That is, $\psi_{\mathrm{M}} < \psi_{\mathrm{A}}$. Thus, we let $\psi_{\mathrm{M}} = \psi_{\mathrm{A}} - \Delta_{\psi}$, where $\Delta_{\psi} \geq 0$. From the data on our focal plasmid, we would have $\Delta_{\psi} = 0$, but we will analyze the more general model here in order to fully consider pleiotropic effects. Importantly, the plasmid-free cells are experiencing net death in our model; i.e., $\delta_{\mathrm{N}} > \psi_{\mathrm{N}}$. For ease, we let the net loss rate of plasmid-free cells be $\alpha_{\mathrm{N}} = \delta_{\mathrm{N}} - \psi_{\mathrm{N}}$, where $\alpha_{\mathrm{N}} > 0$. Thus, the plasmid-free population is assumed to decay, which is what we would expect in the presence of a drug in which the plasmid encoded resistance. However, a slower rate of decay captures the idea of transient resistance, in which a plasmid-free cell is not immediately killed due to the persistence of protein products encoded by an ancestral cell bearing the plasmid. For ease, we will also assume here $\sigma_{\mathrm{A}} = \sigma_{\mathrm{M}} = \sigma$ and $\delta_{\mathrm{A}} = \delta_{\mathrm{M}} = \delta$; that is, the mutation on the plasmid does not affect segregational loss nor the death rate of the host cell.

Using all this information, we now can rewrite the model, as follows:

$$\dot{A} = \psi_{\mathrm{A}}(1-\sigma)A + \gamma_{\mathrm{A}} aN - \delta A, \qquad [2.1]$$

$$\dot{M} = \left(\psi_{\mathrm{A}} - \Delta_{\psi}\right)(1-\sigma)M + \left(\gamma_{\mathrm{A}} + \Delta_{\gamma}\right)mN - \delta M, \qquad [2.2]$$

$$\dot{N} = -\alpha_{\mathrm{N}} N + \psi_{\mathrm{A}}\sigma A + \left(\psi_{\mathrm{A}} - \Delta_{\psi}\right)\sigma M - \gamma_{\mathrm{A}} aN - \left(\gamma_{\mathrm{A}} + \Delta_{\gamma}\right)mN. \qquad [2.3]$$

Our focus will be on how the values of Δ_{γ} and Δ_{ψ} (the effects of the mutation in the plasmid), σ (which affects the rate of creation of plasmid-free cells), and α_{N} (which affects the rate of net loss of plasmid-free cells) impact the invasion potential of a mutant plasmid in a population in which it starts rare.

To start, we will change our dynamic variables from densities to proportions. We can rewrite the system in equations [2] as follows:

$$\dot{a} = \psi_{\mathrm{A}}(1-\sigma)a + \gamma_{\mathrm{A}} an - \delta a - a\left[(\psi_{\mathrm{A}} - \delta)a + \left(\psi_{\mathrm{A}} - \Delta_{\psi} - \delta\right)m - \alpha_{\mathrm{N}} n\right], \qquad [3.1]$$

$$\dot{m} = \left(\psi_{\mathrm{A}} - \Delta_{\psi}\right)(1-\sigma)m + \left(\gamma_{\mathrm{A}} + \Delta_{\gamma}\right)mn - \delta m - m\left[(\psi_{\mathrm{A}} - \delta)a + \left(\psi_{\mathrm{A}} - \Delta_{\psi} - \delta\right)m - \alpha_{\mathrm{N}} n\right], \qquad [3.2]$$

$$\dot{n} = -\alpha_{\mathrm{N}} n + \psi_{\mathrm{A}}\sigma a + \left(\psi_{\mathrm{A}} - \Delta_{\psi}\right)\sigma m - \gamma_{\mathrm{A}} a n - \left(\gamma_{\mathrm{A}} + \Delta_{\gamma}\right) m n - \; n\left[(\psi_{\mathrm{A}} - \delta)a + \left(\psi_{\mathrm{A}} - \Delta_{\psi} - \delta\right)m - \alpha_{\mathrm{N}} n\right]. \quad [3.3]$$

In a community without mutant plasmids present ($m = 0$), there are two equilibria:

$$(\hat{a}, \hat{n}) = (0,1),$$

$$(\hat{a}, \hat{n}) = \left(\frac{\psi_{\mathrm{A}}(1-\sigma) - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}}}{\psi_{\mathrm{A}} - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}}}, \frac{\psi_{\mathrm{A}}\sigma}{\psi_{\mathrm{A}} - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}}}\right).$$

Hereafter, we will focus on the second equilibrium. We are assuming $\psi_{\mathrm{A}} > \delta$, therefore the denominators of $\hat{a}$ and $\hat{n}$ are positive (as $\gamma_{\mathrm{A}} > 0$ and $\alpha_{\mathrm{N}} > 0$). Hereafter, we will assume $\psi_{\mathrm{A}}(1-\sigma) - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}} > 0$, which makes $\hat{a}$ well-defined ($0 \leq \hat{a} \leq 1$). Thus, at this second equilibrium both cells with the ancestral plasmid and plasmid-free cells coexist. Similar to mutation-selection balance, this coexistence is a kind of "segregation-selection" balance. Without mutant plasmids present, this second equilibrium is stable. However, what happens when we consider perturbations that introduce a small proportion of mutant plasmid-bearing cells? That is, is the equilibrium

$$(\hat{a}, \hat{m}, \hat{n}) = \left(\frac{\psi_{\mathrm{A}}(1-\sigma) - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}}}{\psi_{\mathrm{A}} - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}}}, 0, \frac{\psi_{\mathrm{A}}\sigma}{\psi_{\mathrm{A}} - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}}}\right),$$

stable?

Here, we can represent the dynamical system given by equations [3] as two coupled differential equations:

$$\dot{a} = f(a, m), \quad [4.1]$$

$$\dot{m} = g(a, m), \quad [4.2]$$

where

$$f(a,m) = \psi_{\mathrm{A}}(1-\sigma)a + \gamma_{\mathrm{A}} a(1-a-m) - \delta a - a\left[(\psi_{\mathrm{A}} - \delta)a + \left(\psi_{\mathrm{A}} - \Delta_{\psi} - \delta\right)m - \alpha_{\mathrm{N}}(1-a-m)\right],$$

$$g(a,m) = \left(\psi_{\mathrm{A}} - \Delta_{\psi}\right)(1-\sigma)m + \left(\gamma_{\mathrm{A}} + \Delta_{\gamma}\right)m(1-a-m) - \delta m \\ - \; m\left[(\psi_{\mathrm{A}} - \delta)a + \left(\psi_{\mathrm{A}} - \Delta_{\psi} - \delta\right)m - \alpha_{\mathrm{N}}(1-a-m)\right].$$

The Jacobian matrix for equations [4] is

$$\mathbf{J} = \begin{bmatrix} \left.\frac{\partial f}{\partial a}\right|_{\hat{a},\hat{m}} & \left.\frac{\partial f}{\partial m}\right|_{\hat{a},\hat{m}} \\ \left.\frac{\partial g}{\partial a}\right|_{\hat{a},\hat{m}} & \left.\frac{\partial g}{\partial m}\right|_{\hat{a},\hat{m}} \end{bmatrix},$$

which, upon calculating the relevant partial derivatives, is

$$\mathbf{J} = \begin{bmatrix} -\{\psi_{\mathrm{A}}(1-\sigma) - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}}\} & \psi_{\mathrm{A}}(1-\sigma) - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}} - \Delta_{\psi} + \hat{n}\Delta_{\psi} \\ 0 & \hat{n}\Delta_{\gamma} - \Delta_{\psi}(1-\sigma) \end{bmatrix}. \quad [5]$$

Because the matrix in equation [5] is triangular, the eigenvalues line the diagonal:

$$\lambda_1 = -\{\psi_{\mathrm{A}}(1-\sigma) - \delta + \gamma_{\mathrm{A}} + \alpha_{\mathrm{N}}\},$$

$$\lambda_2 = \hat{n}\Delta_{\gamma} - \Delta_{\psi}(1-\sigma).$$

By our earlier assumption $\lambda_1 < 0$. If we fix mutant plasmid-bearing cell proportion at zero, the equilibrium of ancestral plasmid-bearing and plasmid-free cells is stable. The second eigenvalue relates to the stability of the equilibrium to perturbations (i.e., small non-zero introductions) of mutant plasmid-bearing cells. Specifically, this equilibrium is unstable when $\lambda_2 > 0$, or

$$\hat{n}\Delta_{\gamma} - \Delta_{\psi}(1-\sigma) > 0,$$

This gives the following mutant invasion criterion:

$$\hat{n}\Delta_{\gamma} > \Delta_{\psi}(1-\sigma). \quad [6]$$

The criterion in inequality [6] makes sense as the benefit of the plasmid mutation (on the left) needs to outweigh the cost (on the right). All else being equal, as the mutation increases the conjugation rate (Δ_{γ} gets bigger) or as the growth cost of the mutation decreases (Δ_{ψ} gets smaller), the criterion is easier to satisfy. The benefit of the mutation is weighted by the proportion of plasmid-free cells, which is because the effect of the mutation is realized by converting plasmid-free cells at a greater rate. The cost of the mutation is weighted by one minus the segregation probability, which is the fraction of the growing population that remains plasmid-bearing. Because $\hat{n}$ increases as α_{N} decreases, the invasion criterion is easier to satisfy as the plasmid-free cell population decays more slowly (i.e., as transient resistance is stronger). Lastly, as segregation loss probability increases (as σ goes up), the criterion becomes easier to satisfy for two reasons. First, $\hat{n}$ increases as σ increases, so the left side grows as σ increases. Second, the right side

shrinks as σ increases (the growth penalty of the mutant plasmid is more muted when plasmid-bearing cells are lost at division at a greater rate). In Figure 6, we give an example where the criterion does not hold (and thus the mutant cannot invade) and where it does hold (and thus the mutant does invade).

Note that for the plasmid we are analyzing in the current study, it appears that $\Delta_\psi = 0$. In such a case, if $\sigma > 0$ (such that $\hat{n} > 0$), the criterion is guaranteed to hold, and the mutant plasmid should be able to invade. Thus, if a mutation occurred on our plasmid that increased the rate of plasmid transfer without any cost in host growth rate, this mutant plasmid would successfully invade the community.

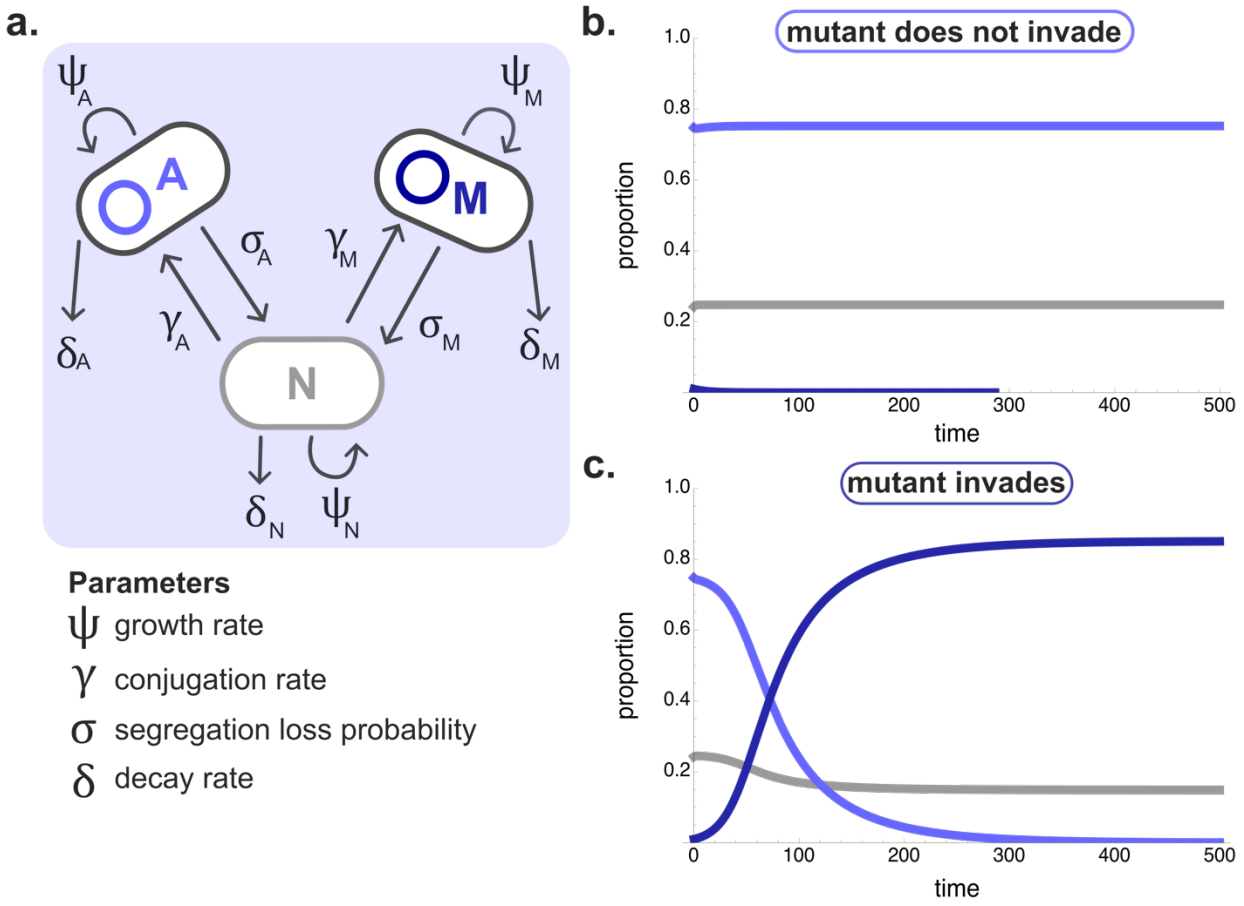

Figure 6. **Model for the direct selection of conjugation under a transient resistance scenario. (a)** A simplified diagram illustrating the structure of our model, including the cell types (A, M, and N) and the key parameters that govern the transition of cell types. A represents the cells carrying the ancestral plasmid (light blue circle), M represents the cells carrying the mutant plasmid (dark blue circle), and N represents the plasmid-free cells. Here we numerically simulate the differential equations [3] starting near (but not at) the equilibrium $(\hat{a}, \hat{m}, \hat{n}) = ((\psi_A(1-\sigma) - \delta + \gamma_A + \alpha_N)/(\psi_A - \delta + \gamma_A + \alpha_N),\ 0,\ \psi_A\sigma/(\psi_A - \delta + \gamma_A + \alpha_N))$. We use the following parametric values: $\psi_A = 1$, $\sigma = 0.25$, $\gamma_A = 0.01$, $\delta = 0.1$, $\alpha_N = 0.1$, and $\Delta_\psi = 0.1$. On **(b)** the top graph $\Delta_\gamma = 0.01$ (where the invasion criterion [6] fails) and on **(c)** the bottom graph $\Delta_\gamma = 0.6$ (where the invasion criterion [6] is satisfied).

Summary

In this study, we investigate the factors influencing plasmid persistence in bacterial populations. As demonstrated in a previous study conducted by Jordt et al. (2020), under selection pressure for plasmid genes, evolution leads to higher plasmid persistence. Plasmids can become more persistent through various mechanisms, such as acquiring mutations that reduce the cost of carriage, lower the rate of segregational loss, or increase the conjugative transfer rate. This study examines the enhancement of conjugation rate, a less-explored trait change that can lead to an increase in persistence. Interestingly, we observed a dramatic increase in conjugation rate after the evolution experiment. We proposed two hypotheses to explain this increase: a pleiotropic effect of cost reduction and direct selection for increased conjugation rates. While pleiotropy is an attractive hypothesis, we lack strong evidence for it, as there was no significant change in the growth rate across the original evolution experiment. We then turned to the direct selection hypothesis. Although no known conjugation-related mutations were found in the plasmid, our theoretical model shows that mutations increasing conjugation rates could potentially lead to the mutant plasmid invasion of the population, assuming certain conditions are met. Our research provides valuable insights into the complex dynamics of plasmid persistence and conjugation rates, contributing to our understanding of antibiotic resistance and microbial evolution. These results suggest that selection under antibiotic pressure not only favors retention of antibiotic resistance genes as well as alleviation of costs associated with the plasmids that carry them, but also may, in some instances, favor an increase in horizontal transmission of the plasmid. This means that selective conditions of antibiotics might favor the greater spread of antibiotic resistance via HGT.

Future directions

Experimental

Further investigations are needed to elucidate the full extent of fitness effects associated with increased conjugation rates and their implications for bacterial adaptation. To gain deeper insights into the influence of fitness effects on the evolution of increased conjugation rates, a competition experiment should be conducted to directly assess the fitness differences between the ancestor and its descendant. Although

we did not observe a significant increase in growth rate in our study, we expect to observe an overall increase in competitive fitness in the descendant E'(p') compared to the E(p) ancestor. However, traditional competition assays will have limitations because of the nature of our system, where the infectious transmission of the plasmid may potentially transfer the growth cost to its competitors, limiting the accuracy of fitness assessment. Therefore, future experiments need to be designed with this consideration in mind. For instance, the system could be manipulated so that the fitness measurements can be corrected for conjugative events. One possibility is gauging transconjugants by putting neutral markers in the hosts and then sequencing the plasmids from colonies at diagnostic positions.

Given the observed mutations in both the plasmid and the host during coevolution, it is unclear which factor or combination of factors contributes to the observed persistence. To differentiate the effects of plasmid evolution and host evolution on persistence, we propose measuring the conjugation rate of the descendant p' plasmid in a new host context. If mutations in the plasmid primarily drive its persistence in the E'(p') population, we anticipate an increase in conjugation rates in E(p') compared to E(p). Conversely, if mutations in the host chromosome account for plasmid persistence, either independently or in conjunction with the descendant plasmid, we expect no such increase. Investigating the conjugation rates of novel host-plasmid combinations will help clarify the relative influences of plasmid and host evolution on the observed persistence.

To further evaluate the mutational changes in the strains that lead to the increase in conjugation rate, it would be beneficial to understand the mutational path that leads to the observed phenotype. Thus, we propose reanimating generations of the evolution experiment to identify whether multiple mutations are required to reach this phenotypic change, as well as the specific order of their occurrence. We note that it may be necessary to engineer some of these mutations into an ancestral background to elucidate causal roles of different mutations. For instance, consider a sequence of mutations, where first mutation A and then mutation B occurred in our evolutionary lineage. Suppose the conjugation rate remained at its ancestral value after mutation A, but increased after the second mutation B occurred. In order to gauge whether the effect of mutation B was epistatic on mutation A, we would engineer mutation B into the ancestral background and gauge the conjugation rate of the plasmid inside such a strain. More generally,

engineering different combinations of the mutations defining our descendant would allow assessment of context-dependent versus context-independent effects of these mutations in terms of phenotypic impact.

Theoretical

There are several theoretical avenues to explore and enhance the current model. First, it would be good to consider incorporating more realistic parameters and modeling the system in a batch culture setup to better align with the laboratory experiment. Additionally, we could introduce a mechanism in the model that allows for a change in growth, along with the loss of resistance when specific proteins are sufficiently diluted by cell division. Furthermore, the current model assumes that a single mutation led to the observed phenotypic change; investigating the possibility of sequential mutations contributing to the phenotype could provide a more comprehensive understanding. Moreover, we could explore the complexities of superinfection scenarios. This would require tracking heteroplasmic states of cells, adding a new layer of intricacy to the model's dynamics. By exploring these directions, we can gain a deeper and more nuanced understanding of the behavior of such microbial systems as well as how such behavior could change under further evolution.

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