Of E. coli and classrooms: Stories of persistence

Hannah Lillian Jordt

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Benjamin Kerr, Chair

Scott Freeman

Beth Traxler

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Abstract

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Hannah Lillian Jordt

Chair of the Supervisory Committee:
Dr. Benjamin Kerr
Biology

Plasmids exist in bacteria and are small, extrachromosomal pieces of DNA that often encode accessory genes such as antibiotic resistance genes. They are largely responsible for spreading antibiotic resistance genes through bacterial populations via their ability to conjugate into different bacterial hosts or species. In environments without selection for the plasmid, the proportion of plasmid-containing cells is expected to decrease in the population due to fitness costs associated with plasmid carriage. Yet because of these costs, we also expect that beneficial genes located on plasmids should eventually transition to the chromosome. Thus, the existence of plasmids is puzzling. I explored reasons for their existence and found that 1) co-evolution of hosts and their plasmid can increase plasmid persistence, which has further consequences for the increased emergence of multi-drug resistance when these co-evolved pairs are in bacterial communities, and

2) environments with alternating selection for the plasmid can allow even costly, conjugative plasmids to be maintained in bacterial populations. I also explored the effects of values-affirmation on the reduction of stereotype threat in introductory biology classrooms, and how completing a classroom exercise in which students affirm values they find important to them can reduce the achievement gap in exam scores between underrepresented minority and white students.

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Chapter 1.

Values affirmation reduces the achievement gap between under-represented minority and white students in introductory biology classes

Hannah Jordt[1], Sarah L. Eddy[2*], Riley Brazil[3], Ignatius Lau[4], Chelsea Mann[5], Sara E. Brownell[6], Katherine King[1], Scott Freeman[1]

[1]Department of Biology, University of Washington, Seattle, Washington, 98195

[2]Department of Biology, Florida International University, Miami, Florida, 33199

[3]School of Medicine, Tufts University, Boston, Massachusetts, 02111

[4]Stony Brook Medicine, Stony Brook, New York, 11794

[5]School of Medicine, University of Washington, Seattle, Washington, 98195

[6]School of Life Sciences, Arizona State University, Tempe, Arizona, 85287

Abstract

Achievement gaps between underrepresented minority (URM) students and their white peers in college science, technology, engineering, and mathematics (STEM) classrooms are persistent across many white-majority institutions of higher education. Attempts to reduce this phenomenon of underperformance through increasing classroom structure via active learning

have been partially successful. In this study, we address the hypothesis that the achievement gap between white and URM students in an undergraduate biology course has a psychological and emotional component arising from stereotype threat. Specifically, we introduced a values affirmation exercise, which counters stereotype threat by reinforcing a student's feelings of integrity and self-worth, in three iterations of an intensive active learning college biology course. On average, this exercise reduced the achievement gap between URM and white students who entered the course with the same incoming GPA. This result suggests that achievement gaps resulting from the underperformance of URM students could be mitigated by providing students with a learning environment that removes psychological and emotional impediments of performance through short psychosocial interventions.

Introduction

Representation of Blacks, Latino/as, Native Americans, and Hawaiian and Pacific Islanders remains low in the STEM workforce, despite their increasing proportion of the general population of the United States (National Science Foundation, 2015). This disparity persists regardless of data indicating that underrepresented minority (URM) students frequently exhibit an equal—if not higher—initial interest in majoring in STEM at the undergraduate level relative to their white peers (Anderson and Kim, 2006; Riegle-Crumb and King, 2010). Longitudinal data demonstrate that retention differences through college are partially to blame for this underrepresentation in STEM careers (Anderson and Kim, 2006). Thus, to diversify the STEM workforce, one critical step is to identify and mitigate the challenges faced by undergraduates from historically underrepresented groups, and create an environment that nurtures their passion

for STEM.

For all students, and STEM undergraduates in particular, one of the best predictors of undergraduate retention across majors is performance in college courses (Riegle-Crumb and King, 2010; Westrick *et al*.,2015; Beasley and Fischer, 2012). Therefore, observed disparities in performance in many STEM classrooms (National Science Foundation, 2015; Eddy and Hogan, 2014; Greene *et al.*, 2008) could help explain why URM students persist in STEM at lower rates. One strategy for increasing retention, and therefore workforce diversity, is to incorporate research-supported pedagogical strategies into the classroom that increase academic performance, such as active learning (Freeman *et al.*, 2014). When paired with out-of-class activities like pre-class reading assignments and post-class review assignments, active learning reduces achievement gaps between Black and white students, first- and continuing-generation students, and students from lower and higher socio-economic backgrounds (Eddy and Hogan, 2014; Haak *et al.*, 2011). These types of teaching innovations may help level the playing field by explicitly modeling strategies and skills needed to succeed in college STEM classrooms. This type of modeling could disproportionately benefit students whose prior educational experiences have not adequately prepared them for college-level work. However, even with these pedagogical interventions, achievement gaps still remain.

Classroom climate is another potential target—beyond changing curriculum—that instructors may need to consider to close achievement gaps. Classroom climate has been defined as the "intellectual, social, emotional, and physical environment in which… students learn" (Ambrose *et al.*, 2010). The impact of classroom climate on students has been documented

across many studies. For example, feeling that the instructor cares about them reduces student apprehension in class and increases motivation, attitudes towards the course, and self-reported learning from the course (Ellis, 2004). In addition, believing that an instructor thinks they can improve increases the likelihood that students will incorporate instructor feedback (Cohen *et al*., 1999) and helps students maintain their interest in the content area of the course (Good *et al*., 2012). Thus, along with considering how learning is structured, considering the climate in a classroom may be critical for closing achievement and persistence gaps.

While there is some evidence that the use of active learning may improve classroom climate (Eddy and Hogan, 2014), the demonstrated changes have been small. In addition, changing classroom climate can be challenging as it involves not only the instructor's behaviors and attitudes but also those of classmates (Bright *et al.*, 1998; Holley and Steiner, 2005). Instead, it may be more efficient to bolster students against negative classroom climate or perceptions thereof, as a way to improve student performance. Fortunately, there is an easily implementable strategy termed values affirmation (Cohen *et al*., 2006; Cohen *et al*., 2009; Sherman *et al.*, 2013; Walton *et al.*, 2014) that is designed to bolster students who may be most likely to experience a negative classroom climate, i.e. students that are often negatively stereotyped in academic settings.

A series of studies suggest students who feel at risk of upholding stereotypes or being judged based on stereotypes (termed stereotype threat) experience lower academic performance (Steele, 1988; Steele and Aronson, 1995; Nguyen and Ryan, 2008). Grappling with the concerns raised by stereotype threat (consciously or unconsciously) can decrease performance by

decreasing working memory (Schmader and Johns, 2003) and can lead to hyper-vigilance (Forbes *et al.*, 2008), which may distract individuals from tasks. Stereotype threat can be especially debilitating to performance on difficult tasks (Neuville and Croizet, 2007; Beilock *et al.*, 2007, Beilock, 2008), such as high stakes examinations that require the entirety of a student's mental faculties. It is not surprising then, that stereotype threat has been shown to impact college level course performance (Miyake *et al.*, 2010; Harackiewicz *et al.*, 2014), as high stakes exams are often the primary contributor to course grades. In addition to short-term impacts on working memory, stereotype threat can have long-term impacts, such as students distancing themselves from a discipline they once identified with (Thoman *et al.*, 2013; Fogliati and Bussey, 2013). This disassociation, coupled with lower performance, could contribute to a student's decision to leave STEM.

Recent work has shown that a values-affirmation exercise can mitigate stereotype threat (Cohen *et al*., 2006; Cohen *et al*., 2009; Sherman *et al.*, 2013; Walton *et al.*, 2014). This simple exercise asks students to identify values that are important to them, and write about how they incorporate these values into their lives. The positive emotions elicited as students consider how their own lives exemplify their own values reduce cortisol levels (Creswell *et al.*, 2005) and are thought to buffer students against the negative emotions caused by stereotype threat (Steele and Aronson, 1995; Cohen *et al*., 2006). In one study among middle schoolers, administering the intervention not only increased the performance of African American students in the term it was given, but had sustained performance benefits two years later (Cohen *et al*., 2006; Cohen *et al*., 2009).

As the use of this intervention has spread beyond the initial researchers, however, the results have become more variable. For example, a more moderate impact was seen for a second group of African-American middle-school students (Borman *et al.,* 2016), with a follow-up replication study showing no effect of the intervention (Hanselman *et al.*, 2016). In an undergraduate physics course, the intervention increased the performance of women (Miyake *et al.*, 2010), but this effect was not replicable during a following semester (Kost-Smith *et al.,* 2012). Similarly, researchers studying the impact of values affirmation on first-generation college students in introductory biology observed an effect of the intervention in one semester (Harackiewicz *et al.*, 2014), but not another (Harackiewicz *et al.*, 2016). The variability of these results suggests that more studies should be conducted in more environmental contexts before we generalize the utility of the values affirmation intervention.

Despite the variable results, there are multiple reasons why this intervention continues to be appealing to instructors. First, the intervention requires little to no (in our study) in-class instructional time and very little student effort. Each application can be administered in-class (or in our case online) and takes 15 minutes at most. Therefore, if an instructor assigns it twice in a term, 30 minutes of student effort outside of class could produce large effects on the performance of historically underrepresented groups. Second, the intervention is easily implemented and scalable. Students primarily write short essays in response to two questions, but the responses are not read by instructors. As a result, the intervention is feasible even in large classes.

Given the potential benefits and the ease of administering the intervention, we contribute to the growing body of research on the values affirmation intervention in specific STEM contexts

by adding our novel context: one of the largest introductory biology classes in the nation (between 450 and 600 students per section) at an R1 institution in the Pacific Northwest with predominately white and Asian American students. To account for potential variability of results we deployed the intervention across three years and six sections of introductory biology. Specifically, we address the question: Can a values affirmation exercise increase the exam performance of students who identify as Black, Latina/o, Native American, Native Hawaiian, or Pacific Islander in introductory biology?

The setting of introductory biology is particularly important, because introductory classes often function as a student's first exposure to their future profession and thus can have a larger-than-average influence on their decision to persist in STEM—making these courses an appropriate target for intervention (Cech *et al.*, 2011). In addition, relative to other STEM fields, biological science majors have the largest number of URM students (National Science Foundation, 2015). Thus, positively impacting the experience of URM students in this setting may have the largest impact on the STEM pipeline. Furthermore, URM students make up on average 12% of the students in these classes (white = ~43%, Asian = ~ 38%, International = 7%, female = ~58%). Although the small number of URM students makes the statistical detection of differences difficult, this context, where URM students are a numerical minority, is where stereotype threat may have the greatest negative effect on their performance (Thompson *et al.*, 2002; Purdie-Vaughns *et al.*, 2008; Hanselman *et al.*, 2014). As a result, this group of students is a logical target for values affirmation. At an institutional level, we were also motivated to test the impact of values affirmation because we had already been successful in reducing the achievement gap for historically underrepresented groups in this course due to the introduction

of highly structured active learning techniques, and we hypothesized that the remaining gap could be further reduced or eliminated by addressing psychosocial barriers to academic success

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Materials and Methods

Course context and study population

The study was conducted in three consecutive fall terms of the first course of a three-quarter-long introductory biology series. In each term a similar number of students were enrolled in the course (~1100) and the class was taught as 2 back-to-back sections to accommodate enrollment numbers. All six sections (labeled A-F) in our study were taught by the same instructor, although in the final two fall terms (sections C, D, E and F) a postdoctoral student worked in partnership with the main instructor, teaching 25% of the class sessions. Each term incorporated a significant amount of student-centered, active learning techniques. These included the use of clickers, practice exams, nightly reading quizzes, and in-class group exercises. The students in these classes were predominately sophomores intending to declare a science major; the course is required for students who intend to major in the life sciences. Information on student gender, URM status, and cumulative college GPA prior to entering the class were obtained from the Office of the University Registrar.

The intervention

Although variable results are often obtained with the values affirmation intervention, there are

general suggestions in the literature for implementing the exercise in ways that would make it more likely to work (Bradley *et al.,* 2015). We implemented our intervention with these aspects in mind, considering the content, introduction, timing and repetition of the exercise.

To determine the effect of values affirmation on academic performance on 6 sections of introductory biology taught across 3 years, students in each section were randomly divided into control and treatment groups, with the instructor blind to the placement of students in each group. In the treatment group, students were given a list of 14 items they might consider valuable in their lives (e.g. independence, athletic ability, membership in a social group, etc.). After selecting the 2-3 values that were most important to them, they wrote a brief response explaining why those values were important, summarized their top two reasons for choosing those values in writing, and answered four Likert-scale questions on the relevance of the chosen values to their lives. In the control group, students selected values from the same list, but instead indicated values that were least important to them, and answered questions on why the values would be important to someone else (for complete exercise, see Supplemental Materials). Students in both the treatment and control groups wrote positively about the values they selected, yet only in the treatment group did students evaluate these values in connection to themselves (Cohen *et al.*, 2006).

The exercise was completed online and outside of class time, and was designed to take approximately 15 minutes. No instructor-mediated introductory or follow-up discussions were provided apart from an initial email from the instructor alerting students to the assignment. As recommended (Bradley *et al.*, 2015), the intervention was framed as a standard class writing

exercise, worth course points based on participation. Students completed the exercise during the first week of the quarter, as sustained benefits of values affirmation are thought to be dependent on early student success (Cohen *et al.*, 2009). Students were assigned the identical exercise again after receiving feedback from their second exam during the sixth week of the quarter, when we hypothesized that stress levels would be especially high for struggling students. The choice to implement the intervention twice in a term was based on prior research. Previous classroom studies that demonstrated positive effects of the values affirmation intervention administered twice (Miyake *et al.* 2010; Harackiewicz *et al.*, 2014). The original creator of this exercise also recommended this approach as standard practice (Geoff Cohen, personal communication, February 22, 2010). We therefore only considered a student to have fully completed the intervention only after completing both rounds of the exercise.

Study population

Students enrolled in these classes were only included in the study if they completed all four course exams (the outcome variable), had a measure of prior academic ability (cumulative college GPA), completed their assigned values affirmation exercise twice during the quarter, and consented, in writing, to the use of their data (University of Washington's Human Subjects Division, application #38240). A total of 2,383 students satisfied these requirements (Table 1). Of this sample, 17.8% were first-year students and thus were not included in our analyses because they did not have a measure of prior demonstrated college achievement. In addition, we did not include Asian-American students and International students in our analyses (38.7% and 5.9% of the overall sample, respectively). International students come from multiple cultural contexts, yet are collapsed into a single category. Given how little information we had about

their backgrounds, we did not feel we could make predictions about their experiences with stereotypes. Similarly, while Asian-American students are often considered overrepresented in STEM, the category comprises many groups with distinct ethnic backgrounds, some of which are underrepresented (Maramba, 2013). Disaggregated ethnic data for Asian-Americans were not available from our institution and thus we were unable to separate this group into underrepresented and well-represented subgroups. Furthermore, combining any Asian-American students within a "majority students" category is potentially problematic as: 1) Asian-Americans may face 'model minority' or other stereotypes in the classroom (Cheryan and Bodenhausen, 2000), and 2) prior work had established an achievement gap between Asian American and white students in these classes (Eddy *et al.*, 2014). Thus, our analysis specifically focused on the intervention's effects on URM students, who are historically the lowest-performing American students, and white students, who are historically the highest performing American students in these classes, for a total of 1,031 students across all three terms.

Statistical Analyses

Outcome variable

Students took four non-cumulative exams worth 100 points each. In each year and section these exams covered the same topics, although the individual questions differed. The exam questions in this course are open response, mostly short answer and, on average, exam items are at the level of application and analysis rather than comprehension and recall (Freeman et al., 2011). Across the years in our study, average exam performance was in the low 70s.

We chose to focus on total exam points rather than course grade because stereotype threat is predicted to be induced in moments of high stress (Neuville and Croizet, 2007; Beilock *et al.*,

2007, Beilock, 2008). Thus, 'high-stakes' exams have a greater potential for inducing stereotype threat relative to lower stakes course assignments. Thus, exam grades are more likely to be affected by the values affirmation exercise (Beilock, 2008). In addition, exams make up at minimum 55% of a student's final grade in these classes and in previous studies exam grades have been shown to explain most of the variation in student course performance ($R^2 = 0.89$ in one study; Freeman *et al*., 2011).

Covariates

Normal variability in exam performance among students has the potential to mask small to moderate impacts of a treatment. Yet effects that might be considered small by statisticians (for example, a three percent change in grade) may be educationally significant to students. To increase our chance of seeing even small effects of the values affirmation intervention, we included covariates in our analyses: course section, student gender, and cumulative college GPA at the start of the term and several interaction terms.

Section, a categorical variable with 6 levels (each being compared to section B, the reference level), was included to help account for differences in exams among the six sections and account for any among-year variation in the student population and course, or any section-specific experiences that could impact performance, such as exam difficulty across sections. In addition, previous values affirmation studies saw variation in the efficacy of intervention in

replication studies (Hanselman *et al.*, 2016; Kost-Smith *et al.,* 2012), so we included a three-way interaction term (Treatment x URM status x Section) to account for that potential variation.

Gender was included in the analysis because historically these classes have shown an achievement gap between males and females (Eddy *et al*., 2014). Although gender is not a binary, at the institution where this research occurred it is collected as a binary variable by the registrar, so in our analysis it is a categorical variable with two levels. In addition to a main effect of gender, the relationship of gender to exam performance may vary by URM status, so we also included a gender x URM interaction. Finally, gender has the potential to impact a student's experience of the treatment (cf. Miyake *et al.* 2010) so we included a gender x URM status x Treatment interaction to account for this potential variation.

Finally, cumulative college GPA at the beginning of the term a student was enrolled in introductory biology was included because it is highly predictive of academic performance in this course (Haak *et al.*, 2011, Freeman *et al.*, 2011; Eddy *et al.*, 2014). Although controlling for a measure of student ability is common in stereotype threat studies (Steel and Aronson, 1995; Miyake *et al.* 2010; Harackiewicz *et al.*, 2014), controlling for cumulative college GPA introduces challenges regarding both interpretation and implementation. From a practical standpoint, use of this control required us to remove students in these classes who did not have a measure of cumulative GPA, reducing our sample by 18%. In addition, some suggest that including any measure of student ability as a covariate may complicate the interpretation of the models (Wicherts, 2005; Yzerbyt *et al*., 2004). One concern is that the student ability covariate may have a different relationship to the outcome variable for students who are and are not under

stereotype threat; i.e. in our case, stereotype threat may have impacted the cumulative GPA of URM and not white students (Wicherts, 2005). Thus, combining these two groups in one model could make interpretation challenging. To address this concern, we added an interaction between cumulative college GPA and URM status, which would reveal whether the relationship between cumulative college GPA varies by URM status. A second concern is that the measure of student ability may be correlated with URM status and thus could confound the results (i.e., one can't know if it's the effect of the treatment on lower cumulative GPA or URM status that drives the interaction; Yzervbyt *et al*. 2004). To control for this concern, we include the interaction of cumulative GPA and treatment.

It should be noted that controlling for these variables has specific implications when interpreting the effects of the values affirmation intervention. For example, any change in performance of URM students in the treatment relative to white students is the average change for a URM student in the same section, of the same gender, and with the same entering GPA as the white student.

Model Selection and Regressions

We employ linear regression models to assess the relationship between the treatment students receive and their exam performance. We chose regressions because they allow us to include covariates to account for other influences on exam performance in addition to the treatment (Theobald and Freeman, 2014).

The initial hypothetical regression model was:

Total Exam Points ~ cumulative college GPA + URM status + Treatment + Section + Gender + URM status x Treatment + Section x Treatment + URM status x Section + cumulative college GPA x Treatment + URM status x cumulative college GPA + URM status x Gender + Gender x Treatment + Gender x Treatment x URM + URM status x Treatment x Section

We used stepwise backward model selection to subtract individual terms from the model until we had a reduced model that was best supported. We used AIC values to determine the best-supported model, which estimate the quality of a given model relative to the other models. Specifically, AIC assesses the goodness of fit of a model to the data while simultaneously including a penalty for each additional term included in the model. The preferred model is the one with the lowest AICc value. If two models have an equivalent AIC value (Δ AIC ≤ 2), then the model with the fewest terms is chosen. Any terms included in the preferred model are considered important for explaining the data even if they do not pass the $p = 0.05$ threshold (Burnham 2002).

Once the preferred model was identified we ran a post-hoc test on the URM status x treatment term to determine if the achievement gap between URM and white students in the treatment remained significant.

Finally, we ran a second set of analyses without cumulative GPA as a covariate using the full data set. We did not expect the analysis without cumulative GPA to be significant given the large variation present in exam scores, but if the results were qualitatively similar to the analyses with GPA it would lend support to the claim that challenges of including cumulative GPA did not meaningfully impair our results.

This results in a second hypothetical regression model:

Total Exam Points ~ URM status + Treatment + Section + URM status x Treatment + Section x Treatment + URM status x Section + URM status x Treatment x Section + URM status x Gender + Gender x Treatment + Gender x Treatment x URM

We followed the same stepwise backwards model selection methods to find the best-supported model.

Analyses were implemented in R (R Core Team, 2016). Model selection via stepwise model selection with AIC was carried out through the package stat (R Core Team, 2016). Post-hoc analyses were implemented in R using the package phia (de Rosario-Martinez, 2015).

Results

Analyses with cumulative GPA as a covariate

In our analysis with cumulative college GPA as a covariate, model selection (Table 2) indicated the preferred model to explain exam performance was:

Cumulative exam performance = $\beta_0 + \beta_1$(Cumulative college GPA) + β_2(URM) + β_3(Treatment) + β_4(Section) + β_5(URM x Treatment) + β_6(Gender) + β_7(Gender x Treatment).

These model selection results suggest several things about our data. First, we did not see evidence that the relationship of cumulative GPA to total exam points varies by URM status (-URM status x cumulative GPA; Table 2) which was one of our primary concerns about including cumulative college GPA as a covariate. Second, the cumulative GPA x treatment interaction was

not included in the final model (-Treatment x cumulative college GPA, Table 2), implying the impact of treatment was not driven by students with lower cumulative GPAs regardless of URM status. Third, we did not see evidence that the impact of the intervention varied largely from section to section (-URM status x Treatment x Section; Table 2), as other research teams have observed upon replicating studies at their own institutions (Kost-Smith *et al.,* 2012; Harackiewicz *et al.*, 2016). Finally, we did not see that the relationship between gender and treatment varied by race (Gender x Treatment x URM; Table 2), indicating that the impacts of gender on how students experienced the treatment were consistent for URM and white students.

This final model also suggests that controlling for cumulative college GPA and section increases the fit of the model. Finally, the final model suggests that the values affirmation intervention impacted the achievement gap between URM and white students, and that student gender mediated this effect. The R^2 of the final preferred model was 0.445.

In the control condition (and conditioned on the final model's covariates), white males were predicted to perform the highest. White women performed only marginally and not significantly lower (0.6% fewer exam points than white males; β_{Gender}= -2.5 ± 2.76, p = 0.363). URM males earned 4% fewer of the possible exam points (β_{Race} = -16.01 ± 3.58, $p < 0.00001$, Table 3) than white males. URM women performed only marginally lower than URM males (earning 4.6% fewer of the possible exam points than white males).

In the treatment group, we observed a reduced achievement gap between white and URM students in the same section and with the same entering cumulative GPA (see Figure 1).

Although white males still received a boost from the treatment ($\beta_{treatment} = 6.45 \pm 3.29$, $p = 0.05$), URM students received a disproportionate boost ($\beta_{treatment \times Race} = 10.29 \pm 4.75$, $p = 0.031$, Table 3). Specifically, male URM students in the treatment earned an additional 4.2% of the possible exam points relative to male URM students in the control, while white male students in the treatment earned only an additional 1.6% of exam points. URM women in the treatment received a more moderate boost than male URM students, as there was a significant interaction between gender and treatment (2.2%; $\beta_{treatment \times Gender} = -8.10 \pm 3.90$, $p = 0.038$, Table 3). This was still a larger boost than white males were predicted to experience and thus slightly reduced the achievement gap between these two groups. There was roughly no difference in performance of white women in the control and treatment groups.

Post-hoc analysis of the interaction demonstrated that there was still a small but significant achievement gap between white and URM students with equivalent prior GPAs in the values affirmation treatment (mean difference = 10.3, F (1, 1026) = 4.7 $p = 0.03$). Thus, averaging across all three terms, the values affirmation reduced but did not eliminate the exam achievement gap between white and URM students with the same college GPA in these introductory biology classrooms.

Analysis without cumulative college GPA as a covariate

In our analysis without cumulative college GPA as a covariate, model selection (Table 4) indicated the best model to explain exam performance was:

Cumulative exam performance = $\beta_0 + \beta_1(URM) + \beta_2(Treatment) + \beta_3(Section) + \beta_4(Gender) +$

β_5(URM x Treatment).

The preferred model for the analysis without cumulative college GPA is qualitatively similar in many ways to the model with GPA. Including section as a control still increases the fit of the model, as does including a URM x Treatment interaction implying that the treatment impacts the performance of URM and white students differently. The major differences are that the gender x treatment interaction does not increase the fit of the model to the data and that the total variance in exam scores explained by the model is much lower ($R^2 = 0.174$).

As we expected, without a control for student ability both the treatment ($\beta = 3.7 \pm 2.370$, $p = 0.117$) and treatment x URM status ($\beta = 8.3 \pm 5.38$, $p = 0.121$) were not significant. However, the treatment x URM status regression coefficient is qualitatively similar to the regression coefficient in the model with cumulative college GPA. This again implies that including this control did not substantially change the pattern of our results; it just accounted for more variance and allowed us to discern more patterns specifically driven by the values affirmation treatment. In addition, the fact that model selection procedure retained treatment and treatment x URM status in the preferred model implies that they remain important for explaining the outcome variable even in this model without cumulative GPA.

Discussion

The use of a values affirmation intervention led to a reduction, but not elimination, of the achievement gap between URM and white students with equivalent college GPAs in three terms of introductory biology. URM males saw the strongest effect of the

treatment, URM women the second strongest, and white men the third strongest. White females were not affected by the intervention.

Previous work has shown that the magnitude of achievement gaps can change depending on course structure and classroom climate. Different instructors and/or instructional strategies can impact achievement gaps between white students and historically underrepresented groups in college STEM courses (Kreutzer and Boudreaux, 2012; Eddy and Hogan, 2014). Prior to our study, several active learning strategies were introduced into the introductory biology courses at this university in an attempt to decrease failure rates (Freeman *et al.*, 2011). While this was accomplished successfully, it had the added benefit of decreasing the achievement gap between educationally and socioeconomically disadvantaged and non-disadvantaged students (Haak *et al.*, 2011). In the current study, we incorporated values affirmation in this same intensely active learning environment with the hope of reducing achievement gaps still further. Based on our belief that gaps in classroom achievement derived from inequitable instructional practices were already mostly reduced due to the intense use of active learning, we hypothesized that the remaining achievement gap between white and URM students was largely due to psychosocial threats in the classroom. Specifically, we focused on the potential emotional or psychosocial threat of being stereotyped. We found that values affirmation benefited URM students and white males, yet disproportionately increased the exam performance of URM students, resulting in yet another reduction of the URM-white achievement gap. Taken together, the result reported earlier in Haak et al. (2011) and the current study suggest that one possible recipe for minimizing achievement gaps between URM and white students in undergraduate biology courses may be 1) presenting content in a way that benefits everyone, but also

disproportionately benefits underrepresented groups (such as the use of active learning), and 2) employing values affirmation or other techniques to bolster students against a negative classroom climate.

The magnitude of the effect of the values affirmation exercise for the average male URM student was a 4.2% increased in exam performance. This is almost half of a standard deviation in raw exam points earned by students in these classes. Female URM students saw more moderate gains from the intervention: a 2.2% increase in exam scores relative to female URM students in the control condition. Learning is a complex task, and thus any intervention intended to impact student performance tends to have moderate to small effect sizes. For example, across over 200 studies of undergraduate STEM courses, changing from a traditional lecture to an active learning classroom increased exam scores on average by 6% for all students, or about half a standard deviation (Freeman *et al*. 2014). Our intervention, which roughly required only 30 minutes of student effort and very little instructor input over the course of an academic term, increased the performance of male URM students half to nearly as much as converting an entire course to active learning. The ease of distributing and completing the exercise makes this intervention a promising tool in addressing the URM achievement gap in undergraduate STEM classrooms.

Prior work with values affirmation in STEM settings other than biology indicates that it can reduce the achievement gap between men and women by raising female achievement. However, in our study we found the opposite for white students: the intervention increased the achievement of white men but not white women. Stereotype threat can stem from many different sources (Shapiro, 2011) and be experienced by any group that feels there are comparatively

negative traits associated with their group. For example, researchers induced stereotype threat in white male undergraduates completing a difficult math test by telling them that their performance would be compared with Asian students (Aronson *et al.* 1999). Because our classrooms are on average 58% female and 38% Asian, it is possible that white males in biology experience stereotype threat in relation to one or both groups. However, evidence from prior studies in this setting does not support this hypothesis as white males do not behave or perform as predicted for groups under stereotype threat. For example, male students in these classrooms participate at higher rates in class and report greater comfort with this participation than females; white males, in particular, out perform all other groups on in-class exams; and peers in the class perceive males to be more knowledgeable about biology than females (Eddy *et al*. 2014; Eddy *et al*. 2015; Grunspan *et al*. 2016). These results suggest that stereotype threat is an unlikely explanation for our observed results. Instead, it may be that the values affirmation intervention, which does not specifically reference stereotype threat in any way in the writing prompt, has some additional value for students beyond stereotype threat reduction. White males may be benefiting from this alternative value. Regardless, URM students in our study disproportionately benefit from values affirmation, leading to a narrowing of the achievement gap between URM and white students.

The only other study to test the effects of values affirmation in a college biology classroom saw an impact on first-generation college students (i.e. students with neither parent having earned a four-year college degree). However, our University Registrar did not collect this demographic information until the final year of our study, and thus we did not explore the effects of our exercise on this group. Future studies exploring this dynamic would be informative.

Limitations of interpretation

Although values affirmation interventions are associated with stereotype threat reduction, the target of the intervention is not specifically stereotype threat. Thus, one could argue the impact of the values affirmation on exam scores was due to the alleviation of some other psychological process impacting student performance. We are unable to rule out this possibility because we did not measure the degree to which individuals either (a) felt they experienced stereotype threat or (b) endorsed views aligned with common academic stereotypes. Furthermore, these measurements might have allowed us to better understand why certain students and not others were impacted by the intervention. However, obtaining a measure of stereotype threat is not common practice in classroom studies that have seen an impact of values affirmation (Cohen *et al*. 2006; Cohen *et al*. 2009; Harackiewicz *et al.*, 2014; but cf. Miyake *et al.*, 2010), and we were therefore wary of introducing such a component into our experimental design. Above all, we wanted to avoid signaling to students that they were taking part in a study designed to reduce stereotype threat, as being aware of the intention of values affirmation has been shown to reduce its effectiveness (Sherman *et al.* 2009).

Although we show an impact of values affirmation on URM student achievement in biology and for white males, these results were obtained in one particular context. Prior studies suggest the effects of values affirmation interventions are sensitive to the environment in which they are used (Hanselman *et al.*, 2014; Cohen and Garcia, 2014; Kost-Smith *et al.*, 2011) or may be affected by the size of the achievement gap they attempt to address (Hanselman *et al.*, 2014). The immediate social climate a student experiences can vary widely across institutions,

classrooms, and years, and interventions that help students cope in these climates may be variably useful. Thus, this study is more a demonstration of potential benefit than a guarantee that biology instructors will see similar impacts of values affirmation in their classrooms.

In addition, college achievement is greatly impacted by past academic preparation, which is highly variable amongst college students. We show that the gap between URM and white students is reduced only after controlling for this variation, and that failing to do so swamps out any signal regarding the psychological benefit of values affirmation. The intervention cannot change a student's preparation, but it can support an environment where students' performance aligns more with their abilities, and ensures that they are less likely to underperform at a disproportionate rate to their white peers with equal incoming GPAs.

Conclusion

A URM student's decision to remain in STEM is impacted by their achievement and by their sense of belonging in the discipline (Chemers *et al.*, 2011; Hausmann *et al.*, 2007). By diminishing psychological threats in an active learning classroom, we may be able to reduce barriers to achievement and empower a student's sense of self-value to encourage retention in STEM. Our study suggests that at least in some cases, these benefits can be achieved with minimal effort by students and instructors through the use of a short, evidence-based psychosocial intervention.

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Figures and Tables

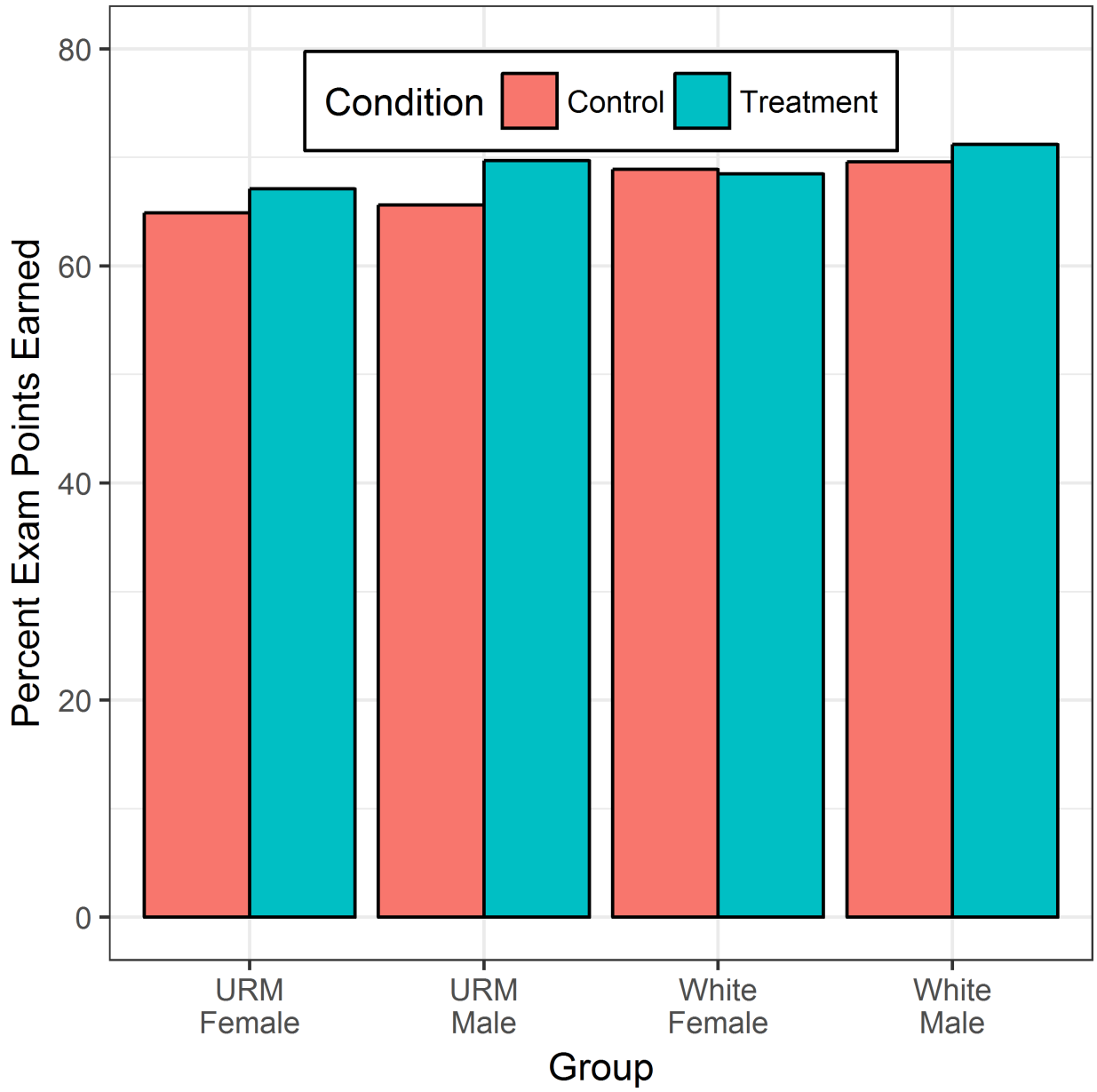

Figure 1

	Full Sample	Sample with a measure of prior demonstrated college ability	Sample with no measure of prior demonstrated college ability
N:	2,383	1,959	424
Gender:			
Female	1,463	1,227	236
Male	920	732	188
Ethnicity/Race/Nationality:			
Asian	922	776	146
Black	53	43	10
Hawaiian and Pacific Islander	31	29	2
Hispanic	144	122	22
International	141	88	53
White	985	837	148
Not Reported	86	48	38
Median exam points earned: (interquartile range)	277 (246 – 304)	278.5 (248 – 305)	270.5 (238 - 299)
Treatment Groups:			
Control Group	1,174	970	200
Treatment Group	1,170	963	211

Table 1

	Analyses with cumulative college GPA				
Initial Model and Terms dropped *Outcome*: Total Exam Points Earned	**Df**	**Deviance**	**Residual Df**	**Residual Deviance**	**AIC**
Initial model: cumulative college GPA + URM status + Treatment + Section + Gender + URM status x cumulative college GPA + URM status x Section + URM status x Treatment + URM status x Gender + Treatment x cumulative college GPA + Treatment x Section + Gender x Treatment + Section x Treatment x URM status + Gender x Treatment x URM status			1006	915723.0	7104.2
- Section x Treatment x URM status	5	3757.7	1011	919480.7	7098.4
- URM status x Section	5	734.9	1016	920215.6	7089.2
- Treatment x Section	5	3085.31	1021	923300.9	7082.7
- Treatment x URM status x Gender	1	650.79	1022	923951.7	7081.4
- URM status x Gender	1	356.0	1023	924307.7	7079.8
- URM Status x cumulative college GPA x Treatment	1	1136.5	1024	925444.2	7079.1
- URM status x cumulative college GPA	1	668.0	1025	926112.2	7077.9
- Treatment x cumulative college GPA	1	656.6	1026	926768.8	7076.6
Final Model: cumulative college GPA + URM status + Treatment + Section + Gender + URM status x Treatment + Gender x Treatment					

Table 2

	Preferred model for students with cumulative college GPA covariate	Preferred model without cumulative college GPA covariate
Coefficients:	β ± se (p-value)	β ± se (p-value)
Intercept:	278.3 ± 3.48 (< 0.001)	275.6 ± 3.51 (< 0.001)
Cumulative college GPA at start of course:	52.81 ± 2.42 (< 0.001)	NA
Racial Group: (*ref: White*) URM	 -16.01 ± 3.58 (< 0.001)	 -25.6 ± 4.07 (<0.001)
Gender: (*ref: Male*) Female	 -2.52 ± 2.76 (0.363)	 -5.4 ± 2.19 (0.0133)
Treatment Group: (*ref: Control*) Treatment	 6.45 ± 3.29 (0.0501)	 3.7 ± 2.37 (0.123)
Gender x Treatment Group: (*ref: Male x Control*) Female x Treatment	 -8.10 ± 3.90 (0.038)	 NA
Race x Treatment Group: (*ref: White x Control*) URM x Treatment	 10.29 ± 4.75 (0.031)	 8.2 ± 5.37 (0.126)
Section: (*ref: Section B*) Section A Section C Section D Section E Section F	 30.8 ± 3.60 (< 0.001) -1.7 ± 3.62 (0.639) -9.4 ± 3.83 (0.014) -0.1 ± 3.52 (0.989) -8.5 ± 3.58 (0.017)	 37.8 ± 3.98 (< 0.001) 6.3 ± 4.00 (0.116) -4.5 ± 4.09 (0.273) 6.9 ± 3.79 (0.071) -1.8 ± 3.87 (0.633)
R^2	0.445	0.174

Table 3

	Analyses without cumulative college GPA				
Initial Model and Terms Dropped *Outcome*: Total Exam Points Earned	**Df**	**Deviance**	**Residual Df**	**Residual Deviance**	**AIC**
Initial model: URM status + Treatment + Section + Gender + URM status x Section + Treatment x Section + URM status x Treatment + Gender x Treatment + URM status x Gender + Section x Treatment x URM status + Gender x Treatment x URM status			1192	1623611	8832.1
- Section x Treatment x URM status	5	10616.7	1197	1634228	8830.1
- Treatment x Section	5	1469.8	1202	1635698	8821.2
- URM status x Section	5	5558.5	1207	1641256	8815.3
- Treatment x URM status x Gender	1	751.4	1208	1642008	8813.9
- URM status x Gender	1	1389.2	1209	1643397	8813.0
- Treatment x Gender	1	2386.7	1210	1645784	8812.7
Preferred Model: URM status + Treatment + Section + Gender + URM status x Treatment					

Table 4

Figure and Table Legends

Figure 1. Predicted student exam scores for different student groups assuming all students had the average cumulative college GPA and was in the reference section. Based on preferred model including cumulative GPA.

Table 1. Sample demographics. Table included the sample of students across the six sections who took all four exams, completed both dosages of the intervention and (a) has a measure of prior academic ability or (b) does not have a measure of prior academic ability.

Table 2. Model selection table for the analyses with cumulative college GPA identifying the preferred model. For each comparison, the term subtracted from the model is listed in the first column. As this is a cumulative table, any terms above the current row have already been removed from the model before the current row is tested. Terms are removed if the AIC of the reduced model is two or less than the AIC value of the fuller model or if the models have equivalent AIC values (Δ AIC is < 2). If removing the term increases the AIC by more than two, the term is retained in the model.

Table 3. Regression coefficients for preferred models for analysis (a) with cumulative college GPA as a covariate and (b) without cumulative college GPA as a covariate. The outcome variable is exam performance. For categorical variables the reference level is in parentheses, indicating the binary comparison that was made (for example, section B compared to section A and section B compared to section C).

Table 4. Model selection table for the analyses without cumulative college GPA identifying the preferred model. For each comparison, the term subtracted from the model is listed in the first column. As this is a cumulative table, any terms above the current row have already been removed from the model before the current row is tested. Terms are removed if the AIC of the reduced model is two or less than the AIC value of the fuller model or if the models have equivalent AIC values (Δ AIC is < 2). If removing the term increases the AIC by more than two, the term is retained in the model.

Chapter 2.

Coevolution of host-plasmid pairs facilitates the emergence of novel multi-drug resistance

Hannah Jordt[1], Thibault Stalder[2], Olivia Kosterlitz[1], Eva Top[2], Ben Kerr[1]

[1] Biology Department, University of Washington

[2] Department of Biological Sciences, University of Idaho

Multidrug resistance (MDR) is of growing public health concern, leading to more than 150,000 deaths each year in the United States alone[1]. The rise of MDR pathogens is in part attributed to the horizontal gene transfer of antibiotic resistance genes via conjugative plasmids[2]. Factors that stabilize antibiotic resistance plasmids in bacterial communities indirectly contribute to a higher incidence of MDR, given the increased likelihood that cells acquire several of these plasmids through conjugation. One such stabilizing factor is host-plasmid coevolution in environments selecting for the plasmid (e.g., antibiotic exposure). Coevolution between hosts and their plasmids can lead to improved plasmid persistence after selection for the plasmid is removed. However, the effect of this phenomenon on the emergence of MDR in a bacterial community of hosts and antibiotic resistance plasmids has not been previously explored. Here we show that host-plasmid coevolution under antibiotic selection can facilitate the emergence of novel MDR in *Enterobacteriaceae* communities consisting of *Klebsiella pneumoniae* and *Escherichia coli* once antibiotics are removed. The increased incidence of MDR is driven by evolution promoting (*i*) more stable sources of

antibiotic resistance plasmids in the community with which to generate MDR cells, and (*ii*) the slower decay of MDR cells once they have arisen.

Plasmids are a common repository of genes encoding antibiotic resistance, and conjugative plasmids can facilitate the spread of such genes via horizontal transfer. The acquisition of a new plasmid can be costly for its host due to the burden imposed by plasmid-related processes such as replication, conjugation and gene expression, or the "interference" associated with interactions between plasmid-encoded proteins and cell housekeeping functions[3–5]. In the presence of an antibiotic, these costs can be outweighed by the benefit of plasmid-encoded resistance. In their absence, however, the costs are predicted to give a competitive advantage to plasmid-free cells. If plasmid loss occurs due to improper segregation of the plasmid during cell division (i.e. segregational loss), such costs could lead to a selective decrease in the fraction of plasmid-bearing cells in the population. In this light, the observation that plasmids encoding antibiotic resistance often persist in the absence of drugs is puzzling[6].

Solutions to this "plasmid paradox"[7] involve processes that counterbalance, disrupt or diminish selection against the plasmid. For instance, high rates of plasmid conjugation can transform plasmid-free cells into plasmid-containing cells, thereby counterbalancing selection for segregants and contributing to plasmid persistence[8]. Low rates of segregational loss or the incorporation of a post-segregational killing mechanism that inhibits growth of plasmid-free cells can disrupt selection against the plasmid; these mechanisms thus contribute to plasmid persistence by ensuring a dearth of (fitter) plasmid-free competitors[9]. Finally, compensatory mutations occurring in the host chromosome or the plasmid can alleviate plasmid costs, which diminishes the strength of

selection against the plasmid and yields improved persistence. This last category may be a particularly important one. Several experiments have shown that compensatory mutations occurring during the coevolution of a host and its plasmid can result in increased plasmid persistence, even after selection for the plasmid is removed[10–13]. Thus, evolution under conditions favoring plasmid-encoded traits can lead to greater persistence of the plasmid in the absence of such conditions.

Compensatory mutations that relieve the fitness cost of plasmids encoding antibiotic resistance allow the trait to persist in the absence of drugs, with the plasmids serving as a stable *source* that can spread resistance to new strains and species within the microbial community. If recipient hosts are already resistant to a different antibiotic, novel MDR results. Thus, in communities of bacteria where prior evolution has led to greater persistence of conjugative plasmids that encode resistance to distinct antibiotics in different hosts, increased incidence of single hosts with multiple plasmids could engender MDR. Given that widespread agricultural, hygienic, and clinical use of antibiotics exerts selection for antibiotic resistance, and that many pathogens, including *K. pneumoniae*[14], frequently contain multiple plasmids, a more formal analysis of these ideas is warranted.

To explore how plasmid dynamics affect the emergence of MDR in bacterial communities, we consider a system involving two bacterial species and two conjugative plasmids, each encoding resistance to a different antibiotic. For any host-plasmid pair, we predict that a plasmid-associated cost will affect the rate of plasmid loss from a population of hosts propagated in the absence of antibiotics. If the magnitude of the cost is high, *de novo* segregants will displace plasmid-bearing cells quickly, producing a steep plasmid decay curve (Figure1b). Yet evolution in the presence of

the relevant antibiotic (Figure 1a) could lead to a reduction in the cost which would then result in greater plasmid persistence (Figure 1c). By incorporating a second host-plasmid pair, we can turn to the emergence of MDR under drug-free conditions where the two bacterial species, each possessing a distinct plasmid, are mixed. We predict that if plasmid costs are high, the incidence of multi-drug resistance will be low (Figure 2b). However, if each bacterial species first coevolved with a distinct plasmid separately before coming together as a community (Figure 2a), the incidence of multi-drug resistance would be high (Figure 2c). Ultimately, we assume that as the two plasmid-bearing populations become more stable, the opportunities for conjugation between host types (i.e. the generation of novel MDR) would increase once the populations were mixed. A more rigorous treatment of these topics, via a mathematical model, supported these predictions (see Supplemental Materials I; Figures S1, S2).

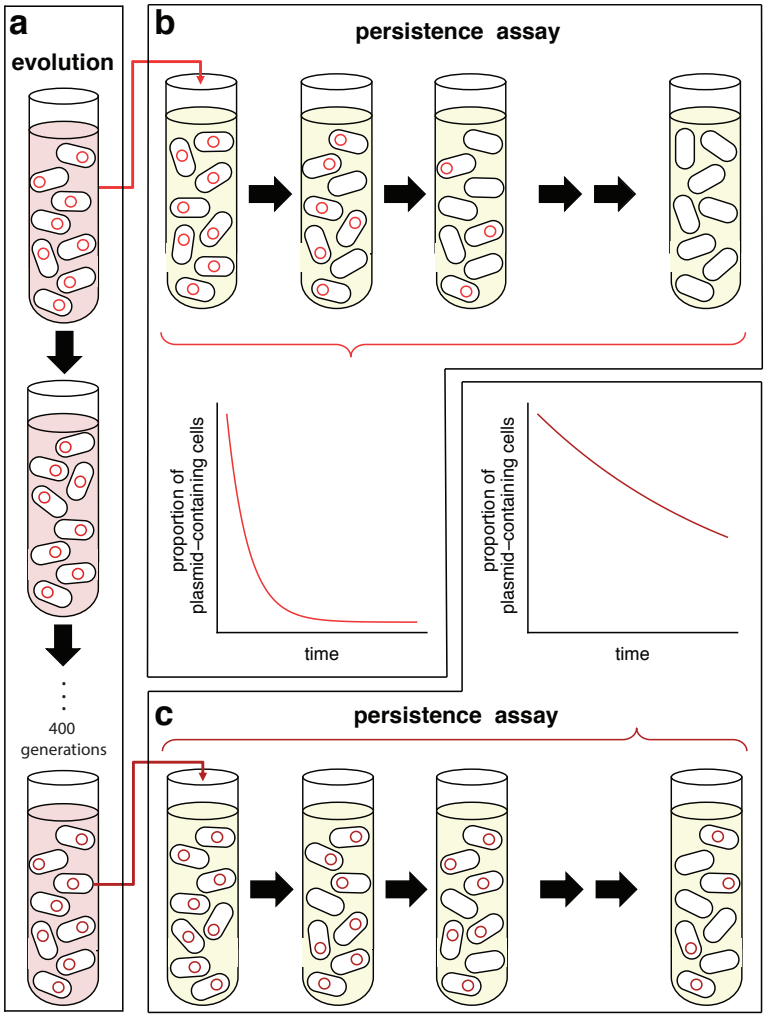

Figure 1. **Predictions on the effects of host-plasmid coevolution on plasmid persistence**. (a) A hypothetical population of bacteria evolving for many generations (represented as a culture propagated via serial batch transfer) in the presence of an antibiotic (red-shaded medium) selecting for maintenance of a plasmid (light red circle) encoding resistance. Evolutionary changes are represented by darker shades at the end of the sequence. (b) An isolate from the ancestral population is grown and propagated without antibiotics for a short time (yellow-shaded medium) and the proportion of the population with the plasmid is tracked. We predict the plasmid is lost rapidly when it is costly. (c) An isolate from the *evolved* population is grown and propagated without antibiotics. If evolutionary changes include mutations that compensate for the cost of plasmid carriage, we predict the loss of the plasmid is slower.

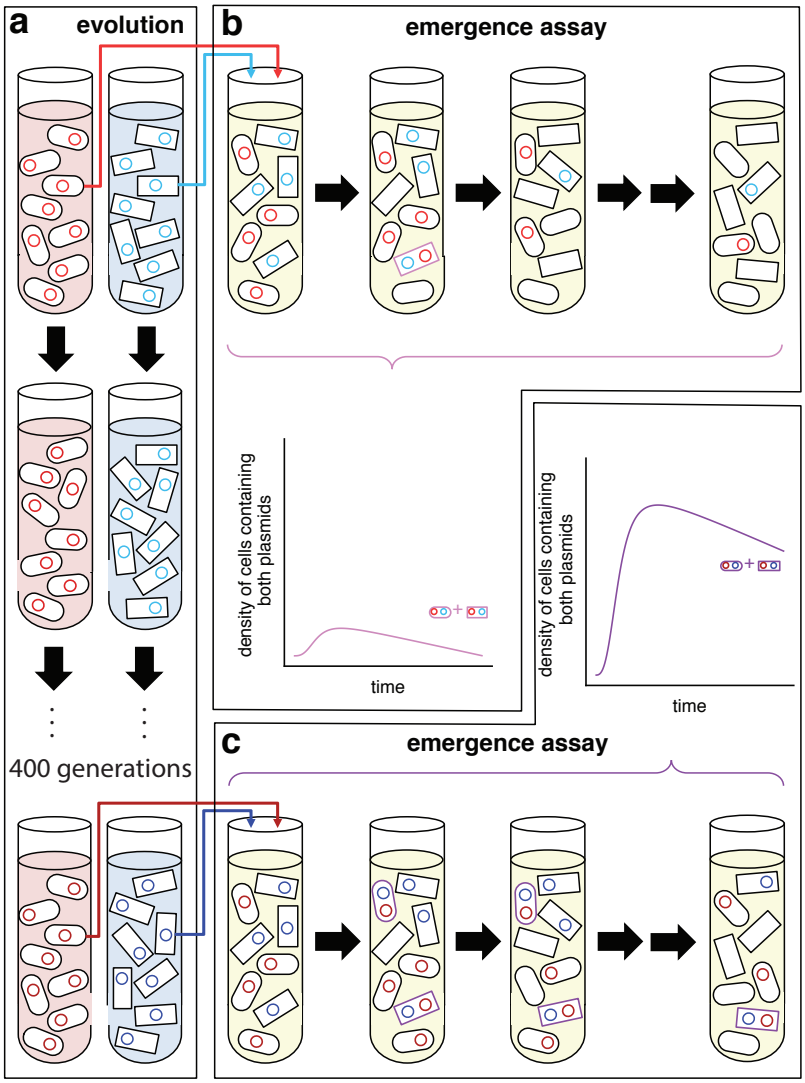

Figure 2. **Predictions on the effects of host-plasmid coevolution on MDR emergence**. (a) Two different species of bacteria (with cells represented as rods and rectangles), each with their own plasmid, evolve independently for many generations in the presence of an antibiotic that selects for maintenance of the plasmid (red-shaded medium selects light red plasmid and blue-shaded media selects for light blue plasmid). Evolution

is indicated by darker shades at the end of the sequence. (b) Isolates from each ancestral population are mixed in media without antibiotics and tracked for a short time, in which case we predict low numbers of MDR cells (containing both plasmids) arising. (c) However, if isolates from the *evolved* populations are mixed in antibiotic-free media, we predict the incidence of MDR cells is higher.

To empirically test our predictions, we introduced conjugative plasmid pALTS28 (hereafter "p1") encoding tetracycline resistance into an *Escherichia coli* host (hereafter "E") and conjugative plasmid pALTS29 (hereafter "p2") encoding chloramphenicol resistance into a *Klebsiella pneumoniae* host (hereafter "K"; for details on strains and plasmids, see Methods). We denote these strains as E(p1) and K(p2), where the host species is listed first and the plasmid inside the host is given in parentheses. Plasmid-free cells are indicated by E(Ø) and K(Ø) and cells that contain both plasmids are denoted E(p1,p2) and K(p1,p2). As shown in Figure 3a, each plasmid-bearing host was propagated in the presence of the relevant antibiotic for approximately 400 generations. To signify that evolution has taken place, and to convey information about the context of evolution, we add subscripts to both the host and the plasmid. Thus, $E_1(p1_E)$ is an *E. coli* cell that evolved with plasmid p1 currently possessing plasmid p1 that evolved in the same *E. coli* host. The reason for the complex notation is that mutations may occur in either the host chromosome or the plasmid, and because we need to specify the evolutionary histories of both hosts and both plasmids as well as new plasmid-host arrangements created by conjugation. For instance, $K_2(p1_E,p2_K)$ is a *K. pneumoniae* cell evolved with plasmid p2, which currently contains both plasmid p2 that evolved with this host and plasmid p1 that evolved in an *E. coli* host.

As predicted, both plasmids were unstable in the absence of antibiotics prior to coevolving with their hosts: p1 was rapidly lost from *E. coli* (Figure 3a) and p2 from *K. pneumoniae* (Figure 3b).

That is, separate populations of E(p1) and K(p2) were rapidly overtaken by E(Ø) and K(Ø) cells, respectively. However, after evolution, plasmids p1 and p2 each persisted to a greater degree in their respective co-evolved hosts across all replicates (Figures 3c and 3d). Quantitative support for these differences was obtained using a maximum likelihood model approach via a plasmid population dynamic model previously described[15–17] (see Methods; Supplemental Materials IV). This entire protocol was also used for the other plasmid-host combinations, E(p2) and K(p1), and the results were similar for 11 of the 12 replicates (see Supplemental Materials IV).

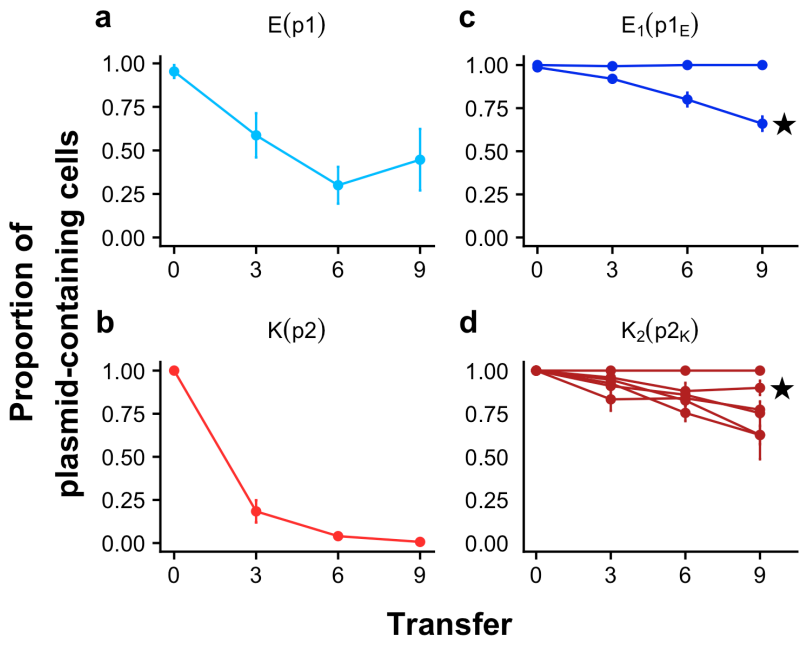

Figure 3. **Plasmid persistence in the absence of antibiotics increases after coevolution of plasmids with their hosts.** Lighter colors (left graphs) indicate ancestral strains. Darker colors (right graphs) indicate evolved strains. (a) Plasmid p1 was lost rapidly in an ancestral E(p1) population. However, coevolution of this host and plasmid in two replicate populations (in the presence of the relevant antibiotic) led to (b) greater plasmid persistence in both resulting $E_1(p1_E)$ populations (in the absence of the antibiotic). (c) Plasmid p2 was similarly lost quickly from an ancestral K(p2) population. Host-plasmid coevolution in six replicate populations resulted

in (d) greater plasmid persistence in all $K_2(p2_K)$ populations. In these graphs, every point is the mean of the three replicate persistence assays conducted for each strain, and bars indicate the standard error. The two "★" symbols denotes the persistence profiles for the evolved strains selected for the remainder of this paper.

With these evolved populations in hand, we then asked how plasmid-host coevolution would affect the emergence of novel multi-drug resistance in mixed-species culture. As diagrammed in Figure 2b, we co-cultured three replicates of our ancestral species (E(p1) with K(p2)) or the evolved species ($E_1(p1_E)$ with $K_2(p2_K)$) in serial batch culture in the absence of antibiotics. We found higher incidence of cells containing both plasmids in the evolved assemblage; a difference of approximately three orders of magnitude (Figure 4). This entire protocol was carried out three times: (i) comparing the E(p2)-K(p1) co-culture to the $E_2(p2_E)$-$K_1(p1_K)$ co-culture, (ii) comparing the E(p1)-E(p2) and $E_1(p1_E)$-$E_2(p2_E)$ co-cultures, and (iii) comparing the K(p1)-K(p2) and $K_1(p1_E)$-$K_2(p2_E)$ co-cultures (see Supp. Materials V). While multi-drug resistant *E. coli* arose in some of the co-cultures, the majority of all MDR cells were *K. pneumoniae*. Regardless, in each comparison, greater MDR emerged in the co-cultures that consisted of evolved pairs, supporting our hypothesis.

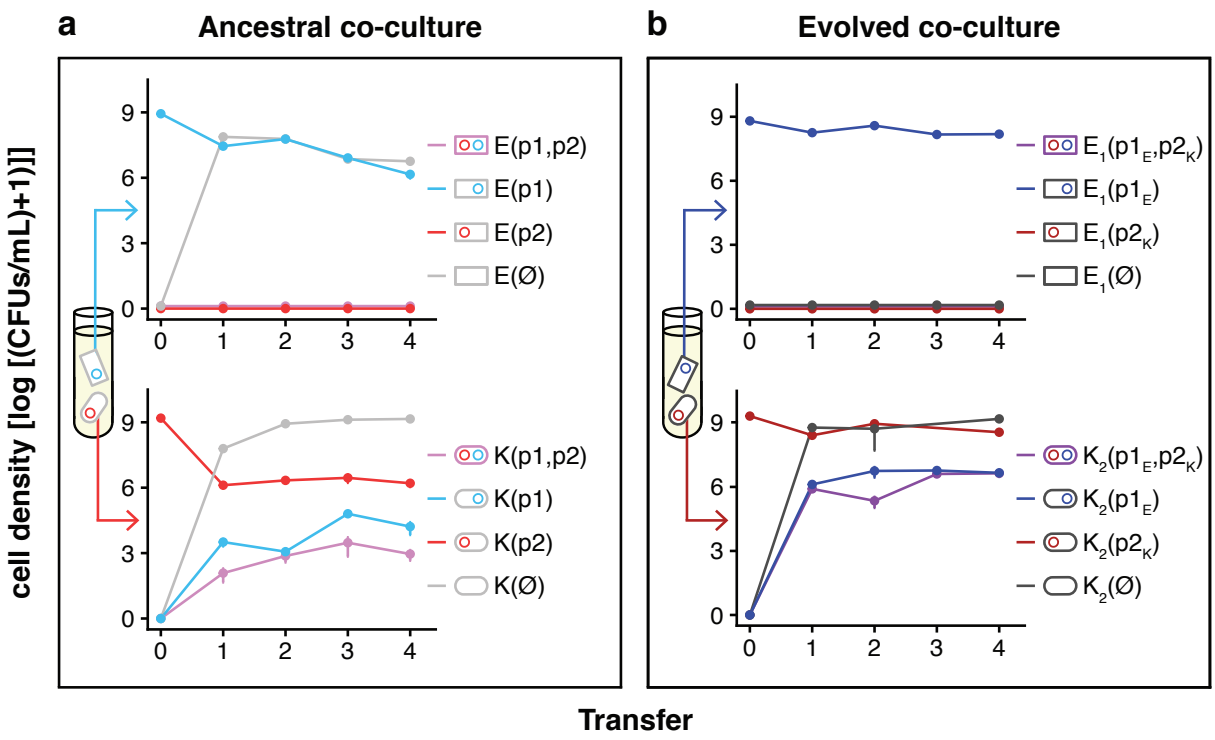

Figure 4. The emergence of MDR in mixed species co-cultures increases after coevolution of plasmids with their hosts. (a) Pairs of ancestral strains (E(p1) with K(p2)) and (b) pairs of evolved strains ($E_1(p1_E)$ with $K_2(p2_K)$) were co-cultured separately across 3 replicates over 4 transfers in the absence of antibiotics. Within the same ancestral strain coculture (a), *E. coli* (upper) and *K. pneumoniae* (lower) were tracked via daily selective plating for each host-plasmid combination. The pair of evolved strains within their own coculture were tracked similarly (b). In all plots, blue shades indicate hosts containing p1 or $p1_E$; red shades indicate hosts containing p2 or $p2_K$; purple shades indicate MDR hosts containing (p1, p2) or ($p1_E$, $p2_K$); and grey shades indicate plasmid free cells.

In our original predictions (Figure 2 and Figure S2), we assumed that mutations that compensated for the cost of the plasmid, whether plasmid-born or chromosomal, only altered fitness of the host-plasmid combination that was coevolving. However, mutant plasmids may persist longer in novel hosts[18] and mutant hosts may retain novel plasmids longer. Indeed, Loftie-Eaton *et al.* (2017)

showed that compensatory chromosomal mutations that increased persistence of a co-evolved plasmid also enabled the same mutant host to better retain alternate plasmids (a phenomenon termed "plasmid permissiveness")[13]. Expanding upon our mathematical model used to support our prior predictions, we explored how such synergistic pleiotropy might affect the emergence of multi-drug resistance in bacterial communities with multiple plasmids. Our model predicted that when compensatory mutations that generate greater plasmid persistence in one host simultaneously improve plasmid persistence in a different host, the incidence of multi-drug resistance is predicted to increase further (Supplemental Materials and Figure S3).

Did synergistic pleiotropy contribute to greater persistence of evolved plasmid-bacteria pairs in our mixed species community? Because the vast majority of cells containing both plasmids in our co-cultures were *K. pneumoniae*, we focused on this species. In addition to evolution increasing the persistence of p2 in its coevolved host (ΔBIC = -866.0; Figure 4b; Supplemental Materials VI), persistence of the p1 plasmid in that same host (i.e. a population of $K_2(p1_E)$) was higher than a population of K(p1); ΔBIC = -413.0; Figure 4c; Supplemental Materials VI). Because we did not find mutations on plasmid p1 in the $E_1(p1_E)$ clone used as a progenitor in our co-cultures (i.e., $p1_E$ = p1; Supplemental materials VII; Table S5), this pattern is consistent with evolved plasmid permissiveness: mutations in the host chromosome stabilized the coevolving, or native, plasmid p2 as well as a novel plasmid p1. We observed similar patterns when $p2_E$ was the novel plasmid in the *K. pneumoniae* host that had evolved with p1 (ΔBIC = -893.7; Figure S8c; Supplemental Materials VI). In this comparison, however, greater persistence of $p2_E$ could be due to changes in the naïve host (K_1), the novel plasmid ($p2_E$), or both, as mutations in both the *K. pneumoniae* host chromosome and plasmid p2 occurred during evolution. In fact, four of our six $K_2(p2_K)$ and five

of our six $E_2(p2_E)$ replicate populations possessed identical deletions in p2, presumably because this mutation conferred an advantage regardless of the host species (Table S5; see also Supplemental Information VII for complete list of mutations for all ancestral and evolved clonal strains). Taken together, these data suggest that evolutionary changes that stabilize plasmid-host relations or increase the fitness of plasmid-host pairs can exhibit synergistic pleiotropy, whereby the same benefits are also experienced in novel plasmid-host combinations. These interactions can then further maintain the source pool of plasmids in the community that are available to generate more MDR cells.

Drug combination therapy is often prescribed in clinical settings to hinder the emergence of strains resistant to single antibiotics[19]. However, when MDR cells exist in a population, applying a drug cocktail containing the relevant antibiotics could result in populations consisting entirely of MDR cells. After such enrichment, how would previous host-plasmid coevolution affect the decay of plasmid-mediated MDR when selection for the plasmids is removed? In our system, this process would include transitions first from double-plasmid containing cells to single-plasmid containing cells and then from single-plasmid containing cells to plasmid-free cells (Figure 5a). To determine the first of these rates, we performed persistence assays on a population of double-plasmid containing cells under single antibiotic exposure, which allowed the population to lose only one of the two plasmids. Specifically, in the presence of tetracycline (selecting for the p1-type plasmid) we measured the persistence of the p2-type plasmid in the strains K(p1, p2) and $K_2(p1_E, p2_K)$ (Figure 5d; note in the evolved strain the persistence being measured is that of the coevolved plasmid). Additionally, in the presence of chloramphenicol (selecting for the p2-type) we measured the persistence of plasmid type p1 in each of the same two strains (Figure 5e; note in the

evolved strain the persistence being measured is that of the newly introduced plasmid). In both cases, the plasmid not under selection was more stable in the evolved strain than the ancestral one. This pattern was meaningful according to the criteria of our population dynamics model for plasmid type p2 (ΔBIC = -23.0; Figure 5d; Supplemental Material VI), and marginally so for plasmid type p1 (ΔBIC = 5.0; Figure 5e; Supplemental Material VI). Thus, coevolution of hosts and their plasmids lowers the transition rate from single-plasmid containing cells to plasmid-free cells (Figures 5b and 5c), as well as the transition rate from double-plasmid containing cells to single-plasmid containing cells (Figures 4d and 4e). This would suggest that the persistence of MDR in an environment with no antibiotics would be greater in the evolved context, $K_2(p1_E, p2_K)$, than in the ancestral context, K(p1, p2), which is indeed what we find (Figure 4f). Thus, prior host-plasmid coevolution not only contributes to increased emergence of MDR (Figure 4), but can also improve the maintenance of MDR in the absence of antibiotics (Figure 5f).

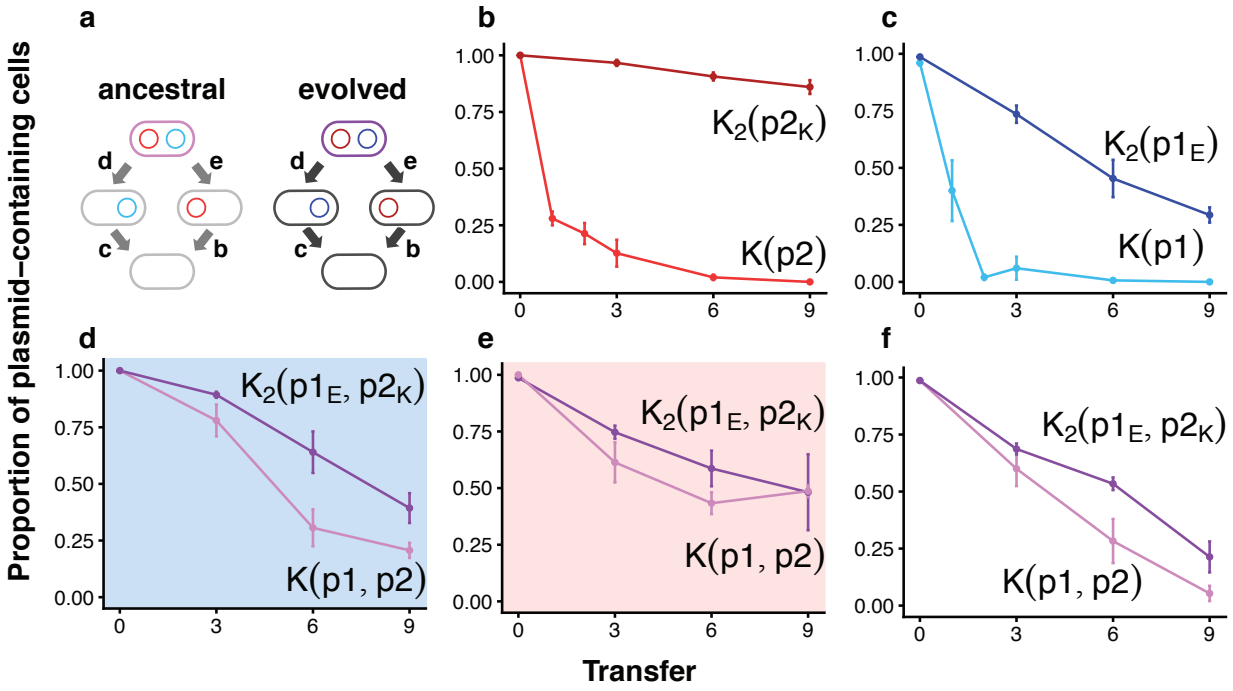

Figure 5. **Maintenance of antibiotic resistance and MDR increases after host-plasmid coevolution, in both coevolved pairs and novel combinations of host and plasmids**. (a) A *K. pneumonia* cell loses MDR by first losing either one of its two plasmid types and then losing whatever plasmid type remains. Here we compare the rates of plasmid loss between ancestral and evolved strains for all single- and double- plasmid containing cells as they transition to further plasmid loss. Plasmid persistence is measured as the transition from single-plasmid containing cells to plasmid-free cells; (b) and (c), as well as the transition from double-plasmid containing cells to single-plasmid containing cells; (d) and (e). All plasmid persistence assays were done with *K. pneumoniae* as the host in an ancestral context (K(p1), K(p2) or K(p1,p2)) or in an evolved context ($K_2(p1_E)$, $K_2(p2_K)$ or $K_2(p1_E,p2_K)$). Note that in all the evolved contexts, p2 is the coevolved plasmid and p1 is recently introduced. Single plasmid persistence in a single plasmid-containing population is higher in the evolved context than the ancestral context for the p2-type plasmid (b) and p1-type plasmid (c). Single plasmid persistence in a double plasmid-containing population (maintained as a double-plasmid containing population via selection for the alternate plasmid) is higher in the evolved context than the ancestral context for the p2-type plasmid (d) and p1-type plasmid (e). The maintenance of MDR in the absence of antibiotics is also higher in the evolved context (f). Background shading indicates the presence of tetracycline (blue; selecting for p1-type), chloramphenicol (red; selecting for p2-type), or no antibiotics (white) during the assay.

Additionally, our study highlights the complex dynamics of plasmid persistence within multi-plasmid communities. Similar to San Millan *et al*. 2014[20], we see that plasmid persistence can increase dramatically when a second plasmid is in the host background (compare ancestral trajectory in Figure 5b to 5d as well as the ancestral trajectory in Figure 5c to 5e). This is consistent with a case in which the fitness cost of one plasmid is reduced epistatically in the presence of a second plasmid. However, we also see that evolution can change the magnitude of such epistasis (compare evolved trajectory in Figure 5b to 5d as well as the evolved trajectory in Figure 5c to 5e; for fuller discussion see Supplemental Materials VIII). This complexity will make further study of communities with multiple species and multiple plasmids a worthwhile pursuit.

Given the prevalence of antibiotic usage across the globe, we expect a wide range of bacteria to commonly experience selection for antibiotic resistance in their natural environments. Our study suggests this type of exposure could lead to stabilized mobile plasmids encoding antibiotic resistance. Such coevolution can then contribute to a higher incidence of novel MDR through at least two mechanisms: by generating multi-plasmid cells at higher frequencies from enriched plasmid sources, and by limiting the rate of loss of these plasmids from MDR cells.

Methods

Detailed methods and strain descriptions can be found in Supplemental Materials.

Hosts and plasmids. Bacterial hosts included strains of two Enterobacteriaceae species *Escherichia coli* and *Klebsiella pneumoniae: strains* MG1655 and Kp08[21]. We use the first letter of the genus name (E and K) to refer to the strains throughout. Plasmids included two conjugative plasmids found in biosolids that are applied on agricultural soil: pALTS28 (61 kb) and pALTS29 (54 kb) (Law *et al*., in preparation). We refer to these plasmids as p1 and p2 throughout.

Evolution of host-plasmid pairs. Replicate populations initiated with either K(p1), K(p2), E(p1), E(p2), K(Ø), or E(Ø) were evolved in 300 µl Lysogeny broth (LB) in microtiter plate wells for 68 culture transfers (approximately 400 generations). Plasmid-containing strains were propagated in the presence of either 10 µg/ml tetracycline or 25 µg/ml chloramphenicol to select for p1 or p2, respectively. At the last transfer, each evolving population was diluted, plated and a randomly

chosen colony served as the "evolved isolate" for the replicate lineage for subsequent assays. All evolved and ancestral strains were archived at -80C in 11% glycerol.

Plasmid persistence assays. All ancestral and evolved plasmid-containing strains were inoculated under antibiotic selection for the plasmid by inoculating the relevant archived cultures into 3 replicate microtiter plate wells. Each culture was then passaged daily for 9 transfers in the absence of antibiotic selection for the plasmid. Every three transfers, each culture was diluted and plated on LB agar to obtain single colonies. The proportion of plasmid-containing colonies out of 50 was then determined via streaking each colony onto (*i*) selective antibiotic plates that allowed for the growth of plasmid-containing cells but inhibited the growth of plasmid-free cells and (*ii*) LB agar plates to make sure the bacteria were being transferred to the plates during the streaking.

MDR emergence assay. Plasmid-containing strains contributing to each mixture were grown in liquid cultures overnight under antibiotic selection for the appropriate plasmid. They were then washed 3 times with saline and *K. pneumoniae* cultures were diluted 10-fold to ensure the mixed-species cultures were started with a similar number of cells per species. Both of the relevant strains were mixed in equal volumes, 5µl of which was used to inoculate 3 replicate microtiter plate wells containing 295 µl fresh LB. All replicates were thereafter grown in the absence of antibiotics and propagated via a 1:60 dilution every 24 hours for 4 days. All possible host- and plasmid-specific cell types were tracked via selective, subtractive plating daily (see supplemental materials IV).

Permissiveness and double-plasmid persistence assays. Plasmid persistence in single plasmid-containing *K. pneumoniae* strains was assayed as above. Double plasmid-containing *K.*

pneumoniae strains were obtained via plate matings between the recipient *K. pneumoniae* host and an *E.coli* donor. They were then tracked for the loss of each plasmid via streaking colonies onto separate antibiotic containing plates. When the double-plasmid containing populations were tracked for the loss of a single plasmid in the background of the maintained alternate plasmid (i.e. the forced presence of the non-focal plasmid), the LB growth media was supplemented with the antibiotic concentration that inhibited the growth of any cells that did not contain the non-focal plasmid.

Statistical analyses. The Bayesian Information Criterion (BIC) was used to determine whether a population dynamic model including parameters for plasmid cost, segregational loss, and conjugation rates better explained the data for all single-plasmid persistence curves in Figure 3, Figure 5, Figure S4 and Figure S8 (compared to a model including just plasmid cost and segregational loss). The results decided the choice of model to use in determining whether there was a meaningful difference between the evolved and ancestral curves; i.e. whether the trajectories were best explained by one set of parameter estimates or two. BIC was also used for this second set of model comparisons.

Supplemental Materials

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a. Sequencing methods

b. Sequencing results

VIII. Extended discussion

a. Broader relevance of hosts and plasmids used in this study

b. The potential for the creation of novel MDR plasmids

c. The effect of plasmid co-residency on plasmid stability

I. Mathematical models: Plasmid persistence and MDR emergence

a. Mathematical Model and Stochastic Simulation

To explore the effects of plasmid-host coevolution on the emergence of novel multi-drug resistance, we developed a simple model involving two bacterial species (denoted **A** and **B**) and two conjugative plasmids (**a** and **b**) each encoding distinct antibiotic resistance. Note that throughout this section, our model notation differs from the experimental notation to allow for greater ease when writing relevant variables in the equations. All combinations of hosts and plasmids are permitted, as indicated by adding a subscript of the plasmid label to the host label (e.g., $\mathbf{A_b}$ is a cell of species **A** with plasmid **b**, and $\mathbf{B_{ab}}$ is a cell of species **B** with both plasmids). We use the letter **X** as a variable for different cell variants (i.e., $\mathbf{X} \in \mathcal{V} \equiv \{\mathbf{A}, \mathbf{A_a}, \mathbf{A_b}, \mathbf{A_{ab}}, \mathbf{B}, \mathbf{B_a}, \mathbf{B_b}, \mathbf{B_{ab}}\}$). Here we assume that bacteria are growing in antibiotic-free batch culture conditions with a limiting resource with concentration given by R. The per capita growth rate for cells of variant **X** follows the Monod equation $\mu_{\mathbf{X}}(R) = \{\mu_{\max}(\mathbf{X})\}R/(K_R + R)$, where $\mu_{\max}(\mathbf{X})$ is the maximum growth rate and K_R is the resource concentration where the growth rate is half its maximum. Before evolution, we assume $\mu_{\max}(\mathbf{A}) = \mu_{\max}(\mathbf{B}) = \mu^*, \quad \mu_{\max}(\mathbf{A_a}) = \mu_{\max}(\mathbf{A_b}) = \mu_{\max}(\mathbf{B_a}) = \mu_{\max}(\mathbf{B_b}) = \mu^* - c$, and $\mu_{\max}(\mathbf{A_{ab}}) = \mu_{\max}(\mathbf{B_{ab}}) = \mu^* - 2c$. Specifically, plasmid-free cells have a maximal growth rate of μ^* and growth rate decreases by the quantity c with the addition of each plasmid (c measures the cost of plasmid carriage). During bacterial division, a plasmid of

either type is lost from a cell of either type with probability λ. Any plasmid-bearing cell transfers its plasmid to another cell via conjugation at a rate τ.

Species **A** evolves with plasmid **a** in the presence of the relevant antibiotic, and species **B** evolves with plasmid **b** with the relevant antibiotic. Here we assume the primary consequence of evolution is an amelioration of the cost of plasmid carriage due to compensatory mutations. Specifically, after evolution, $\mu_{\max}(\mathbf{A_a}) = \mu_{\max}(\mathbf{B_b}) = \mu^* - (1-p)c$ and $\mu_{\max}(\mathbf{A_{ab}}) = \mu_{\max}(\mathbf{B_{ab}}) = \mu^* - (2-p)c$. The parameter p measures how selected mutations affect the growth cost initially incurred by possession of the plasmid. When $p = 0$, there is no reduction in the growth cost (zero compensation). When $0 < p < 1$, there is some reduction in the growth cost (partial compensation). When $p = 1$, there is full reduction in the growth cost (complete compensation). When $p > 1$, the plasmid provides a growth benefit (overcompensation). The larger the value of p, the stronger the effect plasmid-host evolution has on plasmid persistence in the absence of antibiotics.

The community dynamics will be governed by a system of differential equations. Below we show the equations for variants **A**, $\mathbf{A_a}$, $\mathbf{A_b}$, $\mathbf{A_{ab}}$, **B**, $\mathbf{B_a}$, $\mathbf{B_b}$, and $\mathbf{B_{ab}}$ (with densities given by the variables A, A_a, A_b, A_{ab}, B, B_a, B_b, and B_{ab}, respectively) as well as the resource (with concentration R). If ψ is the yield coefficient, the system is:

$$\dot{A} = [\mu_{\mathbf{A}}(R)]A + \lambda\left[\mu_{\mathbf{A_a}}(R)\right]A_a + \lambda\left[\mu_{\mathbf{A_b}}(R)\right]A_b + \lambda^2\left[\mu_{\mathbf{A_{ab}}}(R)\right]A_{ab} - \tau(A_a + A_b + A_{ab} + B_a + B_b + B_{ab})A$$

$$\dot{A}_a = (1-\lambda)\left[\mu_{\mathbf{A_a}}(R)\right]A_a + \lambda\left[\mu_{\mathbf{A_{ab}}}(R)\right]A_{ab} + (\tau/2)\,(2A_a + A_{ab} + 2B_a + B_{ab})A$$
$$- (\tau/2)\,(2A_b + A_{ab} + 2B_b + B_{ab})A_a$$

$$\dot{A}_b = (1-\lambda)\left[\mu_{\mathbf{A_b}}(R)\right]A_b + \lambda\left[\mu_{\mathbf{A_{ab}}}(R)\right]A_{ab} + (\tau/2)\,(2A_b + A_{ab} + 2B_b + B_{ab})A$$
$$- (\tau/2)\,(2A_a + A_{ab} + 2B_a + B_{ab})A_b$$

$$\dot{A}_{ab} = (1-\lambda)^2\left[\mu_{\mathbf{A_{ab}}}(R)\right]A_{ab} + (\tau/2)\,(2A_b + A_{ab} + 2B_b + B_{ab})A_a$$
$$+ (\tau/2)\,(2A_a + A_{ab} + 2B_a + B_{ab})A_b$$

$$\dot{B} = [\mu_{\mathbf{B}}(R)]B + \lambda\left[\mu_{\mathbf{B_a}}(R)\right]B_a + \lambda\left[\mu_{\mathbf{B_b}}(R)\right]B_b + \lambda^2\left[\mu_{\mathbf{B_{ab}}}(R)\right]B_{ab}$$
$$- \tau(A_a + A_b + A_{ab} + B_a + B_b + B_{ab})B$$

$$\dot{B}_a = (1-\lambda)\left[\mu_{\mathbf{B_a}}(R)\right]B_a + \lambda\left[\mu_{\mathbf{B_{ab}}}(R)\right]B_{ab} + (\tau/2)\,(2A_a + A_{ab} + 2B_a + B_{ab})B$$
$$- (\tau/2)\,(2A_b + A_{ab} + 2B_b + B_{ab})B_a$$

$$\dot{B}_b = (1-\lambda)\left[\mu_{\mathbf{B_b}}(R)\right]B_b + \lambda\left[\mu_{\mathbf{B_{ab}}}(R)\right]B_{ab} + (\tau/2)\,(2A_b + A_{ab} + 2B_b + B_{ab})B$$
$$- (\tau/2)\,(2A_a + A_{ab} + 2B_a + B_{ab})B_b$$

$$\dot{B}_{ab} = (1-\lambda)^2\left[\mu_{\mathbf{B_{ab}}}(R)\right]B_{ab} + (\tau/2)\,(2A_b + A_{ab} + 2B_b + B_{ab})B_a$$
$$+ (\tau/2)\,(2A_a + A_{ab} + 2B_a + B_{ab})B_b$$

$$\dot{R} = -\left(\frac{1}{\psi}\right)\sum_{\mathbf{X}\in\mathcal{V}}[\mu_{\mathbf{X}}(R)]X$$

The stochastic process governed by these equations can be simulated using the "Gillespie algorithm"[22].

We start by using our model to explore the effect of host-plasmid coevolution on the persistence of the plasmid. To do so, we focus on a strict subset of the above system consisting of the following three equations:

$$\dot{A} = [\mu_{\mathbf{A}}(R)]A + \lambda\left[\mu_{\mathbf{A_a}}(R)\right]A_a - \tau A_a A$$

$$\dot{A}_a = (1-\lambda)\left[\mu_{\mathbf{A_a}}(R)\right]A_a + \tau A_a A$$

$$\dot{R} = -\left(\frac{1}{\psi}\right)\{[\mu_{\mathbf{A}}(R)]A + \left[\mu_{\mathbf{A_a}}(R)\right]A_a\}$$

Starting with a population fixed for $\mathbf{A_a}$, we simulate these equations across nine 50-fold dilution and growth cycles in the absence of antibiotics. We see that when the host incurs a substantial cost to carry the plasmid, the plasmid is lost from the population rapidly as more fit plasmid-free segregants are generated and subsequently outcompete plasmid-bearing cells (Fig. S1a). Host-plasmid evolution is incorporated by simulating these equations again, but with a reduction in the cost of plasmid carriage. We see that the

plasmid loss is slower, as the rate of competitive displacement is muted (Fig. S6b). These patterns are largely consistent with our experimental results (Fig. 2 and Fig. S4).

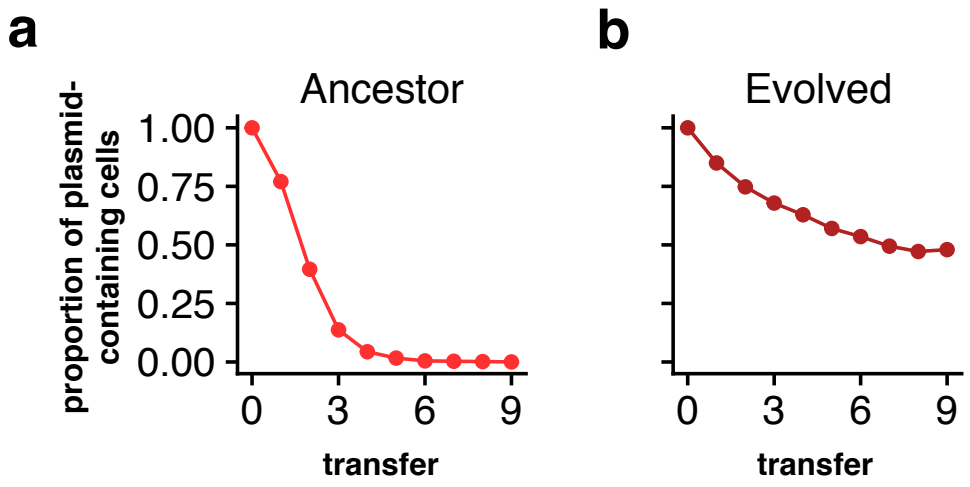

Figure S1: Model simulation of plasmid persistence. (a) The Gillespie algorithm was used to simulate the governing equations with $\mu^* = 0.7$, $c = 0.2$, $K_R = 0.004$, $\lambda = 0.05$, $\tau = 0.00001$, and $\psi = 0.000002$, and the dynamic variables were initialized with $R_0 = 0.02$, $A_0 = 0$, and $A_{a,0} = 200$. The average plasmid-bearing proportion of the simulated population over 10 replicates is shown as points, with each point located at the time closest to the end of the relevant 8-hour transfer period, after which a 50-fold dilution and resource replenishment occurred. (b) Evolution is integrated by a reduction in the cost of plasmid carriage. Here we set $p = 0.99$, which makes the effective cost of the plasmid two orders of magnitude lower. Consequently, the loss of the plasmid from the evolved population is slower compared to its ancestor in part a.

We next move to the full system of differential equations and simulate stochastic realizations starting with a community in batch culture with only $\mathbf{A_a}$ and $\mathbf{B_b}$ cells initially present. From this initial community, it is possible to generate all of the types of cells (by combinations of plasmid loss through segregation and plasmid gain through conjugation). We track the community over four 50-fold dilution and growth cycles in the absence of

antibiotics. We see that MDR cells ($\mathbf{A_{ab}}$ or $\mathbf{B_{ab}}$) rise and decay (the light purple trajectory in Fig. S2a). However, after evolution that reduces the cost of plasmids **a** and **b** to their native hosts **A** and **B**, respectively, the incidence of MDR cells is substantially higher (the dark purple trajectory in Fig. S2b). Again, the basic patterns in these simulated outcomes are similar to our experimental results (Fig. 3, Fig. S5, Fig. S6, and Fig. S7).

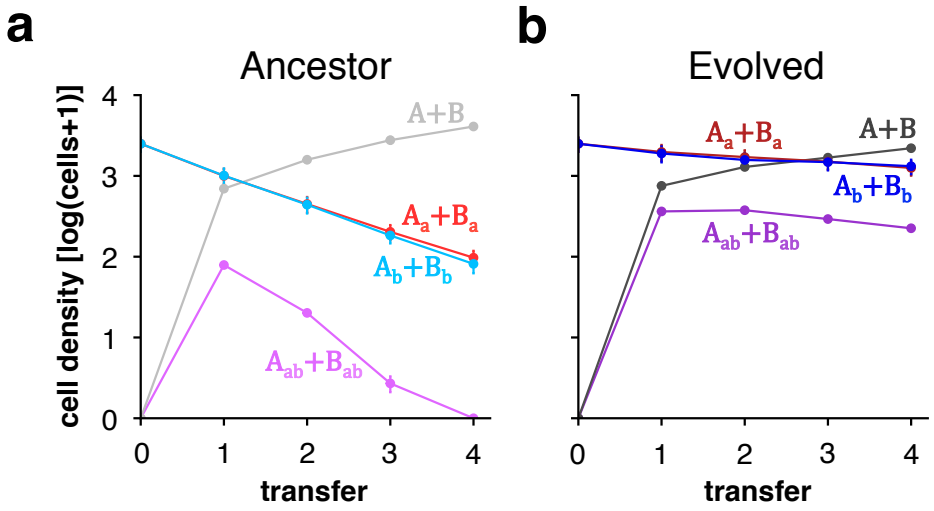

Figure S2: Model simulation of MDR emergence. (a) The Gillespie algorithm was used to simulate the governing equations with parameters as in Fig. S1, and initial non-zero variables set to $A_{a,0} = 200$, $B_{b,0} = 200$, and $R_0 = 0.02$. The average cell densities for the simulated population over 10 replicates is shown as points, with each point located at the time closest to the end of the relevant transfer period. (b) Evolution is incorporated by a reduction in the plasmid cost ($p = 0.99$) for both plasmids in their native hosts. Consequently, the density of MDR cells is higher compared to the ancestral community in part a.

Up to this point, coevolution of the host and plasmid is specific to the native context of the plasmid. For instance, while coevolution between the **a** plasmid and the **A** host transforms the maximum growth rate of the $\mathbf{A_a}$ strain from $\mu^* - c$ to $\mu^* - (1-p)c$, the maximum

growth rate of $\mathbf{A_b}$ or $\mathbf{B_a}$ are assumed to stay constant at $\mu^* - c$. However, mutations in a plasmid reducing the cost it imposes on its native host may also reduce its burden in a novel host[18]. Similarly, mutations in the host chromosome that lower the cost of a native plasmid may also reduce the cost of a non-native plasmid[17]. We can start to incorporate such cases of pleiotropy by introducing another parameter to our model, q, which measures the reduction of costs in non-native plasmid-host configurations. Specifically, we will assume $\mu_{\max}(\mathbf{A}) = \mu_{\max}(\mathbf{B}) = \mu^*$, $\mu_{\max}(\mathbf{A_a}) = \mu_{\max}(\mathbf{B_b}) = \mu^* - (1-p)c$, $\mu_{\max}(\mathbf{A_b}) = \mu_{\max}(\mathbf{B_a}) = \mu^* - (1-q)c$, and $\mu_{\max}(\mathbf{A_{ab}}) = \mu_{\max}(\mathbf{B_{ab}}) = \mu^* - (2-p-q)c$. We note the model above simply assumed $q = 0$. The model could be made more general still by allowing different reductions of cost in different non-native (and native) configurations, but we will move forward with our assumption of symmetry for simplicity.

We again follow the community over 50-fold dilution and growth cycles in the absence of antibiotics. Here we track eight transfers in order to highlight differences. We see that MDR cells ($\mathbf{A_{ab}}$ or $\mathbf{B_{ab}}$) reach higher densities if there are pleiotropic reductions of costs (i.e., if $q > 0$; compare the purple trajectories in Fig. S3a to Fig. S3b). More generally, as either the plasmid cost reduction in native configurations (p) or non-native configurations (q) is increased, the incidence of MDR rises.

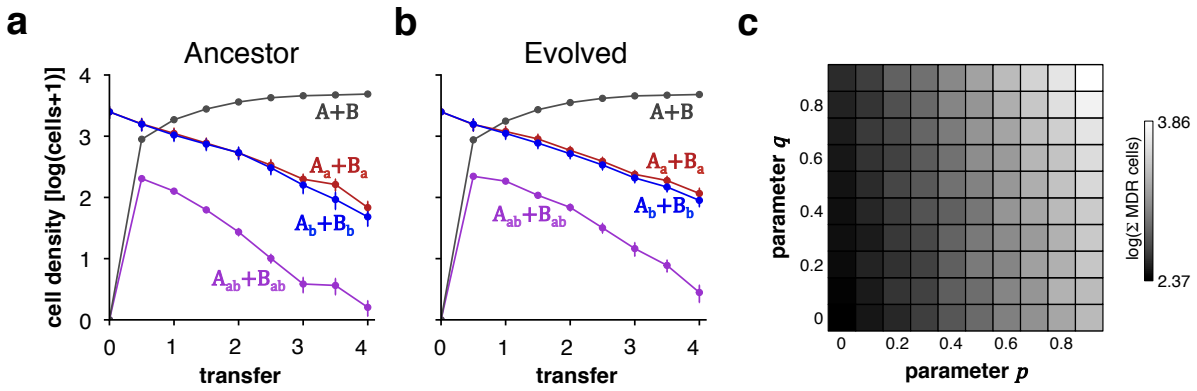

Figure S3: Model simulation of MDR emergence with pleiotropic effects. (a) The Gillespie algorithm[22] was used to simulate the governing equations with parameters and initial variable settings as in Figs. S1 and S2. The average cell densities for the simulated population over 10 replicates is shown as points, with each point located at the time closest to the end of the relevant transfer period. Here we assume that evolutionary changes have led to a reduction in the plasmid cost ($p = 0.5$) for both plasmids in their native hosts, but note for any non-native configuration there is no cost reduction ($q = 0$). (b) Here we allow for pleiotropic effects—namely a reduction of plasmid cost in non-native configurations ($q = 0.5$). Although the difference is not substantial, the density of MDR cells is greater at every transfer. (c) More generally, we show a (literal) density plot for different p - q combinations. The gray level for each square is the log of the cumulative density of MDR cells at the end of 8 transfers for each combination of direct (p) and pleiotropic (q) effects of compensatory mutations. Greater reductions of plasmid costs in either native or non-native contexts correspond to higher incidence of MDR.

II. Strains

a. Supplemental host and plasmid descriptions

Hosts:

An *E. coli* MG1655 mutant strain was generated that contained spontaneous rifampicin and streptomycin resistance mutations (see below). A *K. pneumoniae* mutant of strain Kp08 was similarly generated with resistance to rifampicin. The original Kp08 was isolated from a blood infection[21] and contains 3 native plasmids of varying sizes (162kb, 41 kb, and 5 kb) and average copy number (1, 3, and 75, respectively).

Plasmids:

Focal plasmids included pALTS28 (referred to as 'p1' in the main text; 61 kb) and pALTS29 (referred to as 'p2' in the main text; 54 kb), two self-transmissible plasmids with a broad host range that were found in biosolids that are applied on agricultural soil (Law *et al*., in preparation). They belong to the recently defined broad-host-range (BHR) plasmid family, PromA[23] (pALTS28), and the well-known group of BHR IncP-1beta plasmids (pALTS29). The plasmids were captured from biosolids using a biparental mating protocol (Law et al., in preparation).

b. Antibiotic marking of hosts

A streptomycin-resistant mutant of MG1655, previously generated by us, was grown overnight at 30°C in 5ml LB supplemented with 100 ug/ml streptomycin. 200ul of the resulting saturated culture was then plated onto an LB agar plate supplemented with 50 ug/ml rifampicin and incubated overnight at 30°C. A single rifampicin resistant colony was selected from this plate to be the progenitor of the isogenic freezer stock for that host. A rifampicin resistant version of Kp08 was similarly obtained.

c. Plate mating assays

Each focal plasmid was introduced separately into the E and K hosts (here recipients) via a nalidixic acid resistant mutant of MG1655, K12Nal, which acted as a donor strain. Recipient and donor strains were grown up from freezer stocks in the presence of the appropriate antibiotic. One ml of each saturated culture was spun down for 5 minutes at 6000 rpm and washed with 1 ml 0.086% saline three times. Per host-plasmid combination, a recipient and donor were then mixed in equal volumes, diluted into microtiter plate wells along a gradient of 1:10 dilutions, and 5ul from each well of the dilution series was spotted onto an LB agar plate supplemented with both rifampicin (to select for the recipient host) and either tetracycline or chloramphenicol (to select for the appropriate plasmid). A single transconjugant colony arising on the double-antibiotic plate was re-streaked onto the same plate type. A colony from this streaked plate was selected to be the progenitor of the isogenic freezer stock for that strain.

III. Evolution assay

a. Supplemental methods

Six replicate populations for each strain, i.e. K(p1), K(p2), E(p1), E(p2), K(Ø), and E(Ø) were initiated by transferring 5 µl of an isogenic overnight culture into flat-bottomed microtiter plate wells containing 295 µl Lysogeny broth (LB; a 1:60 dilution). At 12 hour intervals of growth, wells were visually inspected for saturation. If all replicates for a particular strain weren't sufficiently cloudy, all replicates for that strain were incubated for an additional 12 hours (a

common occurrence for replicates initiated with E(p1) and E(p2)). If wells were fully cloudy, 5 µl of each population was transferred into 295 µl of fresh LB. All population were evolved for 68 transfers (approximately 400 generations, considering $\log_2(60) = 5.9$ generations per transfer). Throughout the assay, media supporting growth of strains containing p1- and p2-type plasmids was supplemented with 10 µg/ml tetracycline and 25 µg/ml chloramphenicol, respectively, to select for the plasmid. All cultures were propagated at 30°C in shaken microtiter plate wells, with a subset of each population being preserved at -80°C in 11% glycerol every 100 generations. Additionally, a single colony was obtained from all final populations at generation 400 and preserved at -80°C in 11% glycerol. This stock was used to inoculate all "evolved" populations during future assays. Plating for single colonies at 400 generations revealed *K. pneumoniae* contaminants in four of the six evolved E(p1) replicates. Thus, the remaining two replicates (1 and 4) were the only evolved replicates for this strain carried forward in the remainder of the study. No other occurrences of contamination were experienced during this study.

IV. Persistence assay

a. Supplemental methods

Three replicate populations of each ancestral and evolved plasmid-containing *E. coli* and *K. pneumoniae* lineage were inoculated from the clonal isolates archived at -80°C into microtiter plate wells containing 300 µl of LB supplemented with 20 µg/ml tetracycline or 50 µg/ml

chloramphenicol (for p1- or p2-containing lineages, respectively). Evolved clones included six each of $K_1(p1_K)$, $K_2(p2_K)$, and $E_2(p2_E)$, and two of $E_1(p1_E)$. After the initial overnight growth cycle under plasmid-selective (antibiotic) conditions, 5 µl of each population was then transferred every 24 hours for 9 days into 295 µl fresh LB with no antibiotic supplementation. All cultures in this assay were propagated at 30°C in shaken microtiter plate wells. On transfers 0, 3, 6, and 9, overnight populations were diluted and plated for single colonies onto LB agar plates. Fifty colonies from each plate were phenotypically assayed for the presence of the plasmid-associated ABR gene by stabbing each colony with a toothpick and streaking it first onto an antibiotic-containing plate (LB agar supplemented with either 20 µg/ml tetracycline or 50 µg/ml chloramphenicol) and then onto an antibiotic-free plate (LB agar). The latter was done to ensure that bacteria had been transferred from the colony plate to the streak plate. Streaks producing bacterial growth on the antibiotic-containing plate after overnight incubation were considered 'plasmid-containing'; streaks producing no bacterial growth on the antibiotic-containing plate but *positive* growth on the LB plate were considered 'plasmid-free'.

Generally, in this paper we assessed plasmid presence by plating for antibiotic resistant growth and counting resulting colonies or streaks. We note here, however, it is possible that such growth could occur in a strain without a plasmid (either due to *de novo* resistance or to integration of the antibiotic resistance gene into the chromosome and subsequent loss of the plasmid). In all cases of resistant growth where we checked for the presence of the plasmid via PCR of core plasmid genes *repA* (for p1) or *trfA* (for p2), BAC DNA miniprep kits, or full genome sequencing, we found it.

b. Supplemental results

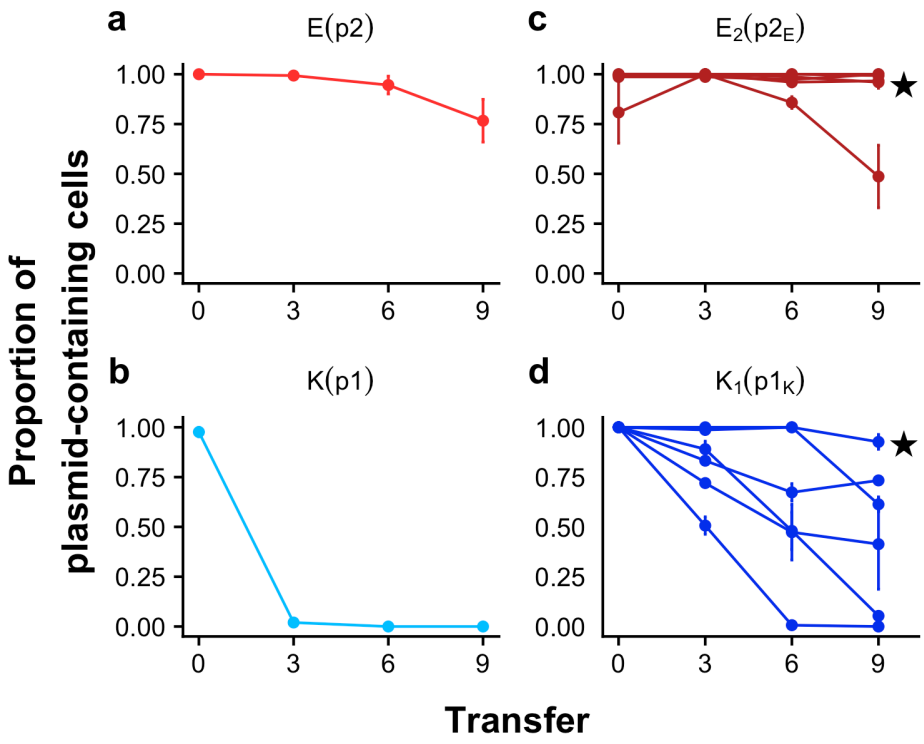

Figure S4. Plasmid persistence in the absence of antibiotic selection for the plasmid. Populations were grown up in the relevant antibiotic and thus start with 100% plasmid-containing cells. Consistent with main text, labels above each graph indicate the host-plasmid pair being assayed. Lighter colors (left graphs; (a) & (c)) indicate ancestral strains. Darker colors (right graphs; (b) & (d)) indicate evolved strains. Blue trajectories indicate the proportion of colonies that were resistant to tetracycline (and thus presumably contained p1). Red trajectories indicate the proportion of colonies that were resistant to chloramphenicol (and thus presumably contained p2). "★" denotes the particular evolved strain used for the subsequent assays outlined in this paper. Each point represents the mean of three replicates per strain; error bars indicate standard error.

c. Statistical analyses and results

Each persistence curve was analyzed via a plasmid population dynamic model that used a maximum likelihood approach to estimate the parameters contributing to plasmid persistence (i.e. plasmid cost, segregational loss, and conjugation)[15–17]. A version of the model without the conjugation parameter (version is denoted 'SS', in alignment with previous work) provided a better fit to the data for 21 out of 24 persistence curves than the full 'HT' model, which includes horizontal plasmid transfer. This suggests that horizontal gene transfer was not an important predictor explaining plasmid persistence rates for most strains (Table S1). This was determined by comparing the Bayesian Information Criterion (BIC) for each of the two models on any given persistence curve, whereby the model with the lowest BIC was preferred[24].

Using the best fitting model, we determined whether evolution had affected plasmid persistence (i.e. the parameter estimates for each evolved strain were different than that of the ancestor). This was done by comparing the BIC from a model in which a single set of parameter values explained any two persistence curves being compared (BIC_joint) and the BIC from a model in which two distinct sets of parameter values explained the data (BIC_separate). In all ancestral versus evolved strain comparisons, the model allowing for two sets of parameter values better explained the data (produced a lower BIC) and all ΔBICs between the models were of a greater magnitude than 10, indicating "very strong evidence" that evolution had affected plasmid persistence in a meaningful way[17,24] (Table S2). ΔBIC from 6-10 would have been considered "strong evidence", ΔBIC from 2-6 would have indicated "positive evidence" and ΔBIC from 0-2 would have indicated "weak" evidence that the models were distinguishable from each other[24]. In our case, ΔBICs ranged from -18 to -1194, with the more negative the ΔBIC (BIC_separate – BIC_joint), the larger the difference between plasmid persistence curves.

Table S1. **SS model better explains the data for most persistence curves.** BIC values based on the plasmid persistence curves for each ancestral and evolved replicate population under either the HT model (parameters include conjugation, plasmid cost, and segregational loss) or the SS model (parameters include plasmid cost and segregational loss). Starred replicates are those used in later assays. Shaded cells indicate incidences when the lower BIC value resulted from the HT model, indicating it was better able to explain the data for that persistence curve. Unshaded cells indicate the SS model was preferred for that curve.

Strain	Replicate	BIC (HT Model)	BIC (SS Model)
E(p1)	Ancestor	145.3	156.1
$E_1(p1_E)$	1★	60.5	55.6
$E_1(p1_E)$	4	18.8	14.4
E(p2)	Ancestor	66.9	61.9
$E_2(p2_E)$	1	190.7	185.7
$E_2(p2_E)$	2	35.3	35.0
$E_2(p2_E)$	3★	31.5	27.2
$E_2(p2_E)$	4	26.6	21.8
$E_2(p2_E)$	5	39.5	35.0
$E_2(p2_E)$	6	16.6	14.4
K(p1)	Ancestor	26.5	21.5
$K_1(p1_K)$	1	53.0	57.3
$K_1(p1_K)$	2	43.1	38.5

$K_1(p1_K)$	3	152.4	149.6
$K_1(p1_K)$	4★	61.3	56.3
$K_1(p1_K)$	5	31.8	26.8
$K_1(p1_K)$	6	27.1	22.1
K(p2)	Ancestor	58.9	54.0
$K_2(p2_K)$	1	65.2	67.2
$K_2(p2_K)$	2★	45.8	41.5
$K_2(p2_K)$	3	12.4	7.5
$K_2(p2_K)$	4	55.3	50.4
$K_2(p2_K)$	5	76.1	72.7
$K_2(p2_K)$	6	64.3	59.3

Table S2. **Predicted parameter estimates are different between the ancestral and evolved single-plasmid persistence curves for every pair.** ΔBIC values between model comparisons in which one or two sets of parameter estimates were used to fit the data generated by an ancestral and evolved pair of persistence curves. All replicates per evolved strain were compared to the ancestor of that strain. Curves are considered different if ΔBIC is less than -10. Starred replicates are those used in later assays. Shaded cells indicate that the HT model was used to compare the two curves, due to the results from Table S1 indicating at least one of the comparison strains was better explained by that model. Unshaded cells indicate the SS model was used.

Strain	Replicate	ΔBIC
		(BIC_seperate - BIC_joint)

E(p1)	Ancestor	
$E_1(p1_E)$	1★	-103.4307
$E_1(p1_E)$	4	-411.7451
E(p2)	Ancestor	
$E_2(p2_E)$	1	-109.9
$E_2(p2_E)$	2	-16.7
$E_2(p2_E)$	3★	-18.9
$E_2(p2_E)$	4	-44.9
$E_2(p2_E)$	5	-55.5
$E_2(p2_E)$	6	-53.2
K(p1)	Ancestor	
$K_1(p1_K)$	1	-786.096
$K_1(p1_K)$	2	-1193.7
$K_1(p1_K)$	3	-483.2
$K_1(p1_K)$	4★	-458.5
$K_1(p1_K)$	5	-104.5
$K_1(p1_K)$	6	-1031.5
K(p2)	Ancestor	
$K_2(pK_2)$	1	-585.6151
$K_2(pK_2)$	2★	-748.1
$K_2(pK_2)$	3	-1061.5
$K_2(pK_2)$	4	-1061.5
$K_2(pK_2)$	5	-522.8

$K_2(pK_2)$	6	-666.7

V. MDR emergence – Experimental assay

a. Supplemental methods

Obtaining initiation co-cultures: Cultures for each plasmid-containing strain were grown overnight in 3ml LB supplemented with appropriate plasmid selecting antibiotic (either 10 μg/ml tetracycline or 25 μg/ml chloramphenicol) at 30°C. One ml of each saturated culture was spun down for 5 minutes at 6000 rpm, the supernatant was replaced with 0.086% saline, and the cells were mixed thoroughly. This washing procedure was repeated 3 times. *K. pneumoniae* cultures were then diluted 1:10 to ensure roughly similar starting densities as *E. coli* cultures. Per co-culture, the two appropriate final washed cultures were mixed in equal volumes.

Initiating and propagating co-cultures: From each initiation co-culture, 5 μl was used to inoculate microtiter plate wells containing 295 μl of LB for each of three replicate starting populations. These populations were grown in shaking microtiter plate wells at 30°C for approximately 24

hours and propagated daily at a 1:60 dilution for 4 days (24 generations) in the absence of antibiotic selection for each plasmid.

Tracking cell types: Initiation co-cultures and each replicate co-culture per day were diluted in saline and plated on a variety of selective antibiotic plates to allow subsets of each community to be tracked. These subsets included plasmid-free cells, and cells containing at least p1 (revealed on tetracycline plates), at least p2 (revealed on chloramphenicol plates), or both plasmids simultaneously (revealed on plates containing both tetracycline and chloramphenicol). Subtractive plating techniques were then used to determine the number of single-plasmid containing cells of a particular type. For example, the number of *K. pneumoniae* cells containing only p1 was calculated by subtracting the number of double-plasmid containing *K. pneumoniae* colonies on a tetracycline/chloramphenicol plate from the number of *K. pneumoniae* colonies on a tetracycline plate). *E. coli* cells were chromosomally marked with streptomycin resistance, and thus could be tracked separately from *K. pneumoniae* cells. In instances where low levels of cross-resistance were observed due to a streptomycin resistance gene in p1, *E. coli* and *K. pneumoniae* cells were easily distinguished via distinct colony morphologies.

Levels of resistance per plasmid-containing cell type were unique to each host species but also depended on whether that host species was the ancestral or evolved strain (i.e. the concentration of the drug at which that strain could no longer grow shifted during the evolution assay). Thus, the antibiotic plate concentrations used to assess cell types for each co-culture were specific to that co-culture. For each co-culture, antibiotic plate concentrations were chosen to allow all cells

containing that plasmid to grow, while maximizing the chance that cells without the relevant plasmid would be inhibited.

Furthermore, the level of conjugation occurring on plates was measured for each co-culture and found to be either non-existent or variable, depending on the co-culture.

Due to the confluence of constraints above, per co-culture we expected the variable detection of two types of false positives: (*i*) colonies lacking the relevant plasmid but growing on the relevant plasmid-selective plate, and (*ii*) colonies containing the relevant plasmid only due to experiencing conjugation on the surface of the selective plate. Thus, for each co-culture we estimated the threshold at which false positives could no longer be detected. Any data points on the graph that fell below this threshold are indicated by an open circle, and should be considered inconclusive (only present in the double-plasmid containing purple ancestral *K. pneumoniae* trajectory in Figure S7).

At time-point 0, the initiation co-cultures (which presumably consisted of only plasmid-containing cells due to growing to saturation under plasmid-selective conditions) were immediately diluted and plated. Thus, it was assumed that any colonies appearing on plates which would have indicated a conjugation event (i.e. a particular host containing an alternate plasmid to the one it started with) or a segregation event (i.e. plasmid-free cells) were false positives or false negatives, respectively. Thus, all values representing the number of these cell types in the population at time-point 0 were considered to be 0.

b. Supplemental results

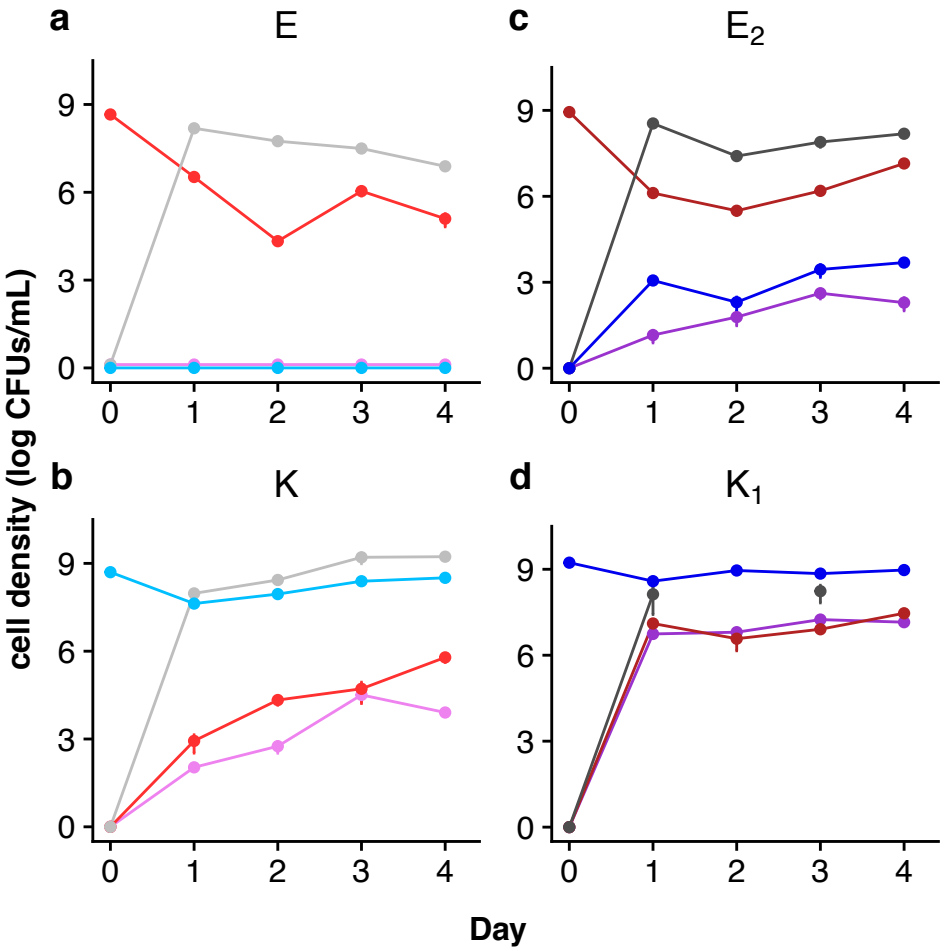

Figure S5. **The ancestral E(p2)-K(p1) co-culture (a & c) compared to the evolved E_2($p2_E$)-K_1($p1_K$) co-culture (b & d).** Fully plasmid-containing populations were initiated into 3 replicate co-cultures which were then grown for 4 days in the absence of selection for the plasmids. Cell types were tracked daily via selective plating for each host-plasmid combination. Blue trajectories: hosts containing p1 or $p1_E$; red trajectories: hosts containing p2 or $p2_K$; purple trajectories: hosts containing (p1, p2) or ($p1_E$, $p2_K$); grey trajectories: plasmid free cells. Lighter shades indicate ancestral trajectories; darker shades indicate evolved trajectories. Labels above each graph indicate host type.

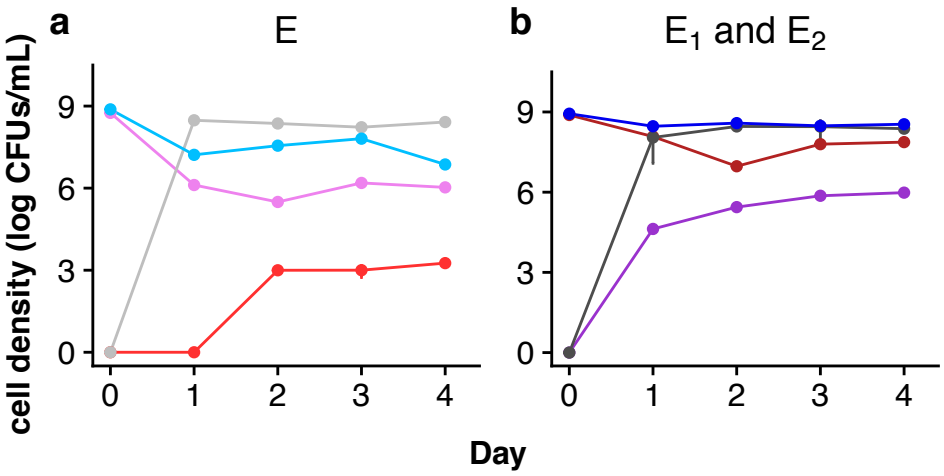

Figure S6. **The ancestral E(p1)-E(p2) co-culture (a) compared to the evolved $E_1(p1_E)$-$E_2(p2_E)$ co-culture (b).** Fully plasmid-containing populations were initiated into 3 replicate co-cultures which were then grown for 4 days in the absence of selection for the plasmids. Cell types were tracked daily via selective plating for each host-plasmid combination. Blue trajectories: hosts containing p1 or $p1_E$; red trajectories: hosts containing p2 or $p2_K$; purple trajectories: hosts containing (p1, p2) or ($p1_E$, $p2_K$); grey trajectories: plasmid free cells. Labels above each graph indicate host type.

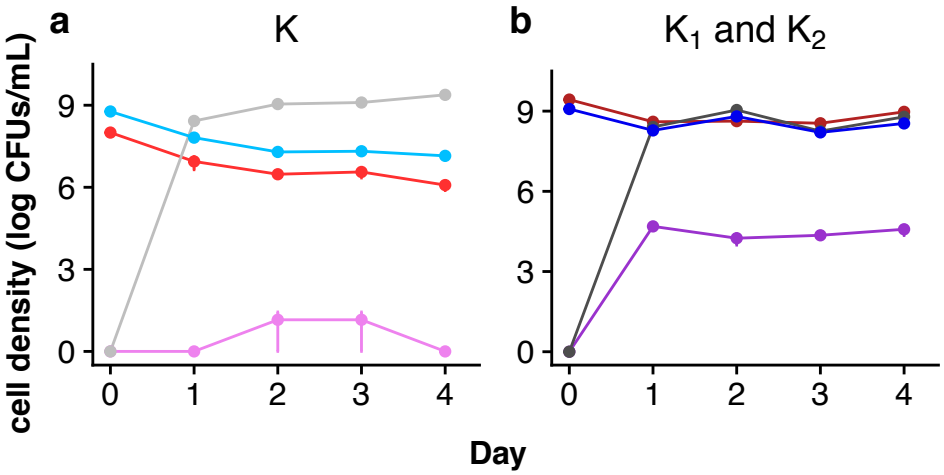

Figure S7. **The ancestral K(p1)-K(p2) co-culture (a) compared to the evolved $K_1(p1_K)$-$K_2(p2_K)$ co-culture (b).** Fully plasmid-containing populations were initiated into 3 replicate co-cultures which were then grown for 4 days in the absence of selection for the plasmids. Cell types were tracked daily via selective plating for each host-plasmid combination. Blue trajectories: hosts containing p1 or $p1_E$; red trajectories: hosts containing p2 or $p2_K$; purple trajectories: hosts containing (p1, p2) or ($p1_E$, $p2_K$); grey trajectories: plasmid free cells. Labels above each graph indicate host type.

VI. Permissiveness and double-plasmid persistence assay

a. Supplemental methods

For reference, strains used to initiate the persistence assays in this section (those shown in Figures 5 and S8) can be succinctly located in Table S4.

K. pneumoniae strains containing both plasmids were obtained from plate mating assays (identical to those described in Supplemental Methods II) between a *K. pneumoniae* plasmid-containing recipient and an *E. coli* donor with the alternate plasmid. From these double-plasmid containing strains, segregants that had lost the coevolved *K. pneumoniae* plasmid but not the newly introduced *E. coli* plasmid were isolated on the 2nd day of a persistence assay to obtain the single-plasmid containing strains used to test persistence of a novel plasmid in the evolved host.

The plasmid persistence curves for all strains in this section were assessed similarly to the persistence assays described in Section S3 with specific exceptions:

(i) For all strains containing both plasmids, each colony tested was streaked onto separate tetracycline and chloramphenicol plates to determine the presence of each plasmid independently, as well as the LB control plate. If no growth occurred after streaking on either plate, but growth appeared on the LB control, the colony was considered plasmid-free.

(ii) Antibiotic concentrations used for initial growth of all strains from the isogenic archived cultures were the lowest concentrations that allowed for growth of the relevant plasmid-containing cells but inhibited growth of plasmid-free cells (i.e. 40 μg/ml tetracycline and/or 50 μg/ml chloramphenicol), as determined by minimum inhibitory concentration assays.

(iii) When the double-plasmid containing populations were tracked for the loss of a single plasmid in the background of the maintained alternate plasmid (i.e. the 'forced' presence of the non-focal plasmid), the LB growth media was supplemented with either 40 μg/ml tetracycline or 200 μg/ml chloramphenicol, depending on the plasmid being forced. These concentrations were determined via MICs to be the concentrations at which cells were unable to grow unless they contained the forced plasmid.

b. Supplemental results

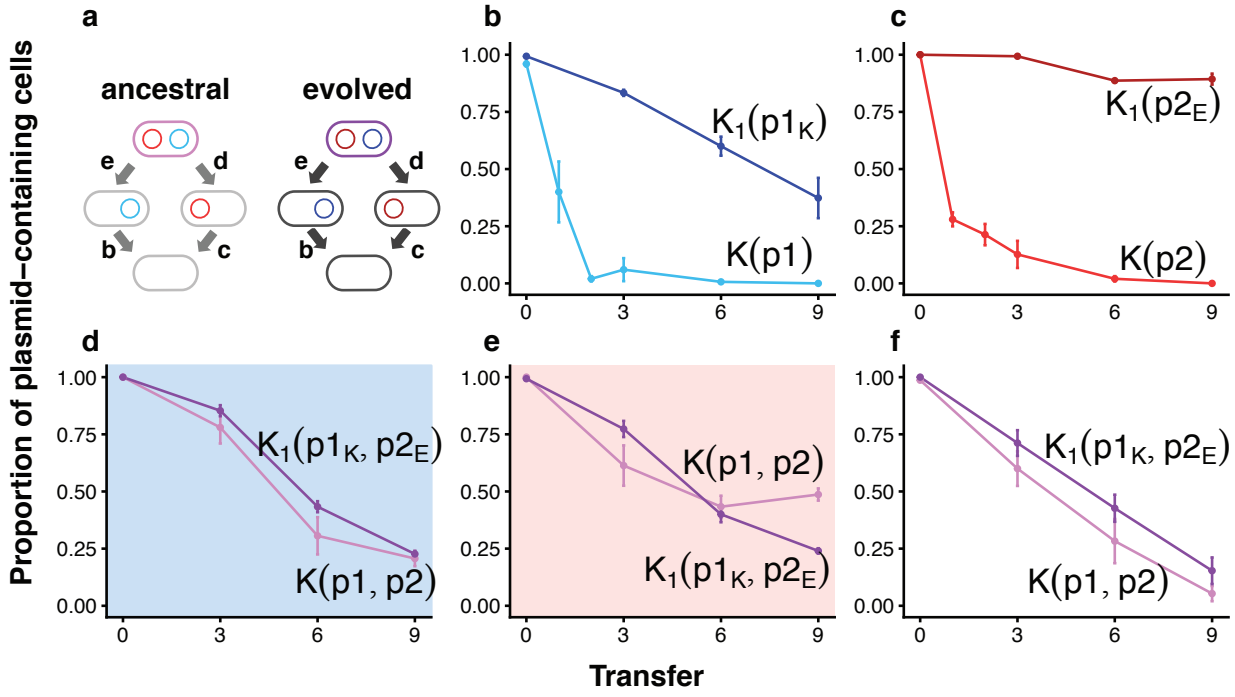

Figure S8. **The maintenance of antibiotic resistance and MDR generally increases after coevolution of plasmids with their hosts.** (a) A *K. pneumonia* cell loses MDR by first losing either one of its two plasmid types and then losing whatever plasmid type remains. Here we compare the rates of plasmid loss between the ancestral and evolved strains/plasmids. Plasmid persistence is measured as the transition from single-plasmid containing cells to plasmid-free cells (see parts b and c) as well as the transition from double-plasmid containing cells to single-plasmid containing cells (see parts d and e). All plasmid persistence assays were done with *K. pneumoniae* as the host in an ancestral context (K(p1), K(p2) or K(p1,p2)) or in an evolved context ($K_2(p1_E)$, $K_2(p2_K)$ or $K_2(p1_E,p2_K)$). Note that in all the evolved contexts, p1 is the coevolved plasmid and p2 is recently introduced. Single plasmid persistence in a single plasmid-containing population is higher in the evolved context than the

ancestral context for plasmid p1-type (b) and plasmid p2-type (c). Single plasmid persistence in a double plasmid-containing population (maintained as a double-plasmid containing population via selection for the alternate plasmid) is higher in the evolved context than the ancestral context for plasmid p1-type (d) and plasmid p2-type (e). The maintenance of MDR in the absence of antibiotics is also higher in the evolved context (f). Background shading indicates the presence of tetracycline (blue; selecting for p1-type), chloramphenicol (red; selecting for p2-type), or no antibiotics (white) during the assay.

c. Statistical analyses

Each persistence curve was analyzed as before, using the plasmid population dynamic model described in Supplemental Methods IV.

Table S3. **No clear pattern emerges as to whether the HT or SS model best predicts the permissiveness and double-plasmid persistence curves.** BIC values based on the plasmid persistence curves found in Figure 5 and Figure S8 under either the HT model (parameters include conjugation, plasmid cost, and segregational loss) or the SS model (parameters include plasmid cost and segregational loss). Shaded cells indicate incidences when the lower BIC value resulted from the HT model, indicating it was better able to explain the data for that persistence curve. Unshaded cells indicate the SS model was preferred for that curve.

Figure	Strain	BIC (HT Model)	BIC (SS Model)
4b	K(p2)	80.1	87.2
4b	$K_2(p2_K)$	45.2	40.4

4c	K(p1)	103.0	109.3
4c	$K_2(p1_E)$	67.7	63.2
4d	K(p1,p2)	60.0	57.7
4d	$K_2(p1_E,p2_K)$	65.7	61.3
4e	K(p1,p2)	77.8	89.0
4e	$K_2(p1_E,p2_K)$	100.5	96.1
S4b	K(p1)	103.0	109.3
S4b	$K_1(p1_K)$	66.2	61.3
S4c	K(p2)	80.1	87.2
S4c	$K_1(p2_E)$	39.3	42.4
S4d	K(p1,p2)	60.0	57.7
S4d	$K_1(p1_K,p2_E)$	51.0	50.0
S4e	K(p1,p2)	77.8	89.0
S4e	$K_1(p1_K,p2_E)$	57.7	55.2

Table S4. **Comparing persistence curves from permissiveness/double plasmid persistence assay.** ΔBIC values between model comparisons in which one or two sets of parameter estimates were used to fit the data generated by two different persistence curves. Evidence that the two curves are different is "very strong" if ΔBIC is less than -10. For ΔBIC values between 2 and 6, as is the case in the comparisons from Figure 4e and S8d, evidence is "positive" that the two curves are better explained by to separate sets of parameter estimates. Shaded cells indicate that the HT model was used to compare the two curves, due to the results from Table S1 indicating at least one

of the comparison strains was better explained by that model. Unshaded cells indicate the SS model was used.

Figure	Strain	ΔBIC (BIC_seperate - BIC_joint)
4b	K(p2)	
4b	$K_2(p2_K)$	-866.0
4c	K(p1)	
4c	$K_2(p1_E)$	-413.0
4d	K(p1,p2)	
4d	$K_2(p1_E,p2_K)$	-23.0
4e	K(p1,p2)	
4e	$K_2(p1_E,p2_K)$	5.0
S8b	K(p1)	
S8b	$K_1(p1_K)$	-552.3
S8c	K(p2)	
S8c	$K_1(p2_E)$	-893.7
S8d	K(p1,p2)	
S8d	$K_1(p1_K,p2_E)$	7.0
S8e	K(p1,p2)	
S8e	$K_1(p1_K,p2_E)$	-17.0

VII. Full-genome sequencing of strains

a. Sequencing methods

Full genome sequencing of clonal populations:

All ancestral and replicate evolved clonal populations were full-genome sequenced. This included all plasmid-containing strains used in this study, as well as clones from 3 replicate plasmid-free host populations for *E. coli* and *K. pneumoniae* that were evolved for 400 generations alongside the plasmid-containing lineages.

Analysis of bre-seq output:

All mutations identified (predicted mutations, unassigned missing coverage, and unassigned new junctions) in the ancestor, evolved controls, and evolved strains were combined, screened and verified using pileup files. After verification, mutations present in all strains were removed from the table. Mutations that are unique to evolved strains and absent from evolved controls are noted (grey shading, see Table S5).

b. Sequencing results

Table S5. Mutations in $E_{Ø}$(Ø) replicates 1, 2, & 3 and E_{1}($p1_{E}$) replicates 1 & 4

Replicon	Gene	EC_EvoCont_1	EC_EvoCont_2	EC_EvoCont_3	EC_28_1*	EC_28_4	Count	Position	Mutation	Description
Chromosome (U00096)	*afuC* ←			X			1	278,438	G>A (A122V)	CP4-6 prophage; putative ferric transporter subunit
	gsk →					X	1	501,342	+GTGAGCTAT[3]	inosine/guanosine kinase
	tolA →				?	X	1	776,650	deletion 371bp	membrane anchored protein in TolA-TolQ-TolR complex
	trxB ← / → *lrp*		X				1	932,392	IS5[1] (intergenic)	thioredoxin reductase, FAD/NAD(P)-binding/leucine-responsive global transcriptional regulator
	stfP → / *stfE* ←					X	1	1,209,602	inversion[2]	e14 prophage; uncharacterized protein/ pseudogene, e14 prophage; side tail fiber protein fragment family;Phage or Prophage Related
	insA ← / → *uspC*		X		X		3	1,979,514	IS2[1] + 5bp (intergenic)	IS1 repressor TnpA/universal stress protein
				X				1,979,486	IS5[1] (intergenic)	
								1,979,633	deletion 4bp (intergenic)	
	yegR ←			X			1	2,168,144	IS5[1] + 4bp	uncharacterized protein
	lrhA ←	X					1	2,406,363	deletion 1bp	transcriptional repressor of flagellar, motility and chemotaxis genes
	rpsD ←		X				1	3,441,528	C>A (D50Y)	30S ribosomal subunit protein S4
	rtcR →					X	1	3,559,861	A>T (S532C)	sigma 54-dependent transcriptional regulator of rtcBA expression
	recF ←					X	1	3,881,173	C>T (A17T)	gap repair protein
	rrfB →		X	X			2	4,171,719	deletion 100-250 bp	5S ribosomal RNA of rrnB operon
	rpoB →			X			2	4,182,791	A>G (D516G)	RNA polymerase, beta subunit
		X						4,182,959	T>G (I572S)	
	fimB → / → *fimE*	X			X		2	4,542,020	IS5[1] + 4bp (intergenic)	tyrosine recombinase/inversion of on/off regulator of fimA/tyrosine recombinase/inversion of on/off regulator of fimA
	fimE →		X				1	4,542,167	IS3[1]	tyrosine recombinase/inversion of on/off regulator of fimA
	fimE → / → *fimA*	X	X		X	X	4	4,542,682	inversion (intergenic)	tyrosine recombinase/inversion of on/off regulator of fimA/major type 1 subunit fimbrin (pilin)
Total number of mutations		4	6	5	3	6				

[1] transposable element was inserted here; [2]reversion back to the reference, [3] insertion but coding sequence remains in frame, *replicate used in other parts of the manuscript

Table X: Summary of variants in clones isolated from each evolved strain with ancestral variants removed. Intergenic regions are given with the upstream and downstream genes. Variants unique to evolved strains and absent in all evolved controls are marked (grey shading).

VIII. Extended discussion

a. Broader relevance of hosts and plasmids used in this study

In a recent report, the World Health Organization outlined recommendations for combatting antibiotic resistance on a global scale. In their first named priority they specifically highlight the concern of multi-drug resistant gram-negative bacteria (WHO, 2017). Gram negative *Enterobacteriaceae* species, including many strains of *E. coli* and *K. pneumoniae*, are recognized as influential contributors of multi-drug resistance across many environments, partly due to the high prevalence of horizontal gene transfer that occurs in these bacteria[25]. While genetic signatures of these events allow us to recognize their occurrence in bacterial populations[26], our study is the first to experimentally mix multiple *Enterobacteriaceae* species and explore the effects on the spread of horizontally acquired antibiotic resistance genes and the emergence of novel MDR. Further, our study suggests an important role for antibiotic stewardship that minimizes the overuse

of antibiotics, so as to prevent coevolution of host-plasmid pairs in the presence of antibiotic selection for plasmid-mediated antibiotic resistance. Our results indicate that this coevolutionary process will only exacerbate the generation and maintenance of MDR in *Enterobacteriaceae* species, even in environments that are free of antibiotics.

Global antibiotic exposure is largely driven by agricultural practices in which antibiotics are spread on crops and given to livestock to boost food production[27]. These practices are only expected to rise[28], and thus concerns are also rising about the increased incidence of plasmid-mediated antibiotic resistance genes spreading from environmental isolates to the clinic. This transition is thought to be somewhat common[29] and is likely the cause of the recent transcontinental spread of resistance to colistin, an "antibiotic of last resort"[30] used in livestock production[31] and to combat clinical MDR infections. The focal plasmids used in this study (p1 and p2; i.e. pALTS28 and pALTS29) were themselves obtained from biosolid waste applied on agricultural soil, and their status as broad host range, antibiotic resistance plasmids make them suitable for helping us better understand how novel MDR might transition from the environment to clinical settings, especially after sustained antibiotic exposure.

b. The potential for the creation of novel MDR plasmids

Our study suggests that host-plasmid coevolution under selection for the plasmid can act to generally stabilize multiple plasmids within a bacterial community, as well as a single host. We note that this could lead to more incidences in which genetic rearrangement between co-residing plasmids could occur. Thus, host-plasmid coevolution could also contribute to increased generation of novel MDR *plasmids* that could then conjugate into new cells as a single

transferrable unit. In Lam *et al*. 2019, the authors specifically voice concerns that MDR and virulence genes could easily become associated on a single plasmid in *K. pneumoniae*, due to the common co-occurrence of multiple plasmid types within that species[32]. Although we did not examine the MDR *K. pneumoniae* cells generated in this study for the incidence of co-integration of plasmids, we propose that this will be a fruitful direction for further research as to how (and how quickly) novel MDR plasmids can originate.

c. The effect of plasmid co-residency on plasmid stability

Our study also suggests an under-appreciated aspect of plasmid persistence when measured in the context of multiple, co-residing plasmids. In naturally occurring bacterial populations, plasmids are often found with other plasmids within a single host[20]. Yet, plasmid persistence dynamics are typically explored experimentally in the context of isogenic, single-plasmid containing strains[10–13]. This simplistic comparison may inflate the typical cost differential between cells containing the plasmid being tracked and the relevant competitor that originates from a segregational loss event. For example, in the context of our system, the fitness difference between K(p1) and K(Ø) may be sizably greater than the fitness difference between K(p1,p2) and K(p2), which would mean that K(p2) would replace K(p1, p2) more slowly than K(Ø) would replace K(p1).

Of the three parameters governing persistence of any one plasmid (i.e. conjugation, plasmid cost, and segregational loss), it is not unreasonable to assume that their absolute values shift in the context of multiple plasmids. For instance, Gama et al., 2017 suggests that conjugation rates decrease for a plasmid when it co-resides with another[33]. However, in the context of additional

hosts and plasmids, we must also consider how relative shifts, and even the addition of new parameters (i.e. the fitness of an intermediate cell type), affects our estimation of plasmid persistence dynamics.

Experimentally, and consistent with the scenario outlined above, we observed a clear increase in ancestral plasmid persistence when it was in the context of the double- vs single-plasmid type (compare ancestral trajectory in Figure 5b to 5d; and S8b to S8d; as well as the ancestral trajectory in Figure 5c to 5e; and S8c to S8e). However, in the evolved context, when there was presumably less cost associated with the plasmid, we did not see this same pattern (compare evolved trajectory in Figure 5b to 5d; and S8b to S8d; as well as the evolved trajectory in Figure 4c to 4e; and 4Sc to 4Se). In fact, in one of the four evolved comparisons, persistence even increased in the single- vs double-plasmid context (i.e. tracking $p2_E$ in the context of $K_1(p1_K,p2_E)$ and $K_1(p2_E)$; evolved trajectories in Figures S8c and S8e). Thus, coevolution appears to determine the magnitude of the epistatic effects of adding multiple plasmids to a host. At least in the ancestral case, the increased stability resulting from co-residing plasmids may provide part of the solution to the plasmid paradox, and help explain why costly plasmids can persist in bacterial populations.

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CHAPTER 3

Chipping away at the 'plasmid paradox': An exploration of plasmid persistence

Hannah Jordt[1], Sylvie Estrela[2], Olivia Kosterlitz[1], Wesley Loftie-Eaton[3], Eva Top[4], Ben Kerr[1]

[1] Biology Department, University of Washington

[2] Department of Ecology and Evolution, Yale University

[3] Roche Sequencing Solutions, Cape Town, South Africa

[4] Department of Biological Sciences, University of Idaho

Bacteria often contain genes on plasmids in addition to those on their chromosome. Typically circular, plasmids replicate independently of their host chromosome, can often be transferred horizontally to other bacteria via conjugation, and range in size from less than a kilobase (kb) to more than 2500 kb[1]. To gauge their pervasiveness, we can consider plasmid prevalence in several bacterial species that are well represented with full genome sequences in the NCBI database. Although certainly not all plasmid-containing, *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* isolates contained an average of 1, 3, and 2 different plasmids per genome, respectively[2]. In an environmental case study, 31% of bacterial isolates from Antarctic seawater and sediment samples were found to contain plasmids[3]. In another, 29% of the bacteria

isolated from the Baltic Sea were plasmid-containing[4]. Collectively, these and other examples suggest that plasmids are generally common across the bacterial domain[1].

This prevalence make sense in some environmental contexts. Plasmid genes primarily encode non-essential but ecologically relevant functions for the host, such as resistance to antibiotics or metal ions, virulence factors, detoxifying agents, or toxins that target surrounding competitors[5]. In environments where these functions are beneficial, or even necessary for bacterial hosts, plasmids can confer a substantial advantage, and thus rise to high frequency in the community. In a study tracking the abundance of plasmids from the incompatibility group IncP-1 in soils over the course of a growth season, higher plasmid levels correlated with increased pesticides in the soil[6]. In another study, plasmid-specific genes were shown to increase in frequency in soil samples treated with manure from antibiotic-treated pigs[7].

The presence of plasmids is puzzling, however, in environments with no known benefit for plasmid genes. Plasmids can impose costs on their hosts in a variety of ways, including inducing the SOS response upon conjugation[14], the conjugation process itself[15], interference with cellular processes of the host[16], and the expression of plasmid-related genes[17,18]. Considering these costs, theoretical[19–21] and experimental[15,22–24] attempts have been made to understand how plasmids can persist in bacterial populations. Ultimately, the idea of the 'plasmid paradox'[25] has resonated across the field. The first tenet of the paradox asks the question: if plasmid-genes do not confer an advantage for their host, why are they not lost to selection?

Part I. Nevertheless, they persist: Plasmids are often maintained in novel hosts

Plasmid persistence is governed by 1) the frequency of mis-segregation events in which the plasmid is lost during cell division, 2) the rate at which conjugation creates new plasmid-containing cells, and 3) the fitness differential between plasmid-containing and plasmid-free cells. If segregative loss occurs regularly, conjugation occurs infrequently, and there is a net fitness cost to possessing the plasmid, plasmids are expected to be lost over time [19,20,26–30]. We tested this expectation by measuring persistence of 18 plasmids in as many as 3 bacterial species each (Table 1). Persistence assays were carried out in triplicate for each strain in nutrient-rich lysogeny broth (LB) under batch culture conditions for 8 culture transfers (see Methods). All plasmids are conjugative and were mated into their hosts via plate conjugation assays (see Methods). Matings were attempted into each host species for all plasmids used in this study but not all host types were able to uptake all plasmids. Thus, our final strain count included 17 plasmid-containing *Escherichia coli* strains, 12 *Klebsiella pneumoniae* strains, and 5 *Shewanella oneidensis* strains.

For a subset of strains in each species, we did not detect a single plasmid-free segregant across the entire assay ('fully persistent' column at left; Figure 1). Of the 17 *E. coli* plasmid combinations, 24% were fully persistent, 41% were highly persistent (populations remained greater than 90% plasmid-containing for every replicate at every time point measured), and 35% were less persistent (the average proportion of plasmid-containing cells across replicates dropped below 90%). Of the 12 *K. pneumoniae* plasmid combination, 50% were fully persistent, 33% were highly persistent, and 17% were less persistent. Plasmids in general were more likely to drop out of the five *S.*

oneidensis hosts: 1 combination (20%) was fully persistent, whereas the remaining four host plasmid combinations (80%) fell into our ‘less persistent’ category (Figure 1).

Table 1: List and description of host strains and plasmids used in this study

Host strains/Plasmids	Incompatibility Group	Plasmid size (kb)[1]	Antibiotic Resistance Markers[2]	Host pairing[3]
Host strains:				
E. coli K12 MG1655	-	-	RifSm	-
K. pneumoniae Kp08	-	-	none added	-
S. oneidensis MR-1	-	-	none added	-
Plasmids:				
pIP135	Inc7/L/M	79	GmSmSu**Tet**Hg	Ec, Kp
Rip71a	Inc9	unknown	Ap**Cm**SmSpSuTc	Ec
R64	IncI1	121	Sm**Tet**	Ec, Kp
pB15	IncI1	50	**Km**	Ec, Kp
pIP175	IncJ2	unknown	**Amp**	Ec
N3	IncN	54	SmSpSu**Tet**Hg	Kp
pALTS27	IncP-1	59	**Tet**	Ec, Kp, So
pALTS35	IncP-1	59	**Tet**	Ec, Kp, So
RP4	IncP-1alpha	60	Ap**Km**Tc	Ec, Kp, So
pBP136.km	IncP-1beta	41	**Km**	Ec, Kp, So

pBP136.gm	IncP-1beta	41	**Gm**	Ec
pALTS29	IncP-1beta	55	**Cm**	Ec, Kp
Rts1	IncT	200	**Km**	Ec, Kp
Rsa	IncW	40	Cm**Km**	Ec
pIP1100	IncX	61	Ap**Gm**Sm	Ec
pALTS28	PromA	61	**Tet**	Ec, So
pUUH239.2	unknown	220,000	GmKmSm**Tet**	Kp
pHH1-43	unknown	unknown	AmpSm**Tet**	Ec, Kp

[1] Plasmid sizes are rounded to the nearest kb.

[2] Host markers listed are those that were added to the original bacterial strains through selection of spontaneous mutants. Plasmid markers were all originally found on the indicated plasmids, with the exception of the kanamycin and gentamycin cassettes inserted into pBP136.km and pBP136.gm, respectively. In some cases, antibiotics listed per plasmid are only those known to the authors and may not include a complete list. Antibiotics in bold are those used by the authors to select for the plasmid in all assays conducted in this chapter. Antibiotic abbreviations are as follows: Ap = ampicillin, Cm = chloramphenicol, Gm = gentamycin, Hg = mercuric ions, Km = kanamycin, Rif = rifampicin, Sm = streptomycin, Sp = spectinomycin, Su = sulfonamides, Tet = tetracycline.

[3] A list of hosts in this study that carry each plasmid. Ec = *E. coli*, Kp = *K. pneumoniae*, So = *S. oneidensis*.

For a given plasmid type, persistence was not consistent across all hosts. Of the plasmids that we tested in all three bacterial species, all belonged to the IncP-1 family of broad-host-range plasmids (Table 1). RP4 was fully or highly persistent in all hosts, whereas pBP136.km was fully persistent in *K. pneumoniae*, but less persistent in *E. coli* and *S. oneidensis*. Plasmids pALTS27 and pALTS35, which are particularly closely related, were fully or highly persistent in *E. coli* and *K. pneumoniae* hosts, but quickly dropped out in *S. oneidensis* (Figure 1).

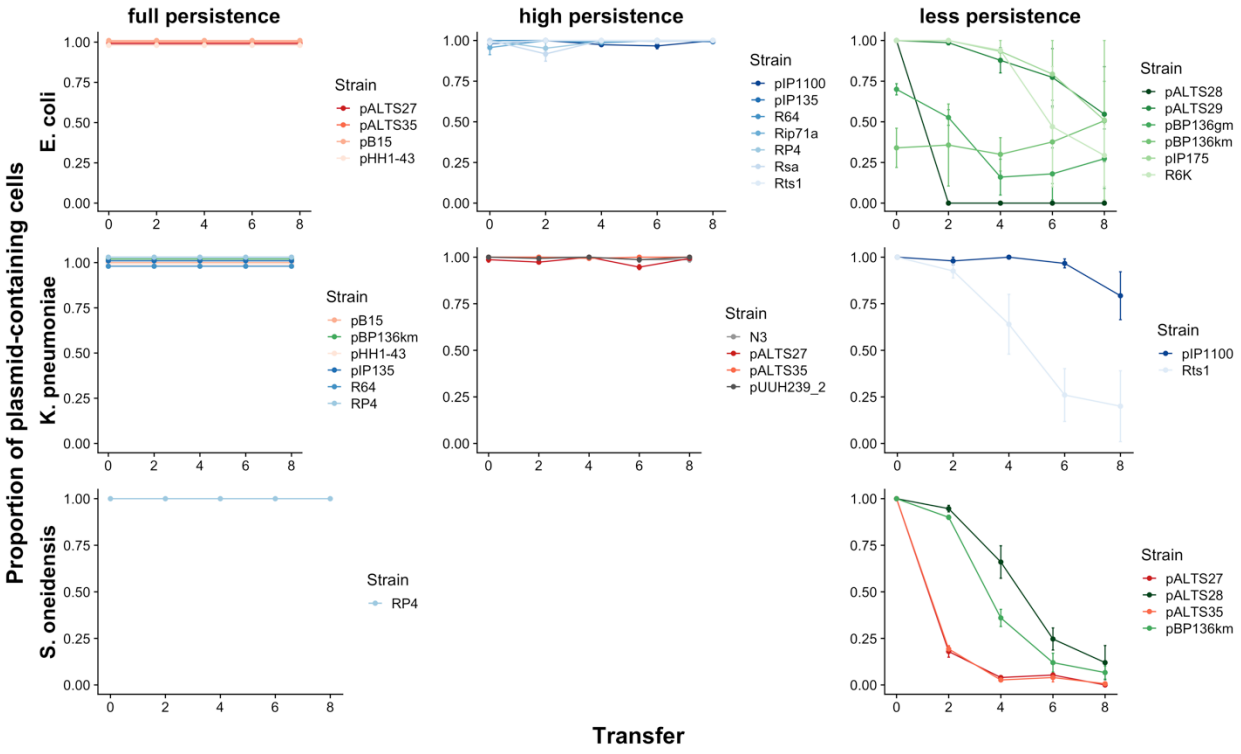


Figure 1. Plasmid persistence varies among three bacterial hosts. Populations began as 100% plasmid-containing and were propagated via serial batch culture for 8 transfers in LB. Every point is the mean of the three replicate

persistence assays conducted for each strain, and bars indicate the standard error. Plasmid persistence profiles fell into three categories: fully persistent (left column), highly persistent (the percentage of plasmid-containing cells maintained in each replicate population was >90% at every time point measured; middle column), and less persistent (the average proportion of plasmid-containing cells across replicates for each strain dropped below 90%; right column). We use the plasmid persistence level of *E. coli* strains (top row) as a color baseline, with red shaded lines indicating strains with full plasmid persistence, blue shades indicating strains with high plasmid persistence, and green shades indicating strains with less plasmid persistence. The colors used for each plasmid type then remain consistent across *K. pneumoniae* and *S. oneidensis* hosts (middle and bottom row, respectively).

Despite expectations that plasmids would be rapidly lost, most plasmids were maintained at high levels in novel hosts under conditions that ostensibly do not select for the genes harbored on the plasmids. Several explanations may be contributing to our observations. For instance, conjugation rates may be high enough to maintain high levels of persistence in some cases. Alternatively, segregative loss rates may be low enough that there is substantial delay in the production of plasmid-free cells (this might include situations in which post-segregational killing mechanisms are employed against all cells that become plasmid-free during cell division). Finally, recently introduced plasmids may not be as costly as assumed for many of the bacterial hosts. Indeed, there may even be subtle conditions favoring plasmid-containing cells (i.e. the plasmid may provide a benefit to its host despite the lack of antibiotics or other obvious plasmid-favoring element in the media). We are currently testing some of these possibilities. Nevertheless, this survey appears to contradict the common assumption that plasmids newly introduced to bacterial hosts will be lost in the (presumed) absence of selection for the plasmid.

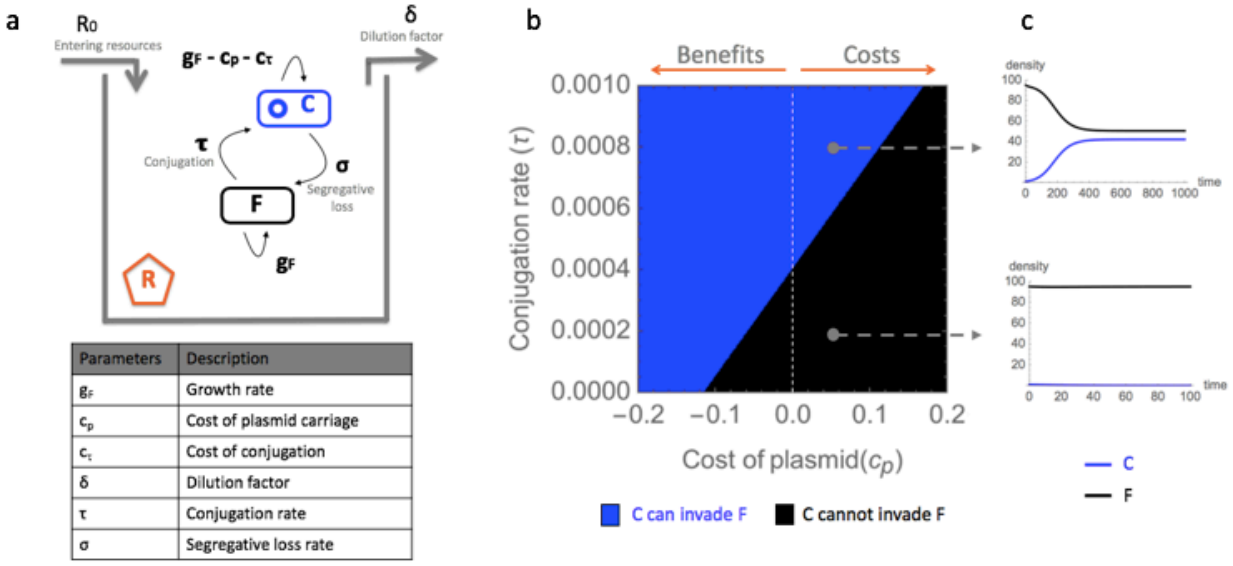
Part II. Use it AND lose it: an ecological solution to the plasmid paradox

How do we explain the continued existence of the plasmids in our experimental survey and in environmental surveys? That is, how can we resolve the plasmid paradox? The question is particularly compelling as a subset of plasmids in our survey were rapidly lost within their host population, and many other plasmids have been shown to place a fitness cost on their host[22,23,31,32] .

We are particularly interested in exploring the conditions under which conjugative plasmids can be maintained, given that conjugation is often a significant contributor to plasmid cost[15]. Conjugative plasmids are thought to make up just 15-28% of plasmids[8], yet they play a significant role in the evolution of bacterial populations[9,10] and the dissemination of genes within both localized areas[11] and around the world[12]. Understanding their persistence is of disproportionate concern for human health, due to the ability of conjugative plasmids to rapidly spread antibiotic resistance genes[12,13,33].

We first consider the conditions under which conjugative plasmids can invade a population of plasmid-free cells from rarity using a simple mathematical model of plasmid population dynamics in a well-mixed (e.g. liquid) environment. This model includes the known parameters governing conjugative plasmid/host interactions, namely cost of plasmid carriage, cost of conjugation, rate of conjugation, and rate of segregational plasmid loss. Our hypothetical population consists of just two cell types: plasmid-free cells (F) that can receive the plasmid via conjugation, and conjugative plasmid cells (C) that carry and can transfer the plasmid (see Supplemental Materials for the differential equations that govern these dynamic variables). We find that conjugative plasmid-

containing cells are able to invade a population of plasmid-free cells as conjugation rates increase and as the cost of the plasmid decreases (Figure 2).



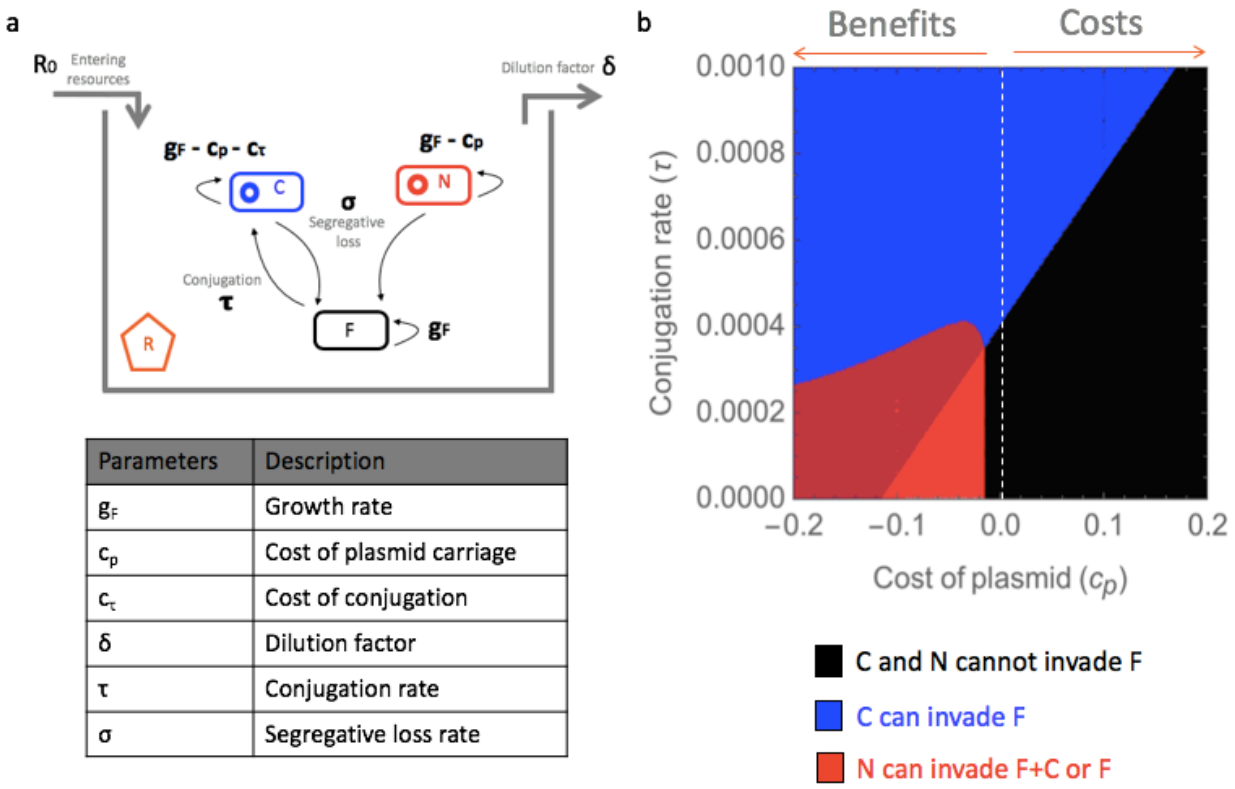
Parameters	Description
g_F	Growth rate
c_p	Cost of plasmid carriage
c_τ	Cost of conjugation
δ	Dilution factor
τ	Conjugation rate
σ	Segregative loss rate

Figure 2. Conjugative plasmid-containing cells can invade a population of plasmid-free cells as conjugation rates increase and plasmid costs decrease. a. Diagram illustrating the dynamic variables of our model (C, F, and R) and the key parameters that govern the transition of cell types (alongside arrows; for full model description see Supplemental Materials). C carries a conjugative plasmid that encodes a trait that *can* be beneficial to their host (e.g. antibiotic resistance). F refers to plasmid-free cells. R refers to the resources available in the environment. All dynamic variables can be thought of as being contained within a chemostat-like environment, with resources entering the system at R_0 and cells being diluted at factor δ in a continuous manner. **b.** Results of an invasion analysis highlighting the parameter values for cost of plasmid and conjugation rate under which C can invade F (blue). As cost of plasmid increases and conjugation rate decreases, C cannot invade F (black). We consider plasmid costs (c_p) less than 0 to indicate that the plasmid confers a benefit to the host. **c.** Results of numerical simulations showing the density of cells as a function of time under the specific parameter values indicated by the grey dot, i.e., for $\tau = 0.0008$ and $c_p = 0.05$,

C will invade F (upper graph). For $\tau = 0.0002$ and $c_p = 0.05$, C cannot become established in the population (lower graph). Note $c_\tau = 0.1$ for all graphs.

It is clear from our survey of parameter space (Figure 2b) that conjugative plasmids can be readily maintained when plasmid costs are negative (i.e. confer a benefit), even considering a consistent influx of plasmid-free cells from plasmid-containing cells via segregational loss. However, from this situation arises another aspect of the 'plasmid paradox': given the costs associated with plasmid carriage, why are beneficial genes located on plasmids not eventually incorporated into the host chromosome[20]?

To explore this question, we incorporate non-conjugative plasmid-containing cells (N) as a third cell type into our mathematical model. These cell types are also non-mobilizable and can only transmit the plasmid vertically (i.e. during cell division). Thus their growth rates are not hindered by the costs associated with conjugation (c_p in our model) and their plasmid genes serve as a proxy for an accessory region on the chromosome. We find that, from rarity, N is able to invade a population of F cells or a population of F and C cells when conjugation rates of C are sufficiently low, and when plasmid costs are negative (i.e. plasmids confer a benefit to their hosts). Furthermore, at such lower conjugation rates, C is never able to establish in a population (Figure 3).



Parameters	Description
g_F	Growth rate
c_p	Cost of plasmid carriage
c_τ	Cost of conjugation
δ	Dilution factor
τ	Conjugation rate
σ	Segregative loss rate

Figure 3. Conjugative plasmid-containing cells cannot establish in a population of plasmid-free cells or plasmid free cells and non-conjugative plasmid-containing cells if conjugation rates are relatively low. **a.** Diagram illustrating the dynamic variables of our model (N, C, F, and R) and the key parameters that govern the transition of cell types (for full model description see Supplemental Materials). C carries a conjugative plasmid that encodes a trait that *can* be beneficial to its host (e.g. antibiotic resistance). N carries a non-conjugative version of the same plasmid. F refers to plasmid-free cells. R refers to the resources available in the environment. All dynamic variables can be thought of as being contained within a chemostat-like environment, with resources entering the system at R_0 and cells being diluted at factor δ in a continuous manner. **b.** Results of an invasion analysis highlighting the parameter values for cost of plasmid and conjugation rate under which C can invade F (blue), N can invade F or F+C (red) or C and N cannot invade F (black). We consider plasmid costs (c_p) less than 0 to indicate that the plasmid confers a benefit to the host. Note c_τ is held constant at 0.1

From this simple model we might conclude that genes can only be maintained on conjugative plasmids when the gene confers a benefit to the host and/or conjugation rates are relatively high. Empirically, there are examples of high conjugation rates driving plasmid persistence in the absence of selection for the plasmid[34]. Indeed, for this study, we attempted to experimentally compete C, N, and F type *E. coli* cells in a plasmid disfavoring environment (i.e. lacking antibiotic selection for the plasmids) and found that C was as prevalent as F throughout the duration of the assay due to sufficiently high conjugation rates (see Supplemental Materials). However, high conjugation cannot fully account for the maintenance of conjugative plasmid-mediated genes in all cases[20,35]. In other words, some conjugative plasmids exist in the area of parameter space where we would not expect conjugative plasmids to be able to establish. We use our mathematical model to test a hypothesis for how genes could be maintained on conjugative plasmids in the absence of selection on those genes, even when conjugation rates are low.

Specifically, we hypothesize that *costly, conjugative plasmids are maintained in an environment with alternating selection*. Furthermore, this can occur even when non-conjugative, less costly versions of the same plasmid (in our study acting as a stand-in for the 'chromosomal variant') are competing in the same community. The rationale for this prediction is as follows: when switching from an environment that does not favor the plasmid-encoded genes (plasmid-disfavoring environment) to an environment that favors the genes (plasmid-favoring environment), cells with conjugative plasmids will increase relative to cells with non-conjugative plasmids, as long as there are many plasmid-free cells to move into. When the environment switches back to a plasmid-disfavoring environment, plasmid-free cells (e.g., generated through segregative loss of the plasmid) will be selected over both types of plasmid-containing cells. As long as the cycle is renewed, the population will be replenished with new plasmid-free cells (potential hosts), ensuring

that conjugation remains advantageous. Thus, a gene on a conjugative plasmid is predicted to be favored, relative to the same gene in a non-conjugative state, when it alternates between environments in which cells can "use it" and "lose it."

To test our hypothesis theoretically we use our mathematical model involving C, N, and F. We consider three environmental scenarios: a constant plasmid-favoring environment (*env 1*); a constant plasmid-disfavoring environment (*env 2*), and an alternating environment (*env 3*) where *env 1* and *env 2* are applied alternatively and cyclically. As expected, we find that N dominates in *env 1* (Figure 4a) while F dominates in *env 2* (Figure 4b). In agreement with our prediction, we find that C is maintained in the alternating environment (*env 3*). If the period of switch is short, all strains are maintained in the population (Figure 4c). Interestingly, if the period spent in each environment is increased, C can displace N (Figure 4d). Thus, in systems which could not previously have supported conjugative plasmid-containing cells (recall lower area of Figure 3b), alternating selection can allow them to periodically dominate and thus be maintained.

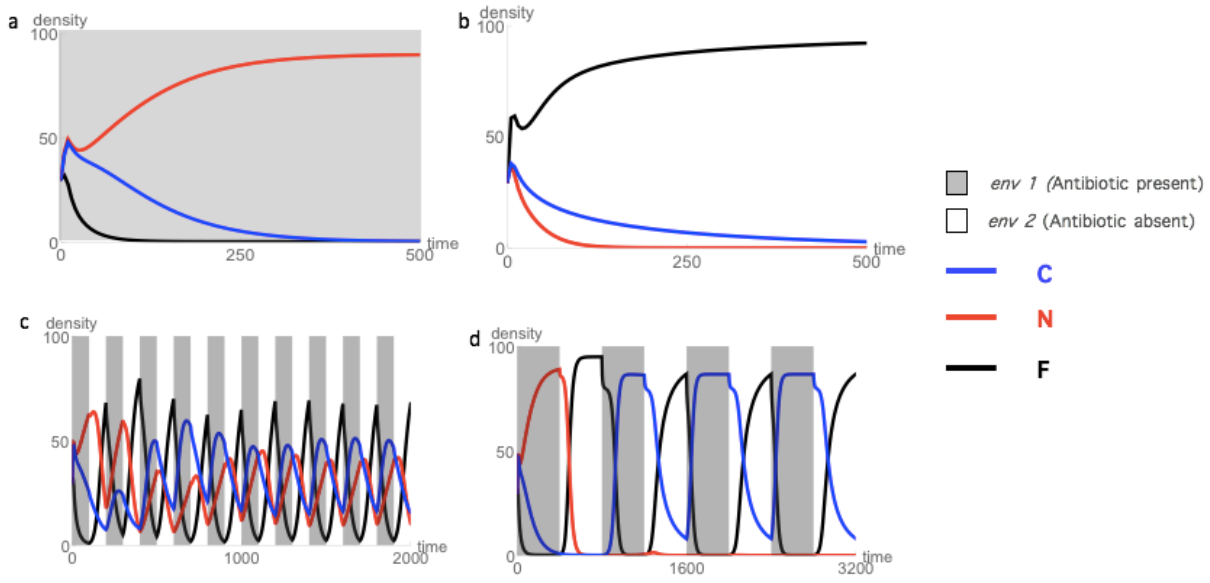


Figure 4. Conjugative plasmids can be maintained under alternating selection. Results of our numerical simulations showing the density of cells as a function of time given the parameter values $\tau = 0.0005$, $\sigma = 0.00005$, $c_\tau = 0.025$, c_p in *env 1* = -0.05, c_p in *env 2* = 0.125, g_F in *env 1* = 0.15, g_F in *env 2* = 0.3. **a.** Cell types either compete in a constant environment which selects for the trait encoded in the plasmid (*env 1*; grey background), **b.** in a constant environment that selects against the trait encoded in the plasmid (*env 2*; white background); **c. & d.** or in an alternating environment (*env 3*) that switches and cycles between *env 1* and *env 2* (each environment lasts for a period of time p_s).

Our findings in Part II have direct implications for the practice of antibiotic cycling in the medical field as a means of avoiding drug resistance. Indeed, if experimental findings agree with the predictions derived from our hypothesis and model, this would strongly suggest that antibiotic cycling, a current practice to minimize evolution of antibiotic resistance[36], may actually contribute to persistence of mechanisms to horizontally transmit resistance genes.

Methods

Mating assays. To obtain our plasmid-containing strains, each plasmid was introduced separately into the *E. coli*, *K. pneumoniae*, and *S. oneidensis* hosts (here recipients) via various *E. coli* host strains (here donors) that originally contained each plasmid. Recipient and donor strains were grown up from archived stocks in the presence of the appropriate plasmid-selecting antibiotic. One ml of each saturated culture was spun down for 5 minutes at 6000 rpm and washed with 1 ml 0.086% saline twice. Five µl of each donor and recipient were added as a pair to the top of LB agar-filled microtiter wells and allowed to incubate at 30°C for 3 hours. One hundred µl of 0.086% saline was then gently pipetted up and down on the surface of the well to dislodge the bacteria and resuspend the cells. A subset of these cells were then diluted and plated for single colonies on LB agar supplemented with the appropriate plasmid-selecting antibiotic. If the recipient was E. coli, 50µg/mL rifampicin and 100µg/ml streptomycin were also added to the selective plate to select for transconjugants, as the recipient cells were resistant (and the donor host cells sensitive) to these

antibiotics. For *K. pneumoniae* and *S. oneidensis* recipients, Kosar citrate agar plates and Pseudomonas isolation agar plates were used, respectively, to select for transconjugants, as *E. coli* cells are inhibited by both media types. A single colony was selected from amongst the transconjugants for each mating and streaked onto fresh agar plates. From these, a single colony was again selected and preserved at -80°C in 11% glycerol to serve as the archived population from which we innoculated our persistence assays.

Plasmid persistence assays. All plasmid-containing strains were inoculated from clonal isolates archived at -80°C into microtiter plates wells containing 300 µl of LB supplemented with the appropriate antibiotic to select for the plasmid for that strain. After the initial overnight growth cycle under plasmid-selective (antibiotic) conditions, 5 µl of each population was then transferred every 24 hours for 9 days into 295 µl fresh LB with no antibiotic supplementation. On transfers 0, 2, 4, 6, and 8, overnight populations were diluted and plated for single colonies onto LB agar plates. Fifty colonies from each plate were phenotypically assayed for the presence of the plasmid-associated ABR gene by stabbing each colony with a toothpick and streaking it first onto an antibiotic-containing plate and then onto an antibiotic-free plate (LB agar). The latter was done to ensure that bacteria had been transferred from the colony plate to the streak plate. Streaks producing bacterial growth on the antibiotic-containing plate after overnight incubation were considered ‘plasmid-containing’; streaks producing no bacterial growth on the antibiotic-containing plate but *positive* growth on the LB plate were considered ‘plasmid-free’.

Supplementary Materials:

Mathematical Model

Differential equations governing each of our dynamic variables: resource (R) and 3 types of cells: plasmid-free cells (F), non-conjugative plasmid-carrying cells (N) and conjugative plasmid-carrying cells (C).

$$\frac{dR}{dt} = \delta R_0 - \Upsilon\left(\frac{g_C R}{\kappa_C + R} C + \frac{g_N R}{\kappa_N + R} N + \frac{g_F R}{\kappa_F + R} F\right) - \delta R$$

$$\frac{dF}{dt} = \frac{g_F R}{\kappa_F + R} F - \tau CF + \sigma(C + N) - \delta F$$

$$\frac{dC}{dt} = \frac{g_C R}{\kappa_C + R} C + \tau CF - (\sigma + \delta) C$$

$$\frac{dN}{dt} = \frac{g_N R}{\kappa_N + R} N - (\sigma + \delta) N$$

where $g_F = g_{max}\,; g_C = g_{max} - \mathrm{c}_p - c_\tau; g_N = g_{max} - \mathrm{c}_p$.

Table S1: Description of model parameters and variables

Symbol	Definition
R	Resource
F	Plasmid-free cell
C	Cell carrying a conjugative plasmid
N	Cell carrying a non-conjugative plasmid
g_{max}	Intrinsic maximum growth rate
τ	Conjugation (transfer) rate
σ	Segregative loss rate
c_p	Cost of plasmid maintenance
c_τ	Cost of conjugation
δ	Dilution rate
κ	Half-velocity constant of growth
Y	Yield coefficient

Experimentally testing the prediction that conjugation rates alone can explain the maintenance of conjugative plasmids

We tested our full model experimentally using *E. coli* K-12 strain BW25113 and the conjugative IncF plasmid F'42. To create the non-conjugative version of the plasmid, we used lambda red recombineering to knock out *traB*, a plasmid gene that encodes for an essential structural protein

of the conjugative pilus. We simultaneously recombineered a tetracycline-resistance cassette into both the conjugative and non-conjugative versions of the plasmid to serve as the mode of selection in the plasmid-favoring environment, and a chloramphenicol-resistance cassette into the non-conjugative version so we could distinguish the two plasmid types. All genetic manipulations were confirmed for the appropriate insertions and deletions via Sanger sequencing.

To pre-adapt our host strain to the appropriate media conditions, we evolved it for 100 generations prior to introducing our IncF plasmids through mating. We obtained our non-conjugative strain by using a traB complement plasmid expressing *traB* to assist the conjugation of the non-conjugative plasmid into the host during the mating procedure.

Plasmid persistence was assessed in the final versions of each strain. The non-conjugative plasmid had higher persistence than the conjugative version, suggesting it was less costly for the host by being able to save on the costs associated with conjugation (Figure S1). These results are consistent with the assumptions made in our mathematical model.

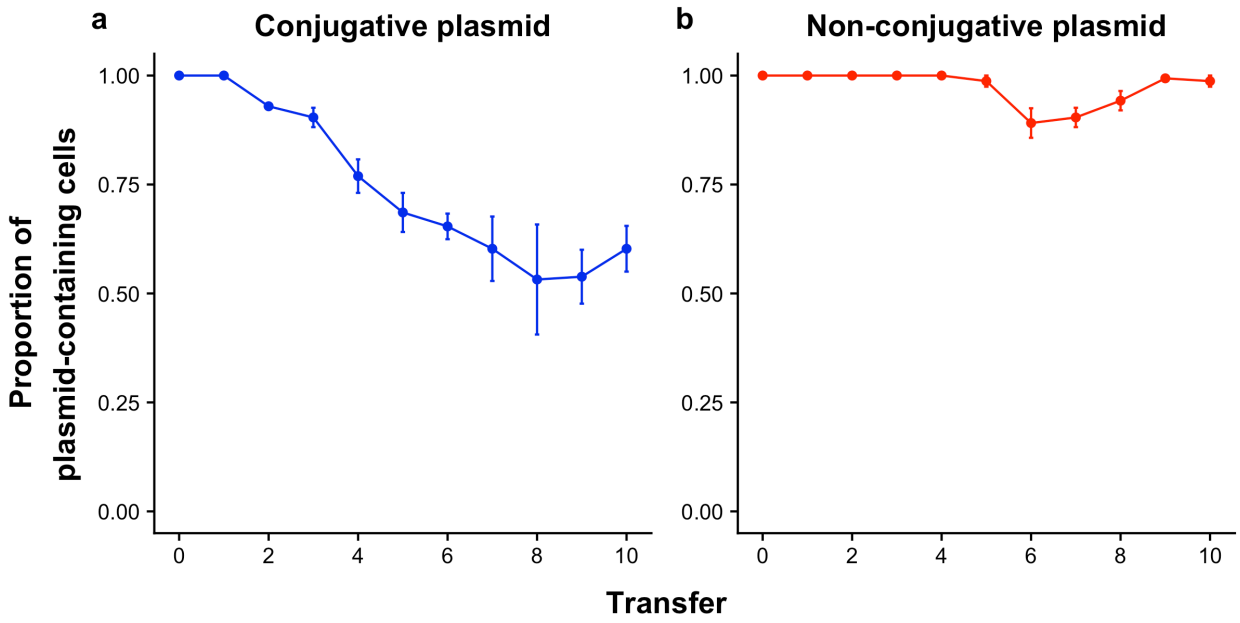


Figure S1. The conjugative IncF plasmid was less persistent than the non-conjugative version. Populations began as 100% plasmid-containing and were propagated via serial batch culture for 10 transfers in LB (see Methods from

main text for fuller description of persistence assay). Every point is the mean of the three replicate persistence assays conducted for each strain, and bars indicate the standard error.

To test whether conjugative plasmid-containing cells were maintained at the lowest frequency in a non-plasmid-favoring environment compared to plasmid-free and non-conjugative plasmid-containing cells, we mixed all types and propagated the mixture for 5 transfers. Contrary to our expectations, conjugative plasmid-containing cells were maintained at higher frequencies than plasmid-free or non-conjugative plasmid-containing cells for the entire duration of the assay (Figure S2). This result is inconsistent with our model predictions, which points to a mismatch between our experimental system and the hypothesis we wish to test. Given the mismatch, we did not proceed to test the system under alternating conditions. Instead, our experimental results suggest that the rate of conjugation was much higher than in our model runs and we come to an entirely different conclusion: some conjugative plasmids can be maintained, at least in the short term, by high levels of conjugation, even in environments that are not plasmid-favoring. This suggests a plasmid-as-parasites solution to the plasmid paradox, in which plasmids can spread rapidly through populations despite the costs associated with their maintenance.

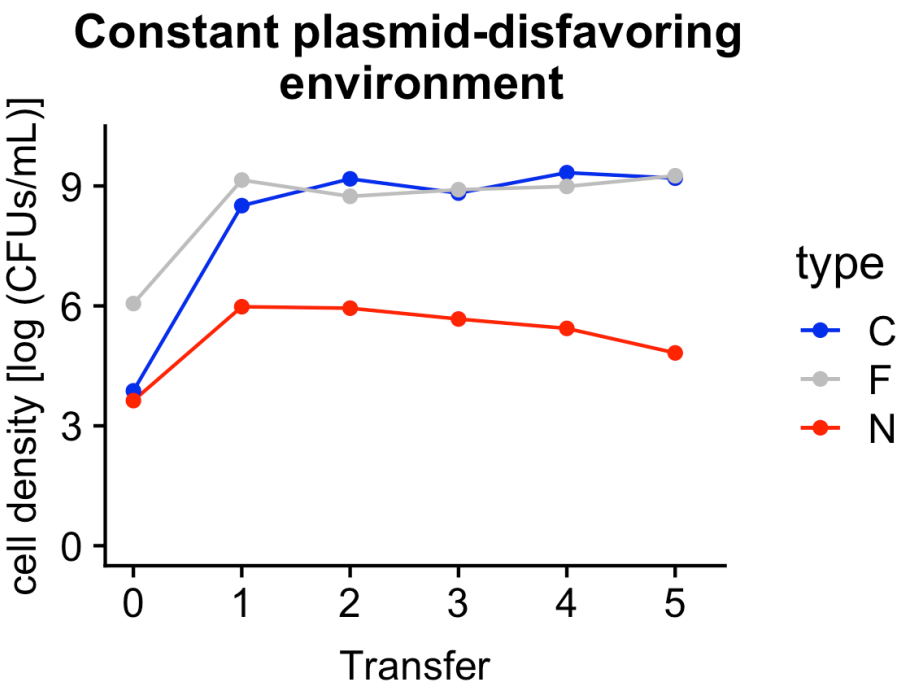


Figure S2. Conjugative plasmid-containing cells (C) were readily maintained in a mixed culture of C, F and N cell types, even without antibiotic selection for the plasmid. Cells were grown from archived isogenic populations of C, F, and N overnight in antibiotic selection for the plasmid, if appropriate. One ml of each saturated culture was spun down for 5 minutes at 6000 rpm and washed with 1 ml 0.086% saline twice, then diluted and mixed at a 1:100:100 volume (for F:C:N) in triplicate at Transfer 0. Replicate mixtures where then propagated via serial batch culture for 5 transfers in microtiter plates in non-selective media for the plasmids (i.e. LB). After each growth cycle, cultures were diluted and plated for single colonies on agar plates containing either LB, chloramphenicol, or tetracycline. Density of C was determined by subtracting the number of cells that grew on the chloramphenicol plates from the number of cells that grew on the tetracycline plates (given that N was resistant to tetracycline and chloramphenicol. The number of F cells were calculated by subtracting the number of cells that grew on the tetracycline plates from the number of cells growing on LB plates.

In summary of Chapters 2 and 3:

Understanding why plasmids exist is vital if we hope to understand how and when plasmids transmit genes throughout the prokaryotic world, especially given the urgency with which we must work to combat the spread of plasmid-mediated antibiotic resistance. Given the costs associated with plasmid carriage, I have focused my dissertation on addressing the question: 'what allows plasmids to exist?' I have found that there are at least four answers:

(i) Host-plasmid coevolution under sustained selection for the plasmid can alleviate plasmid costs and allow plasmids to persist in the absence of selection for the plasmid (chapter 2).

(ii) Initial fitness differentials between plasmid-containing cells and plasmid-free cells may be less than expected. This could be either because the intrinsic cost of the plasmid is low (a potential explanation for the results in chapter 3: Part I) or because co-residency of multiple plasmids in the same host cell doesn't allow the host to experience a significant gain in fitness when one of the plasmids is lost during segregation and thus it takes longer for the double-plasmid containing cells to be displaced by single-plasmid containing cells and then ultimately plasmid-free cells (chapter 2).

(iii) A plasmids-as-parasites model can explain the maintenance of conjugative plasmids if conjugation rates are high enough (for theoretical evidence, see chapter 3: Part II; for experimental evidence, see chapter 3: Supplemental Materials).

(iv) Conjugative plasmids can be maintained in environments with alternating selection by maintaining an advantage for genes that are able to spread via horizontal gene transfer over genes that can only be transmitted vertically (chapter 3, Part II).

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VITA

Hannah Jordt received her training in biology from the University of Washington, both as an undergraduate and graduate student. She received her training in education from the University of Mississippi as a masters student, and from her students in the Mississippi Delta and the classrooms of UW. She is especially grateful to her students for teaching her the most.