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TRADEOFF BETWEEN HORIZONTAL AND VERTICAL MODES OF TRANSMISSION IN BACTERIAL PLASMIDS

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Abstract.—It has been hypothesized that there is a fundamental conflict between horizontal (infectious) and vertical (intergenerational) modes of parasite transmission. Activities of a parasite that increase its rate of infectious transmission are presumed to reduce its host's fitness. This reduction in host fitness impedes vertical transmission of the parasite and causes a tradeoff between horizontal and vertical transmission. Given this tradeoff, and assuming no multiple infections (no within-host competition among parasites), a simple model predicts that the density of uninfected hosts in the environment should determine the optimum balance between modes of parasite transmission. When susceptible hosts are abundant, selection should favor increased rates of horizontal transfer, even at the expense of reduced vertical transmission. Conversely, when hosts are rare, selection should favor increased vertical transmission even at the expense of lower horizontal transfer. We tested the tradeoff hypothesis and these evolutionary predictions using conjugative plasmids and the bacteria that they infect. Plasmids were allowed to evolve for 500 generations in environments with different densities of susceptible hosts. The plasmid's rate of horizontal transfer by conjugation increased at the expense of host fitness, indicating a tradeoff between horizontal and vertical transmission. Also, reductions in conjugation rate repeatedly coincided with the loss of a particular plasmid-encoded antibiotic resistance gene. However, susceptible host density had no significant effect on the evolution of horizontal versus vertical modes of plasmid transmission. We consider several possible explanations for the failure to observe such an effect.

Key words.—Antibiotic resistance, conjugation, *Escherichia coli*, experimental evolution, host density, parasite, plasmid, tradeoff, transmission mode, virulence.

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Horizontal transmission occurs when a parasite moves from an infected to an uninfected individual, whether by direct contact or an infectious particle. Vertical transmission occurs when an infected individual reproduces (either sexually or asexually), giving rise to progeny that also harbor the infection. Activities of a parasite that increase its rate of infectious transmission (e.g., greater production of infectious particles) are often thought to reduce host fitness and thus limit the vertical transmission of the parasite. Consequently, an evolutionary tradeoff between vertical and horizontal modes of transmission has been hypothesized (Levin and Lenski 1983; May and Anderson 1983; Bull 1994; Ewald 1994).

Parasite virulence is a relative term and can be defined as the reduction in host fitness due to infection (Levin and Lenski 1983; May and Anderson 1983; Bull et al. 1991; Herre 1993; Bull 1994; Ewald 1994). Informal arguments relying on group selection once led to widespread acceptance of the notion that parasites inevitably evolve toward a state of attenuated virulence (e.g., Burnet and White 1972). However, more recent studies contradict the idea that parasites inevitably evolve toward benign coexistence with their hosts (Levin and Pimentel 1981; Ewald 1983, 1987; May and Anderson 1983; Herre 1993; Bull 1994; Lenski and May 1994). Many of these studies indicate that the availability of uninfected hosts in the environment is a key factor in determining the evolution of parasite virulence. When the density of uninfected hosts is high, the expected time for parasite transmission from an infected to an uninfected host is short. Hence, it is advantageous for the parasite to maximize its transmission to new hosts, regardless of the effect of this transmission on its current host's fitness, and so selection favors more virulent forms of the parasite. However, when the density of uninfected hosts is low, the time required for transmission to a new host is greater or the probability of transmission lower. In that case, a parasite benefits from doing little damage to its present host, and any parasite that harms its present host too much may reduce its own potential for infectious transmission. By the same logic, if a parasite can be transmitted vertically as well as horizontally, then vertical transmission becomes relatively more important, and thus selectively favored, when uninfected hosts are rare.

Experimental System

Plasmids are typically circular, extrachromosomal DNA molecules able to replicate autonomously within a bacterium. Many bacterial traits important for medicine, agriculture, commerce, and the environment are, in fact, plasmid-encoded: antibiotic resistance, nitrogen fixation, antibiotic production, and pollutant biodegradation (Hardy 1986; Kinashi et al. 1987). Although plasmids are ubiquitous in natural populations of bacteria, they are dispensable to their hosts under many conditions. All plasmids replicate using the cellular machinery of their hosts and are thus transferred vertically. Sometimes vertical transmission is uneven, such that one of the two daughter cells does not receive a copy of the plasmid, a process known as segregation. However, various plasmid-encoded mechanisms exist to promote fidelity of vertical transfer, and so spontaneous segregation usually occurs with a low probability (Helinski et al. 1996). In the absence of selection for plasmid-encoded traits such as antibiotic resistance, most plasmids reduce host fitness relative to plasmid-free counterparts (Levin 1980; Dykhuizen and Hartl 1983; Levin and Lenski 1983; Lenski and Bouma 1987; Bouma and Lenski 1988; Lenski and Nguyen 1988; Nguyen et al. 1989;

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Modi and Adams 1991). Most plasmids are also able to transfer horizontally from an infected cell (donor) to an uninfected cell (recipient) through a process called conjugation (Lederberg 1956). Although some aspects of conjugation remain poorly understood, it requires contact between donor and recipient cells, and it is mediated by plasmid-encoded structures called pili. Thus, conjugative plasmids can be transmitted by two distinct modes: horizontal transmission occurs by conjugation, whereas vertical transmission occurs by host cell division.

A Priori Expectations

We define plasmid "virulence" as the magnitude of reduction in host fitness due to plasmid carriage. Activities of a plasmid that increase its horizontal transmission (such as production of more pili) should generally increase its virulence, thereby reducing the plasmid's rate of vertical transmission. Thus, there exists a conflict between the two modes of transmission available to conjugative plasmids.

A simple mathematical model shows how susceptible host density should influence the evolution of a plasmid's mode of transmission. Five assumptions are implicit in the model: (1) the density of susceptible hosts is constant; (2) hosts cannot be multiply infected, so within-host competition is unimportant; (3) horizontal transfer follows mass-action kinetics; (4) plasmid segregation occurs at a negligible rate; and (5) there is a genetic tradeoff between the rates of horizontal and vertical plasmid transmission. Let H and P be the densities of plasmid-free and plasmid-bearing hosts, respectively. If γ is the rate of conjugative transmission and m is the growth rate of plasmid-bearing cells, then the growth rate of the plasmid-bearing population is $dP/dt = mP + \gamma HP$, and the corresponding per capita rate is $r = m + \gamma H$. The components of r show that the rate of vertical spread (m) is independent of susceptible host density, whereas the rate of horizontal spread (γH) is proportional to the density of potential recipients. (The effect of segregation, if any, is essentially equivalent to a reduction in m.) A plasmid can be maintained in a bacterial population provided that the rate of horizontal transfer is sufficiently high to offset losses due to its harmful effects on host fitness and segregation (Stewart and Levin 1977; Levin 1980; Levin and Lenski 1983).

To illustrate the importance of the tradeoff between horizontal and vertical transmission, consider two hypothetical plasmids, A and B. Plasmid A conjugates at the rate $\gamma_A = 1$, and the growth rate of hosts infected by A is $m_A = 1$. Plasmid B conjugates at the higher rate $\gamma_B = 1.5$, but it reduces the growth rate of its infected hosts to $m_B = 0.5$. Figure 1 shows their horizontal, vertical, and net rates of transmission as a function of the density of susceptible hosts. If $H < 1$, then $r_A > r_B$ and plasmid A prevails by virtue of its greater vertical transmission. But if $H > 1$, then $r_A < r_B$ and plasmid B prevails due to superior horizontal transmission. Whether selection favors a more or less virulent plasmid thus depends on the abundance of susceptible hosts.

Lipsitch et al. (1995, 1996) extended this simple analysis to include equilibrium conditions as well as rates of invasion. They found that increased opportunities for horizontal transmission may actually *reduce* the optimal virulence at equilibrium. This counterintuitive effect occurs because "as horizontal transmission increases, the fraction of the population infected increases; thus, vertical transmission actually becomes more important" (Lipsitch et al. 1996, p. 1731). If one sought to increase the opportunity for horizontal transmission simply by raising the overall host density, one might fail because a higher fraction of the host population would be infected at equilibrium. However, to avoid precisely this complication in our experiments, we increased the opportunity for horizontal transmission by providing an *influx of uninfected hosts.*

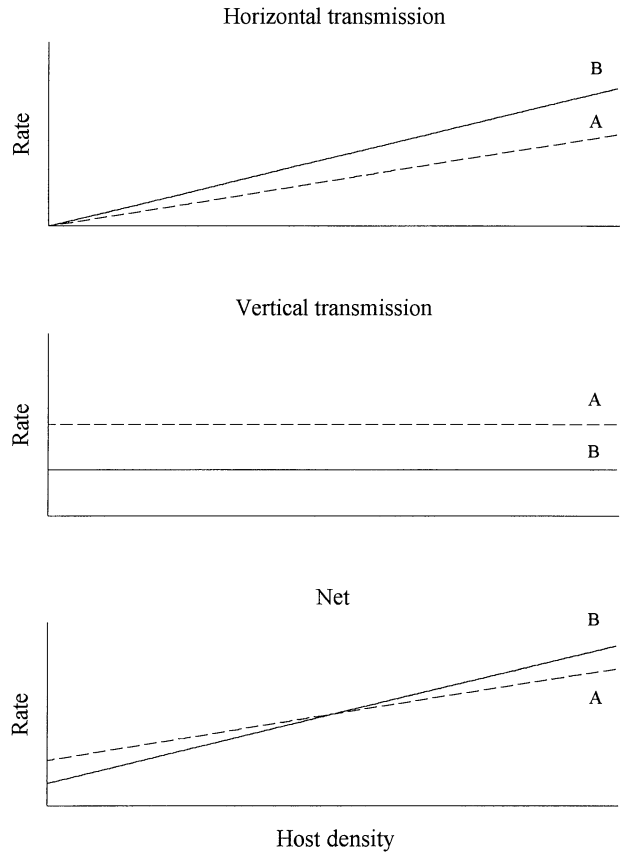

FIG. 1. Hypothesized horizontal, vertical, and net rates of increase for two plasmid genotypes, A and B, as a function of susceptible (uninfected) host density. The more virulent plasmid B is favored only when susceptible hosts are sufficiently abundant. See text for details.

Experimental Overview

Bacteria such as *Escherichia coli* provide an excellent system for testing evolutionary theories. They have short generation times and large population sizes, so that one can observe evolution in action. They can be stored in a state of suspended animation, thus allowing direct comparisons between ancestral types and their evolutionary descendants. Also, because they have been widely used in genetic and molecular research, much is known about the mechanistic basis of their interactions with parasitic elements such as plasmids. Thus, bacteria and their plasmids provide an attractive system to study host-parasite interactions and the

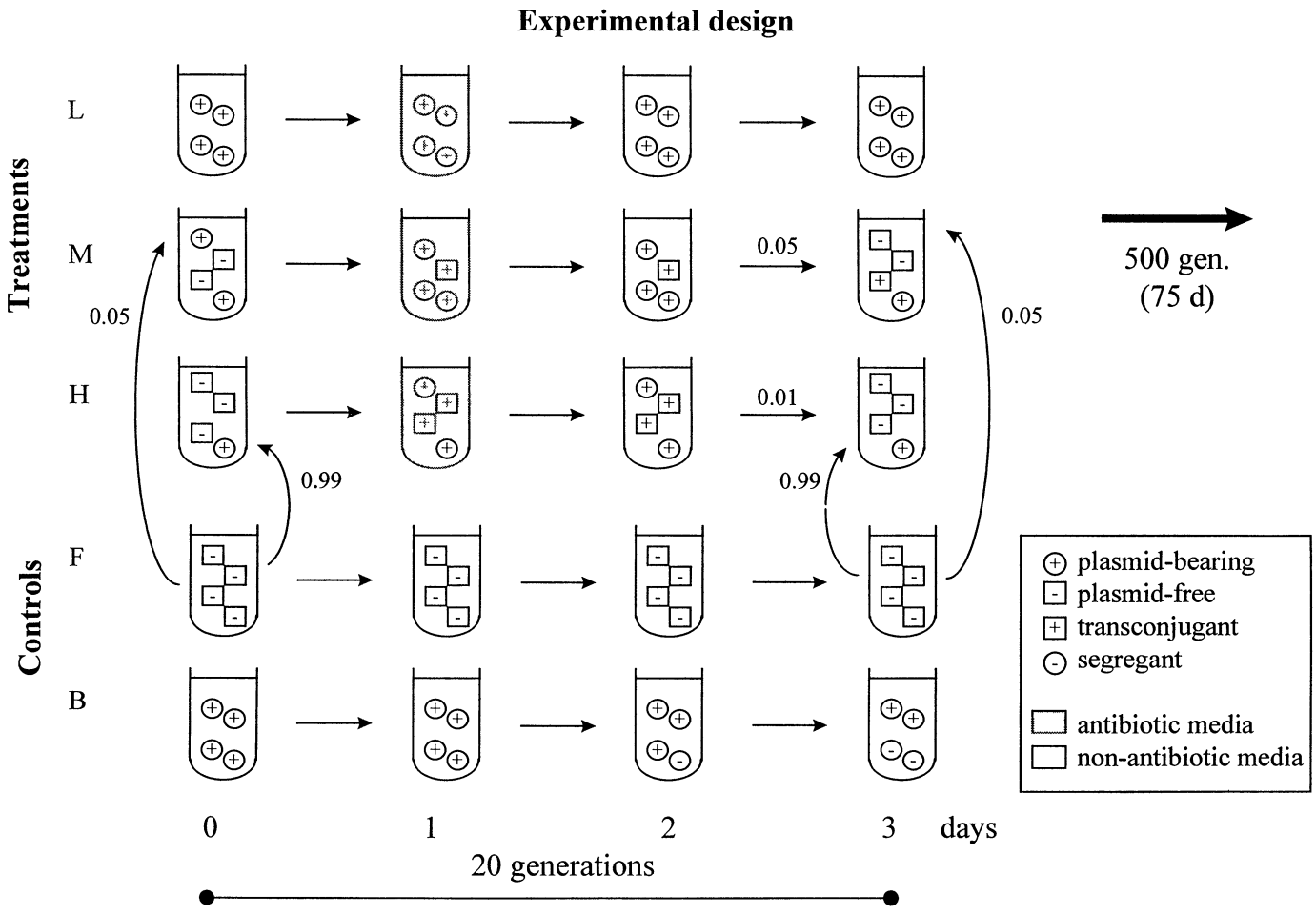

FIG. 2. Summary of the propagation schemes for the control and experimental treatment groups. The schematic shows the composition of each population prior to serial transfer. All arrows indicate standard serial transfer (0.1 mL into 9.9 mL of fresh medium) unless otherwise indicated. There were three replicate populations in each group, and all of the populations were propagated for 75 days. See text for details.

evolution of virulence (Levin and Lenski 1983; Bouma and Lenski 1988; Bull et al. 1991; Modi and Adams 1991).

We performed a 500-generation experiment to examine tradeoffs between vertical and horizontal modes of plasmid transmission and the influence of susceptible host density on the evolution of plasmid virulence and mode of transmission. We used pB15, a plasmid that was reported to be stably maintained in chemostat populations of *E. coli* by a high rate of horizontal transmission (Lundquist and Levin 1986). In our study, replicated populations of *E. coli* infected with pB15 were serially propagated in batch culture environments and were subjected to different levels of immigration of susceptible (plasmid-free) hosts. One treatment never received plasmid-free immigrants, while in two other treatments the ratio of susceptible immigrants to infected residents was 1:1 or 100:1 at 20-generation intervals. Thus, we expected the first treatment to favor vertical transmission exclusively, whereas the other two treatments should favor horizontal transmission to different degrees. To ensure that the resident bacteria retained their plasmids, all three treatments were periodically exposed to an antibiotic, which prevented take-over by plasmid-free immigrants or segregants. Our experiment also had two controls, neither of which received any immigrants or was ever exposed to antibiotic. One control contained plasmid-free *E. coli* that served as the source of immigrants for the treatments described above. The other contained cells that were initially infected with pB15, but the population was vulnerable to takeover by plasmid-free segregants that arise spontaneously and may have a selective advantage in the antibiotic-free medium (Lenski and Bouma 1987). Figure 2 summarizes the five treatment and control groups, each of which had three replicate populations. To evaluate whether the availability of susceptible hosts affected plasmid evolution, we measured changes in conjugation rate (Simonsen et al. 1990) and in the cost of plasmid carriage to the host (Bouma and Lenski 1988; Lenski et al. 1994). We expected both to increase as the result of evolution when susceptible hosts were abundant (due to high rates of immigration) relative to when susceptible hosts were rare.

MATERIALS AND METHODS

Bacteria and Plasmid

Table 1 gives pertinent features of the bacterial strains used in this study. All were derived from a single clone of *E. coli*

TABLE 1. Key bacterial strains used in this study.

Strain	Relevant properties[a]	Abbreviation
REL1206	plasmid-free *Escherichia coli* B	Ara^-_0
REL1207	spontaneous Ara^+ mutant of REL1206; ancestral immigrant	Ara^+_0
REL5382	transconjugant of REL1206 using pB15; ancestral resident	Ara^-_0/p_0
REL5384	transconjugant of REL1207 using pB15	Ara^+_0/p_0
PET319	spontaneous Nal^r mutant of REL1206	Ara^-_0/Nal^r
PET318	spontaneous Nal^r mutant of REL1207	Ara^+_0/Nal^r
PET354	transconjugant of PET318 using pB15	$Ara^+_0/Nal^r/p_0$

[a] Ara^+ indicates the ability to grow on L-arabinose. Nal^r denotes resistance to nalidixic acid; all strains are Nal^s unless otherwise indicated.

B (REL1206), which evolved previously for 2000 generations in a defined environment; this strain cannot grow on L-arabinose (Lenski et al. 1991). A spontaneous arabinose-utilizing mutant (REL1207) was obtained previously (Bennett et al. 1992). Henceforth, REL1206 and REL1207 are designated Ara^-_0 and Ara^+_0, respectively, where the subscript 0 denotes their ancestral status in this evolution experiment. Ara^- and Ara^+ strains form red and white colonies, respectively, on tetrazolium-arabinose (TA) indicator plates (Levin et al. 1977). The Ara marker has been shown previously to be selectively neutral, or nearly so, under culture conditions similar to those in this study (Bennett et al. 1992). In the Results, we show that this marker is also effectively neutral in the context of our experiment.

The conjugative plasmid used in this study, pB15, was obtained from B. R. Levin (Emory University, Atlanta, GA). It is a large plasmid (~80 kb), but the average number of plasmid copies per cell is unknown (Lundquist and Levin 1986). This plasmid was isolated from an *E. coli* sampled from a human under antibiotic treatment; it confers resistance to kanamycin (Kan^r) and tetracycline (Tet^r). Kanamycin binds to the 70S ribosomal subunit and is bacteriocidal; tetracycline inhibits protein synthesis and is bacteriostatic. We confirmed pB15-encoded resistance to these antibiotics by spreading plasmid-bearing and plasmid-free cells onto TA plates supplemented with 25 μg/mL Kan or 1 μg/mL Tet. Plasmid pB15 was moved into Ara^-_0 and Ara^+_0 by mixing each strain with a donor and selecting a transconjugant after overnight matings. These matings generated clones Ara^-_0/p_0 (REL5382) and Ara^+_0/p_0 (REL5384), which carried pB15 but were otherwise isogenic to Ara^-_0 and Ara^+_0, respectively.

Media and Culture Conditions

The basic medium employed in all experiments was Davis minimal broth (Carlton and Brown 1981) supplemented with 2 μg/mL thiamine hydrochloride and 1000 μg/mL glucose; this medium is designated DM1000. In some cases, the medium was further supplemented with 25 μg/mL of kanamycin and is designated DM1000+Kan. Culture volume was 10 mL in 18-×-150-mm glass tubes, which were held in a nonshaking incubator at 37°C. Cultures were serially propagated daily by vortexing them and then transferring 0.1 mL of the old culture into 9.9 mL of fresh medium. During this 24-h cycle, the bacteria attained their stationary-phase density, at which point they had depleted the available glucose. The resulting 100-fold growth of the population encompasses ~6.64 (= $\log_2 100$) generations of binary fission per day.

Experimental Treatments

Control Populations.—Three clones of Ara^+_0 were used to initiate the three populations in the plasmid-free control, designated "F." These populations were propagated in DM1000 for 75 d. Every third day, each F-population also served as an immigrant pool for the treatment populations (see below). Three clones of Ara^-_0/p_0 were used to found three populations in the plasmid-bearing control, designated "B," which were also propagated in DM1000 for 75 d. The B-populations were not subjected to antibiotic selection and were therefore vulnerable to possible takeover by spontaneous plasmid-free segregants.

Manipulations of Susceptible Host Density.—Three clones of Ara^-_0/p_0 were used to initiate the three replicate populations in each treatment group. At the start of the experiment (day 0), each population in the "L"-treatment (denoting low opportunity for horizontal transfer) was grown in DM1000. On day 1 each L-population was serially transferred into DM1000+Kan, while on day 2 it was transferred back into DM1000. This three-day cycle was repeated for 75 d. The L-treatment differed from the B-control only insofar as the populations received antibiotic every third day, which ensured plasmid retention by selecting against any segregants.

On day 0, each resident population in the "M"-treatment (denoting medium opportunity for horizontal transfer) and a paired immigrant population from the F-control were mixed at a 1:1 volumetric ratio and 0.1 mL of this mixture was put into 9.9 mL of DM1000. Populations in the "H"-treatment (denoting high opportunity for horizontal transfer) were founded in the same way, but at a 100:1 volumetric ratio of immigrants to residents. These mixed populations were allowed to grow and conjugate during a standard growth cycle. On day 1, all M- and H-populations were then transferred into DM1000+Kan, which eliminated immigrants that did not acquire the plasmid by conjugation (along with spontaneous segregants). (During the experiment, we saw no changes in the susceptibility of plasmid-free cells to kanamycin.) On day 2, populations were transferred back into DM1000, which diluted the antibiotic before the next round of immigration. On day 3, each M- or H-population underwent an identical immigration event, with the same plasmid-free F-population, as on day 0. This three-day cycle was repeated for 75 d. Aside from manipulation of the immigrant-to-resident ratio (and the concomitant effect on the size of the resident population), all other aspects of the environment were the same for the L-, M-, and H-treatments. The treatment and control groups are summarized schematically in Figure 2.

Population Samples.—Cells from each population were periodically spread onto TA, TA+Kan, and TA+Tet plates to determine their arabinose phenotype and antibiotic-resistance profiles. Every 15 d, after serial transfer, glycerol was added to each population, which was stored in a freezer at −80°C for future study. Clones were randomly chosen from each population at generation 500 and stored at −80°C.

Fitness Assays

To assess their relative fitness, two strains competed in DM1000 or DM1000+Kan. So that they could be distinguished, one competitor was Ara^+ and the other was Ara^-. The strains were grown separately for one day in the experimental medium to ensure they were in comparable physiological states. They were then mixed at a 1:1 ratio, diluted 1:100 into fresh medium, and allowed to grow and compete for 24 h. Initial and final densities of each strain were estimated by spreading samples on TA plates. Let the initial densities of the two competitors be $N_1(0)$ and $N_2(0)$, respectively; and let $N_1(1)$ and $N_2(1)$ be their densities after one day. The time-average rate of increase, m_i, for each competitor was then calculated as $m_i = \ln[N_i(1)/N_i(0)]/(24\text{ h})$. The fitness of one strain relative to another is expressed simply as the dimensionless ratio of their rates of increase (Lenski et al. 1991): $W_{ij} = m_{i/mj}$.

Cost of Plasmid Carriage

The cost of plasmid carriage, c, was determined by allowing a plasmid-bearing strain to compete against an isogenic plasmid-free strain that differed only by the neutral marker. Plasmid-bearing and plasmid-free strains were first grown in DM1000+Kan and DM1000, respectively; the two strains were then separately preconditioned for one day in DM1000. They were then mixed at a 1:1 ratio and allowed to compete for 24 h in DM1000. The fitness of the plasmid-bearing strain relative to the plasmid-free strain was calculated (as above). The plasmid's cost of carriage, c, is simply the difference between 1.0 and the estimated fitness, so that $c > 0$ indicates that a plasmid reduced the fitness of its host. (Estimation of c is complicated, in principle, by segregants and transconjugants that arise during the competition. However, these cell types were very small minorities of the total population and did not appreciably affect our estimates; see Results.)

The *change* in the cost of plasmid carriage, Δc, was measured by competition between a strain carrying the ancestral plasmid and an isogenic strain with an evolutionarily derived plasmid. To simplify construction of the relevant genotypes, we first obtained mutants of Ara^+_0 and Ara^-_0 that were resistant to nalidixic acid (Nal^r); Ara^+_0/Nal^r (PET318) and Ara^-_0/Nal^r (PET319) are otherwise isogenic to Ara^+_0 and Ara^-_0, respectively (Table 1). We moved the ancestral plasmid p_0 to the Ara^+_0/Nal^r background, making $Ara^+_0/Nal^r/p_0$ (PET354). Each evolved plasmid was then moved onto Ara^-_0/Nal^r, and the resulting strain competed against $Ara^+_0/Nal^r/p_0$. The two plasmid-bearing competitors were grown separately in DM1000+Kan for one day and then in the competition environment (either DM1000 or DM1000+Kan) for a second day. The strains were mixed at a 1:1 ratio, and they competed over the usual cycle. The fitness of the strain bearing the evolved plasmid relative to the strain bearing the ancestral plasmid was estimated; Δc is obtained by subtracting this fitness value from 1.0. If $\Delta c > 0$, then the derived plasmid was more costly to its host than was the ancestral plasmid. If $\Delta c < 0$, this implies that a derived plasmid became less costly (or even beneficial if $c + \Delta c < 0$) to its host. Note that the sum of the ancestral cost and the change in cost should equal the cost of carriage of the derived plasmid.

Segregation Rate

The segregation rate of a plasmid, τ, was estimated by allowing a clone of the plasmid-bearing strain to grow in DM1000+Kan; the culture was then diluted 1:100 into DM1000 and allowed to grow for 24 h, during which time segregants could grow and accumulate due to the absence of antibiotic. The culture was plated onto TA agar, and 50 colonies were then tested on TA+Kan to estimate the fraction of plasmid-free segregants, which cannot grow on this medium. The segregation rate (per cell generation) was then estimated as: $\tau = s/g$, where s is the fraction of segregants and g is the number of cell generations during the assay (here, $6.64 = \log_2 100$). The logic of this formula is as follows. Each segregant observed may be a primary segregant that occurred during the last generation, the daughter cell of a segregant that appeared in the previous generation, and so on. The population doubles each generation, and so there are twice as many segregation events each generation, but each event in an earlier generation leaves twice as many descendant segregants as an event one generation later; hence, these two effects cancel one another. This formula slightly overestimates τ if segregants grow faster than plasmid-bearing cells in the absence of antibiotic, but this bias is opposed by conjugal reinfection of segregants.

Conjugation Rate

To measure rates of horizontal plasmid transfer, we used Ara^+_0/Nal^r and Ara^-_0/Nal^r as recipients because the second marker facilitated detection of transconjugants. The conjugation rate, γ, was estimated by mixing a plasmid-bearing donor with a plasmid-free Nal^r recipient having the opposite Ara marker. Donors and recipients were grown to stationary phase in DM1000+Kan and DM1000, respectively, and then each was grown for another day in DM1000. Donors and recipients were mixed at a 1:1 volumetric ratio, diluted 1:100 into fresh medium, and then grew and conjugated during a standard 24-h cycle. Initial and final densities of donors, recipients, and transconjugants were estimated from the numbers of colonies formed on appropriate agar plates. We also estimated Ψ, the exponential-phase growth rate (h^{-1}) of each culture, by regressing the natural logarithm of total cell density versus time during the period of log-linear increase. Total cell density was measured using a Coulter electronic particle counter. We then calculated the plasmid's rate of horizontal transfer (mL/cell h) using the end-point formula of Simonsen et al. (1990): $\gamma = \Psi \ln[1 + (T/R)(N/D)]/(N - N_0)$, where D, R, and T are the final densities of donors, recipients, and transconjugants, respectively; and N_0 and N are the initial and final total densities, respectively. Unlike other approaches to quantifying rates of plasmid transfer (e.g., Watanabe 1963; Curtiss et al. 1969; Bale et al. 1987), the end-point method is largely unaffected by such factors as donor-to-recipient ratio or subtle differences in growth rate among the cell types because it estimates the actual rate-constant of transfer rather than simply the resulting frequency of transconjugants (Simonsen et al. 1990).

RESULTS

Selective Neutrality of the Ara Marker

We ran three experiments to measure the effect, if any, of the Ara marker on fitness. In one, Ara^-_0 and Ara^+_0 competed in medium without antibiotic (DM1000). Twenty assays gave a mean fitness of Ara^-_0 relative to Ara^+_0 of 1.008, which did not differ significantly from 1.0 (t_s = 1.068, df = 19, P = 0.299). In the second experiment, Ara^-_0/p_0 and Ara^+_0/p_0 competed in the absence of antibiotic, with 10-fold replication. The fitness of Ara^-_0/p_0 relative to Ara^+_0/p_0 was 0.996, which again did not differ significantly from 1.0 (t_s = 0.256, df = 9, P = 0.804). In the third experiment, these same two plasmid-bearing strains competed in medium that contained antibiotic (DM1000+Kan), also with 10-fold replication. This gave a mean fitness of Ara^-_0/p_0 relative to Ara^+_0/p_0 of 1.003, which yet again did not differ significantly from 1.0 (t_s = 0.113, df = 9, P = 0.912). Thus, the Ara marker was effectively neutral under all three experimental conditions.

Ancestral Plasmid Traits

Ancestral Cost of Plasmid Carriage.—We performed experiments to measure the fitness, W, of the ancestral plasmid-bearing strain, Ara^-_0/p_0, relative to the plasmid-free ancestor, Ara^+_0, in the absence of antibiotic (DM1000). To address whether plasmid segregation and conjugation might seriously bias fitness estimation, we sampled the competitions to measure the proportion of segregants (Ara^-_0) and transconjugants (Ara^+_0/p_0). We estimated relative fitness in two ways, first adjusting the data by excluding segregants and transconjugants and then without adjustment. With 10-fold replication, the fitness of Ara^-_0/p_0 relative to Ara^+_0 was 0.981 when segregants and transconjugants were excluded; without such adjustments, relative fitness was 0.979. Both segregants and transconjugants were small minorities, and so their inclusion did not significantly affect fitness estimation (paired t_s = 0.566, df = 9, P = 0.585). Because screening for these rare genotypes required much more work, but did not improve estimation, we performed two more blocks without screening. Combined results from all three blocks gave a mean fitness of plasmid-bearing relative to plasmid-free of 0.989. There was no block effect (P = 0.655) and fitness did not differ significantly from 1.0 (t_s = 1.226, df = 29, P = 0.230). The cost of plasmid carriage, c, is estimated as $1 - W = 0.011$. Evidently, the ancestral plasmid imposed only a small cost.

Ancestral Segregation Rate.—We measured the segregation rate, τ, of the ancestral plasmid-bearing strain, Ara^-_0/p_0, in antibiotic-free medium (DM1000) with 10-fold replication. The mean estimate was 0.0042 (± 0.0023 SE) per cell generation. We also measured τ for the ancestral plasmid in the alternative background, Ara^+_0/p_0, again with 10-fold replication, which gave a mean estimate of 0.0033 (± 0.0012 SE). The two estimates are not significantly different (t_s = 0.364, df = 18, P = 0.720). These data indicate that the ancestral plasmid is fairly stable, with fewer than one in 200 cell divisions yielding a segregant. ("Postsegregational killing" [Helinski et al. 1996] is not responsible for the observed stability of pB15 because segregants made colonies.)

Ancestral Conjugation Rate.—We ran 20 assays to measure the transfer rate of pB15 from the infected ancestor, Ara^-_0/p_0, to the uninfected recipient, Ara^+_0/Nal^r, in antibiotic-free medium (DM1000). The conjugation rate, γ, was calculated using the method of Simonsen et al. (1990), as described in Materials and Methods. After $\log_{10}$-transformation, the mean value of γ for the ancestral plasmid was -12.161 (± 0.040 SE) mL/cell h. That is, during one hour, each plasmid-bearing cell can effectively "search" a volume of $\sim 10^{-12}$ mL and infect any plasmid-free cell therein. (Because cell densities are $\ll 1/\gamma$, a donor is unlikely to encounter two recipients in close temporal proximity, or vice versa; hence, the system is unsaturated.)

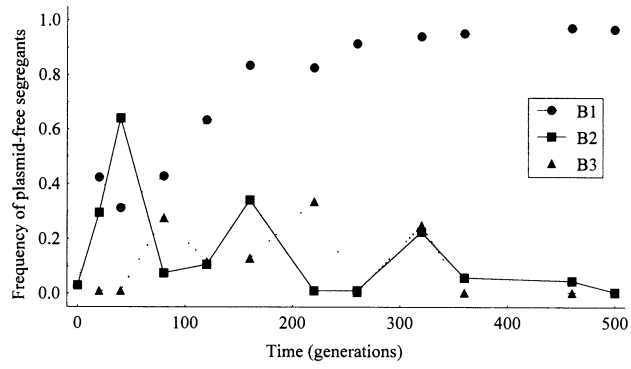

FIG. 3. The frequency of plasmid-free segregants in three B control populations. These populations received neither plasmid-free immigrants nor antibiotics (see Fig. 2).

Control Populations

Plasmid Dynamics.—The plasmid-bearing (B) control populations never experienced antibiotic selection, and hence they were at risk for overgrowth by spontaneous plasmid-free segregants. Figure 3 shows the frequency of segregants in the three B-populations during 500 generations. In two populations, segregants remained at relatively low frequencies, suggesting that plasmid transfer was sufficiently frequent to offset losses due to segregation and the cost of carriage. This outcome is similar to results obtained in chemostat culture (Lundquist and Levin 1986). In the third population, however, segregants became essentially fixed.

Fitness Dynamics.—We sought to determine whether the plasmid-free (F) and plasmid-bearing (B) control populations differed in their adaptation to antibiotic-free medium (DM1000). We measured each population's fitness relative to the reciprocal ancestor, either Ara^-_0 or Ara^+_0, at 100-generation intervals. Assays were repeated twice, and the grand mean fitness over the three populations in each group was calculated at every time point. Figure 4 shows that F- and B-populations underwent nearly parallel fitness gains during the first half of the experiment, with the B-group trailing by a small amount consistent with the cost of plasmid carriage. However, the B-populations fell behind the F-populations in their adaptation during the last 200 generations or so. The possible relevance of this discrepancy will become apparent in the next section.

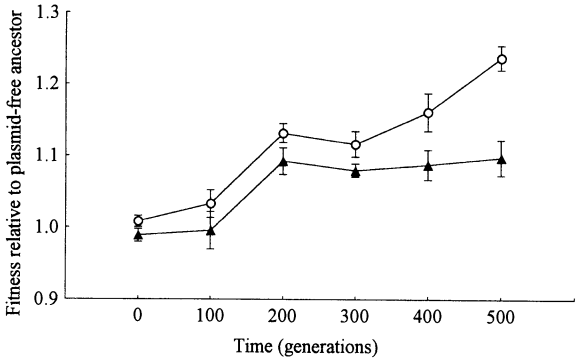

FIG. 4. Fitness trajectories for plasmid-bearing and plasmid-free populations during evolution in the antibiotic-free environment. Plasmid-free (F) control populations (○) continued to adapt to the environment throughout the 500 generations, whereas plasmid-bearing (B) control populations (▲) did not. Fitness was measured relative to the plasmid-free ancestor. Each point represents the grand mean (± SE) of three populations, except the plasmid-bearing and plasmid-free ancestors which are based on 30 and 20 assays, respectively.

Manipulations of Susceptible Host Density

Evolutionary Dynamics.—Plasmid-bearing cells in the M- and H-treatments were exposed to plasmid-free (susceptible) immigrants at regular intervals, which provided the plasmids an opportunity for horizontal transfer. The immigrant genotypes could not take hold unless they acquired a plasmid from a resident host, because antibiotic selection was imposed every third day. The initial resident and immigrant bacteria were Ara^- and Ara^+, respectively, which enabled us to distinguish primary from secondary infections. Figure 5 shows that infected immigrants remained at low frequency until the latter half of the experiment, at which time they spread quite rapidly in five of the six M- and H-populations. This shift appears to coincide with the divergence of fitness trajectories for the F- and B-controls (Fig. 4). That is, plasmids were prevalent on the immigrant background only after the fitness gains for the plasmid-free immigrants had begun to outpace the gains for the plasmid-bearing lines. Also, the higher immigration rate in the H-treatment did *not* lead to a higher proportion of immigrant hosts. After 500 generations, all three M-populations contained nearly 100% immigrants, whereas the three H-populations had roughly 5%, 60%, and 100% immigrants (Fig. 5).

Fitness Changes.—After 500 generations, we sought to determine whether the extent of fitness gains in the treatment populations depended on the level of susceptible host immigration. To do so, we first allowed a heterogeneous sample from each of the nine evolved L-, M-, and H-populations to compete against the reciprocally marked plasmid-free ancestor, either Ara^+_0 or Ara^-_0, in DM1000. (This experiment would not have been possible for population H2, which contained a substantial mixture of both resident and immigrant genetic backgrounds [see Fig. 5], except that the colony morphology of the derived cells on TA agar was sufficiently distinct from the ancestral state that they could be readily distinguished. Also, to ensure accurate fitness estimates for all populations, we sampled a subset of cells to track plasmid losses and gains due to segregation and conjugation that occurred during the competition. As we saw when estimating the ancestral cost of plasmid carriage, segregants and transconjugants were small minorities, and their inclusion or exclusion from the calculations had little effect on fitness estimates [data not shown].) Five fitness assays were run for each evolved population, and the grand mean for the three populations in each treatment was then calculated. Figure 6

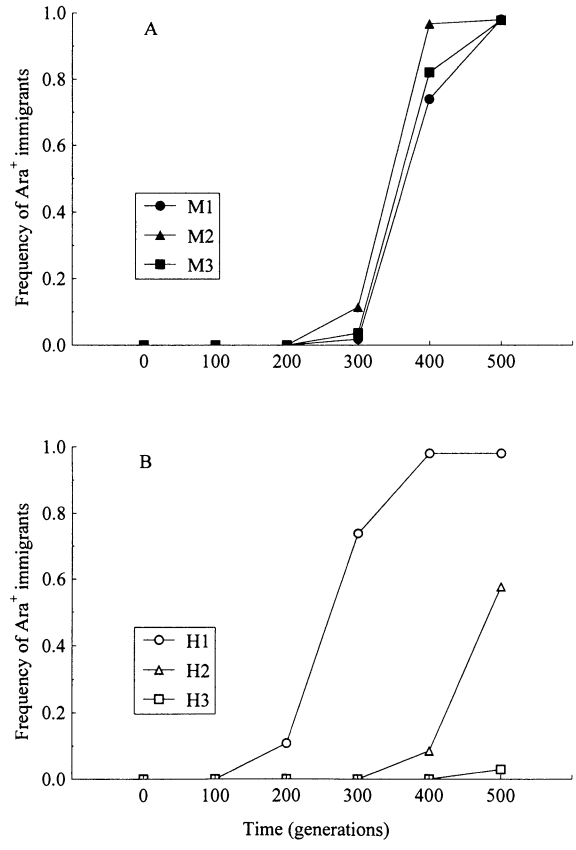

FIG. 5. Changes in the frequency of Ara^+ immigrant backgrounds in the plasmid-bearing populations subjected to medium (M: panel A) or high (H: panel B) levels of immigration by plasmid-free cells. Each curve represents a replicate population; the two treatments do not differ systematically in the frequency of immigrants.

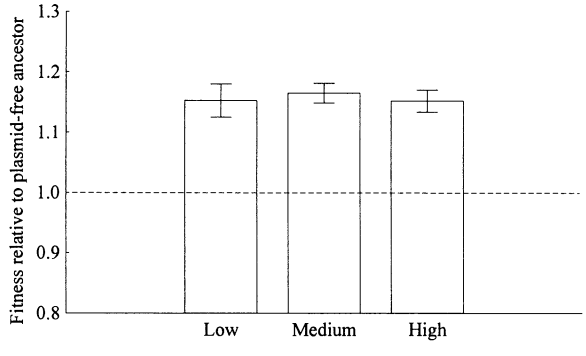

FIG. 6. Mean fitness in treatment populations subjected to low, medium, and high levels of immigration by plasmid-free cells. Each bar shows the grand mean (± SE) of three replicate populations. Fitnesses were measured relative to the plasmid-free ancestor in the antibiotic-free environment. See Table 2 for statistical analysis.

TABLE 2. Nested ANOVA to examine the effects of susceptible-host-density treatment, and population within treatment, on the fitness of evolved populations relative to their ancestor.

Source	SS	df	MS	F	P
Treatment	0.0016	2	0.0008	0.119	0.890
Population	0.0405	6	0.0067	0.787	0.586
Error	0.3082	36	0.0086		

shows that fitness relative to the plasmid-free ancestor increased significantly in all three treatments, which indicates that the populations had adapted genetically and, moreover, that adaptation carried over to the antibiotic-free medium. A nested ANOVA indicates no significant variation in fitness among the L-, M-, and H-treatments or the three replicate populations within each group (Table 2).

We also ran fitness assays in which individual clones (instead of heterogeneous samples) from each L-, M-, and H-population competed with the reciprocally marked plasmid-free ancestor. We isolated a clone of the majority genotype (either Ara^- or Ara^+) at generation 500 from each population. In close agreement with the heterogeneous samples, the clones had a grand mean fitness of 1.169 (± 0.016 SE), and there was no significant effect of treatment (data not shown). We conclude that the differences in susceptible host density among the treatments did not lead to differential increases in fitness relative to the plasmid-free ancestor.

Fidelity of Vertical Transmission.—Using the clones from each L-, M-, and H-population, we asked whether the segregation rate, τ, was different from the ancestral value of ~0.004 per cell generation. We performed three assays for each evolved clone, which were run in parallel with three of the 10 assays on the ancestral strains reported earlier. Among the 1350 samples tested (9 clones × 3 assays per clone × 50 colonies per assay), there was only a single segregant, which gives an average estimate of τ equal to 0.00011 (± 0.00011 SE) per cell generation. The fidelity of vertical transmission, already high at the outset, evidently increased under all three treatments. This outcome may reflect selection on either the plasmid or the host to minimize segregation, the former to avoid outright loss and the latter to avoid the lethal effect of antibiotic that was imposed every third day (Fig. 2).

Changes in Plasmid Traits

The fitness values reported above reflect evolutionary change in both the plasmids and their bacterial hosts. To quantify any change that can be attributed specifically to a plasmid's evolution requires that it be moved back to the ancestral host and its cost of carriage estimated there. We used the plasmids from the clones sampled at generation 500 from each L-, M-, and H-population; henceforth, we refer to each plasmid by its source population. We moved each one into the ancestral strain Ara^-_0/Nal^r, in which Nal^r facilitated identifying transconjugants. (As shown below, one derived plasmid, H1, was unable to conjugate and could not be moved into this strain.) These constructs were used to address the following questions: Were there evolutionary changes in the cost of carriage of the derived plasmids and/or in conjugation rate? Were changes in either trait affected by the level of susceptible-host immigration? Were changes in these two traits genetically correlated?

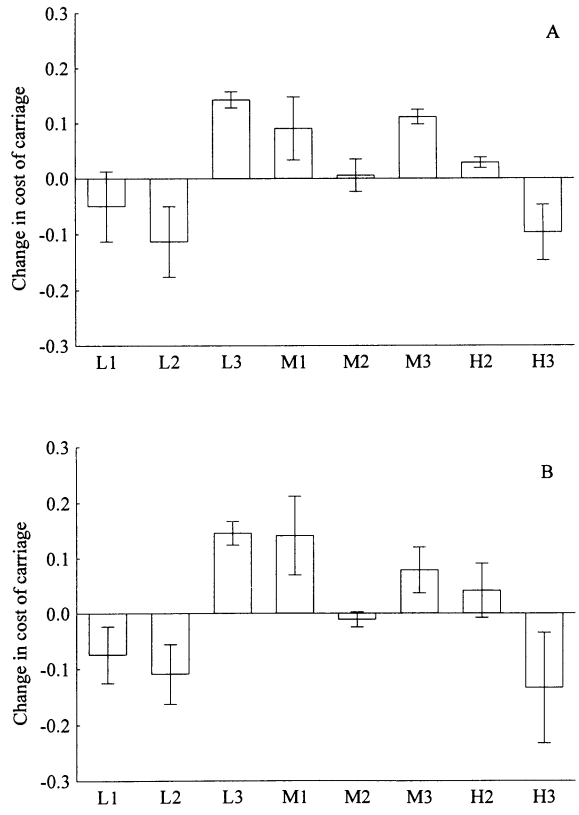

FIG. 7. Change in cost of carriage (Δc) for the eight evolved plasmids that retained their ability to conjugate. To estimate Δc, each evolved plasmid was transferred to the ancestral host, which competed against a marked, isogenic host carrying the ancestral plasmid. If $\Delta c < 0$, then an evolved plasmid is less costly than the ancestral plasmid, and it may even have become beneficial. (A) Competition assays performed in antibiotic-free medium. (B) Assays performed in medium containing kanamycin. Error bars show SE. See Table 3 for statistical analysis.

Cost of Plasmid Carriage.—Each of the eight Ara^-_0/Nal^r constructs that carried one of the derived plasmids was placed in competition with the reciprocally marked strain carrying the ancestral plasmid, $Ara^+_0/Nal^r/p_0$. These competitions were performed in both the presence and absence of antibiotic (DM1000+Kan and DM1000, respectively), and they were replicated threefold. We calculated the fitness of the host carrying the evolved plasmid relative to the isogenic host carrying the ancestral plasmid; subtracting this value from 1.0 provides a direct estimate of the *change* in the plasmid's cost of carriage, Δc. Recall that the ancestral cost of carriage plus any change in cost should equal the cost of carriage of the derived plasmid.

As shown in Figure 7, the quantity Δc was highly variable among the evolved plasmids, including even those from the same treatment. For example, in both the L- and H-treatments, one plasmid underwent a significant increase in its cost of carriage whereas another plasmid showed a significant reduction in cost. Values for Δc were virtually identical in the absence and presence of antibiotic (Figs. 7A and 7B,

TABLE 3. Two-way ANOVA to examine the effects of plasmid genotype and competition environment on the change in cost of plasmid carriage. Genotype is a random effect and environment is a fixed effect.

Source	SS	df	MS	F	P
Genotype	0.4366	7	0.0624	8.321	< 0.001
Environment	0.0003	1	0.0003	0.258	0.627
Interaction	0.0087	7	0.0012	0.166	0.990
Error	0.2399	32	0.0075		

respectively), which indicates that the variation reflects differences in cost of carriage rather than changes in the level of resistance to kanamycin. A two-way ANOVA confirms that Δc was heterogeneous among the derived plasmids, whereas neither the competition environment nor the genotype-environment interaction had any significant effect (Table 3).

Conjugation Rate.—We measured, with threefold replication, the conjugation rate, γ, for all nine evolved clones. As recipient, we used a reciprocally marked strain, either $Ara^+{}_0$/Nal^r or $Ara^-{}_0$/Nal^r. As shown in Figure 8, five of the evolved plasmids conjugated at rates somewhat higher than that of the ancestral plasmid, while four others conjugated at rates much lower than their ancestor. All three plasmids from treatment M conjugated at high rates, whereas there was tremendous variation among replicates within L and H. Treatment H, for example, yielded one plasmid with higher transmissibility than the ancestor (H2), another with reduced transmissibility (H3), and one that was unable to conjugate at all (H1). A Kruskal-Wallis test finds significant differences among the treatments with respect to conjugation rates of the derived plasmids ($K = 10.662$, df = 2, $P = 0.005$). This test was significant because the three replicates of M, which was the intermediate treatment, had the (marginally) highest values of γ. However, we had expected that the highest conjugation rate would evolve under treatment H, which had the highest level of immigration of susceptible hosts and thus the most opportunity for horizontal transfer.

Tradeoff between Modes of Transmission

Correlation between Conjugation Rate and Cost of Carriage.—Figure 9 depicts the relationship between the rate of conjugation ($\log_{10}\gamma$) and the cost of plasmid carriage (Δc in DM1000) for the eight evolved plasmids that could be moved onto the same host background. (The change in cost of carriage, Δc, provides a suitable measure of the cost of carriage, because the change was always measured relative to the same ancestral plasmid.) The positive correlation between these two traits is highly significant ($r = 0.867$, $n = 8$, $P = 0.005$), which indicates that a plasmid's cost of carriage to its host is tightly coupled with its conjugation rate. The results of the evolution experiment thus support the hypothesis that there is a systematic tradeoff between horizontal and vertical modes of transmission, even though neither trait alone responded to the susceptible-host manipulations as we had expected.

Some Further Experiments.—Recall that the ancestral plasmid had only a small cost of carriage, on the order of 0.01,

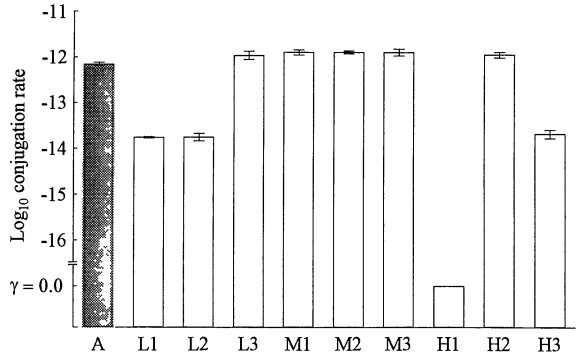

FIG. 8. Conjugation rates (γ) for the ancestral (filled bar) and nine evolved plasmids. Each bar represents the mean (± SE) of three measurements, except for the ancestor, which is based on 20 assays. One plasmid (H1) had a conjugation rate equal to zero, since transconjugants were not detected. See text for statistical analysis.

or 1%. Three plasmids (L1, L2, and H3) that had evolved lower conjugation rates underwent *changes* in their cost of carriage that were on the order of −0.08 (Fig. 7); that is, relative to isogenic strains carrying the ancestral plasmid, the bacteria that carry these derived plasmids have about 8% higher fitness. These data suggest that carriage of these plasmids should, in fact, be *beneficial* to the ancestral host, since a 1% initial cost is much more than offset by the 8% fitness advantage of the evolved plasmid relative to the ancestral plasmid.

While testing this conjecture, we obtained some anomalous results. Despite substantial effort, we cannot fully explain these results but summarize them for the sake of completeness. In some subsequent experiments, the ancestral plasmid imposed a very high cost of carriage, on the order of 20–35%, rather than the mere 1% reported above. We initially suspected that these discrepancies involved interactions between the plasmid and genetic markers in different host backgrounds; for example, in a later experiment $Ara^+{}_0$/Nal^r/p_0 competed against $Ara^-{}_0$/Nal^r, whereas in our earlier experiment $Ara^-{}_0$/p_0 had competed with $Ara^+{}_0$. But when we de-

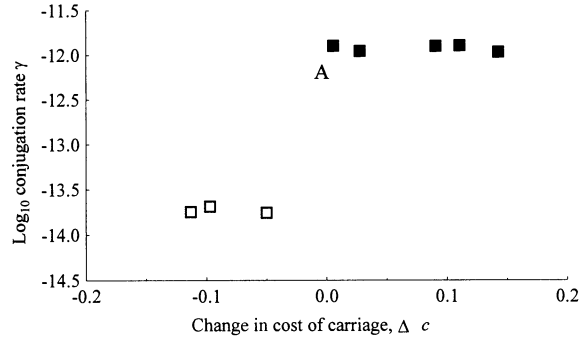

FIG. 9. Genetic correlation between horizontal and vertical transmission in pB15 and its evolved derivatives. A is the ancestral plasmid. Filled squares show five evolved plasmids that conjugate at a high rate, but that impose a high fitness cost on their host. Open squares show three evolved plasmids that are less costly, but which conjugate at lower rates. The correlation between conjugation rate (Fig. 8) and change in cost of carriage (Fig. 7A) for the eight evolved plasmids is highly significant ($r = 0.867$, $P = 0.005$).

signed experiments to look for such interactions, they were not seen. We also tested for block effects, such that the cost of carriage might vary from one week to another, but they too were elusive.

Finally, we reconstructed the critical strains by independently moving pB15 onto the ancestral backgrounds, Ara^-_0 and Ara^+_0, and measuring simultaneously the cost of carriage for the original constructs (REL5382 and REL5384) and the new, supposedly identical, constructs. We obtained a striking, but perplexing, result. The several "fresh" constructs all had small costs, consistent with our initial estimates for the original constructs. However, the original constructs now had much higher costs, which they did not have when they were freshly made. It seems that the plasmid-bearing strains "decayed" in fitness during storage. How this could occur is unclear, since the strains were stored as clones at −80°C. This anomaly seems peculiar to the plasmid-bearing strains; if the plasmid-free strains had also decayed, then *relative* fitness would not have changed. A possible explanation is that the plasmid causes the slow accumulation (or depletion) of some cytoplasmic factor, such that freshly made constructs underestimate the cost of plasmid carriage to the bacterial host (D. Dykhuizen, pers. comm.).

Despite this uncertainty regarding the *absolute* cost of plasmid carriage, the *relative* costs for the various derived plasmids have been quite reproducible. Over the course of several separate experiments (with different host genotypes, performed at different times and by different persons), hosts that carried the derived plasmids with low conjugation rates (L1, L2, and H3) were always more fit than isogenic hosts that carried the derived plasmids with high conjugation rates (L3, M1, M2, M3, and H2). Thus, we are confident of the tradeoff between horizontal and vertical modes of transmission that is shown in Figure 9.

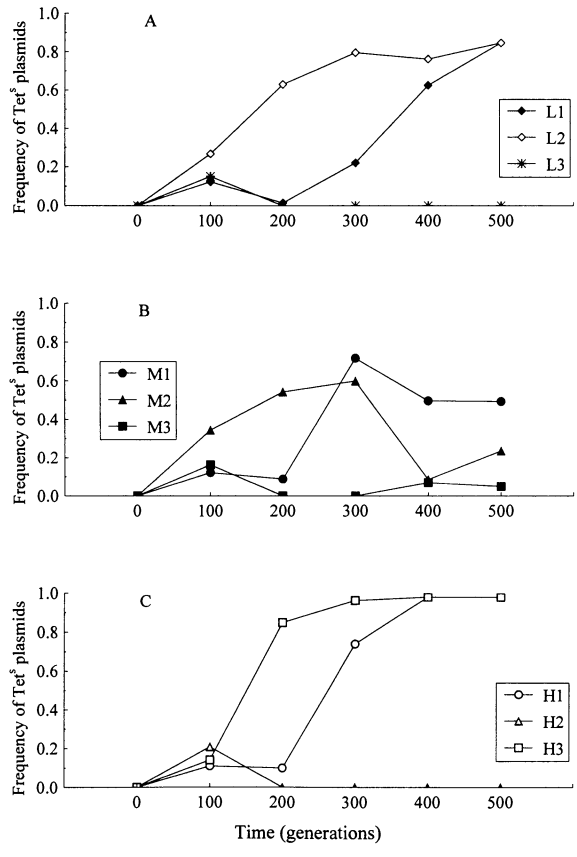

FIG. 10. Changes in the frequency of Tet^s variants in treatment populations subjected to low (L: panel A), medium (M: panel B), and high (H: panel C) levels of immigration by plasmid-free cells. Each curve represents a replicate population. See text for statistical analysis.

Association between Tet Phenotype and Evolved Mode of Transmission

Appearance of Tet^s plasmids.—The ancestral plasmid, pB15, conferred resistance to both kanamycin and tetracycline. Populations in the L-, M-, and H-treatments were exposed periodically to kanamycin to ensure plasmid retention (Fig. 2), but they were not exposed to tetracycline, and so tetracycline resistance could be lost by drift or by selection. We screened each population for resistance every 100 generations. As shown in Figure 10, tetracycline sensitive (Tet^s) plasmids appeared in all nine populations, although their fate was highly variable. In four populations (L1, L2, H1, and H3) Tet^s derivatives became dominant by 500 generations; in four populations (L3, M2, M3, and H2) Tet^r plasmids remained dominant; and in one population (M1) Tet^r and Tet^s plasmids were almost equally common. (We never saw Tet^s plasmids at any frequency in the B control populations, which were never exposed to kanamycin. This suggests that the loss of tetracycline resistance was promoted by selection to retain kanamycin resistance.)

Correlation between Tet Phenotype and Transmission Properties.—Recall that we saw a significant correlation between the conjugation rate of a derived plasmid and the change in its cost of carriage (Fig. 9). Plasmids that evolved higher conjugation rates were more harmful to their hosts than was the ancestor, whereas those that evolved lower conjugation rates had become less harmful. (One of the latter plasmids could not be formally tested for its cost of carriage because it was nontransmissible; however, it had clearly become more dependent on vertical transmission.) Interestingly, there was a perfect correspondence between the plasmids' transmission properties and their Tet phenotypes: all five that improved their horizontal transfer remained Tet^r, whereas all four that evolved greater dependence on vertical transmission became Tet^s. A Fisher's exact test shows that this association is highly significant (two-tailed $P = 0.008$). (Tet^r and Tet^s plasmids also differ when each property is considered separately. A Mann-Whitney test indicates that the difference in conjugation rate between the Tet^r and Tet^s derived plasmids is significant [$U_s = 0$, $P = 0.016$]. A t-test shows a significant difference in the cost of carriage, in DM1000, between the five Tet^r and three Tet^s plasmids that were moved into the ancestral host [$t_s = 4.382$, df = 6, $P = 0.005$].) Table 4 summarizes the relevant characteristics for these nine derived plasmids, and for the treatment populations from which they were sampled.

Eight populations were dominated by one Tet phenotype or the other (Fig. 10), and the plasmid used to measure conjugation rate and cost of carriage was representative of the

TABLE 4. Summary of relevant properties of the evolved plasmids and the populations from which they were isolated.

Plasmid	Plasmid trait[a]					Population profile[b]	
	$\log_{10}\gamma$	W	Δc	Kan	Tet	Immigrants	Tet^s
L1	−13.755	1.050	−0.050	r	s	0%	85%
L2	−13.746	1.113	−0.113	r	s	0%	85%
L3	−11.970	0.857	0.143	r	r	0%	0%
M1	−11.901	0.909	0.091	r	r	99%	49%
M1s	−∞[c]	ND	ND	r	s	99%	49%
M2	−11.896	0.994	0.006	r	r	98%	24%
M3	−11.899	0.889	0.111	r	r	98%	5%
H1	−∞[c]	ND	ND	r	s	100%	100%
H2	−11.953	0.972	0.028	r	r	58%	0%
H3	−13.685	1.097	−0.097	r	s	3%	100%

[a] $\log_{10}\gamma = \log_{10}$ conjugation rate; W = fitness in DM1000 of a strain bearing the evolved plasmid relative to an isogenic strain carrying the ancestral plasmid; Δc = change in cost of carriage ($1 - W$); r and s indicate resistance and sensitivity, respectively, to antibiotics kanamycin (Kan) and tetracycline (Tet); ND = not determined because the plasmid could not be moved into common host background.

[b] Final percentages of immigrant hosts and tetracycline sensitive (Tet^s) plasmids in the treatment populations from which each evolved plasmid was isolated.

[c] Plasmid was unable to conjugate.

majority type in its population (Table 4). However, population M1 had a nearly equal mix of Tet^r and Tet^s plasmids, and the plasmid that was initially chosen happened to be Tet^r. We chose a second Tet^s clone, designated M1s, from this population. When we attempted to move plasmid M1s to the ancestral host, we found that it was nontransmissible. M1s thus represents another independent association between the Tet^s phenotype and (partial or complete) loss of conjugative function, on the one hand, and retention of Tet^r and increased transmissibility, on the other hand. Yet, there was no effect of susceptible host density on the frequency of the two syndromes (Fig. 10). An ANOVA confirms the lack of a significant treatment effect on the final proportion of Tet^s plasmids (F_s = 0.491, df = 2,6, P = 0.635, using arcsine-transformed values).

DISCUSSION

Many parasites can be transmitted both horizontally (by infection) and vertically (via host reproduction). In such cases, it has been hypothesized that there is a genetically based tradeoff between horizontal and vertical transmission (Levin and Lenski 1983; May and Anderson 1983; Bull 1994; Ewald 1994). This tradeoff presumably occurs because activities of a parasite that increase its infectious transmission are generally harmful to its host. If this tradeoff hypothesis is correct, and given certain other assumptions (see below), then a simple model predicts that the density of uninfected (susceptible) hosts will determine whether a parasite evolves to be more or less infectious and, concomitantly, more or less virulent to the host (Fig. 1). If uninfected hosts are common, then the potential for horizontal transmission is great and selection on the parasite should favor increased infectiousness, even at the expense of reduced vertical transmission due to lower host fitness. But when uninfected hosts are scarce, horizontal transfer becomes infrequent relative to vertical transmission, and selection on the parasite should favor reduced virulence. We tested the tradeoff hypothesis and model predictions by allowing pB15, a conjugative plasmid that infects the bacterium *E. coli*, to evolve for 500 generations in replicated environments in which we manipulated the density of uninfected hosts.

The two main findings of our study can be summarized as follows. First, our experiments demonstrate a tradeoff between the plasmid's conjugation rate, which governs horizontal transfer, and its virulence (cost of carriage), which determines vertical transmission (Fig. 9). We observed 10 evolutionarily independent changes in the plasmid's conjugation rate relative to the ancestral state, including five increases and five decreases. When conjugation rate increased, the cost of plasmid carriage increased relative to the ancestral cost; and when conjugation rate declined, the cost of carriage fell. Second, despite this genetic tradeoff, our experiments provide no support for the predicted effect of susceptible host density on the evolution of these alternative modes of transmission. Although some plasmids evolved higher conjugation rates and others became less costly to their hosts, the density of susceptible hosts did not affect the outcome.

Taken together, these two findings are rather puzzling. The epidemiological model's key evolutionary assumption—that there is a tradeoff between the two modes of transmission—was met, but the prediction that susceptible host density would determine the plasmid's evolutionary direction failed to materialize. In the next section, we discuss a number of potential explanations for this failure to observe an effect of host density manipulation on evolutionary outcomes. These include possible deviations from assumptions of the epidemiological model and possible artifacts of our experimental design. Many of these explanations are not mutually exclusive.

Potential Reasons for the Failure to Observe an Effect of Susceptible Host Density

Insufficient Replication or Duration.—It is always possible, of course, that we might have seen a significant treatment effect with greater replication or a longer experiment. However, the execution and analysis of evolution experiments are demanding and time consuming, which limits what is feasible. Moreover, the replication and duration of our experiments were quite sufficient to observe evolution in the traits of interest and to demonstrate a consistent correlation between these trait changes, whereas any effect of host density on the outcome was highly inconsistent.

Varying Densities of Susceptible Hosts.—The epidemiological model in Figure 1 assumes a constant density of susceptible hosts. In a closed population, the equilibrium density of uninfected hosts may not correlate in a simple way with total host density. For example, one would expect more frequent infectious transmission during a parasite's initial invasion of a dense population of susceptible hosts than during invasion of a sparse population. But as the parasites invaded, they would concomitantly reduce the density of the remaining uninfected hosts until some equilibrium was achieved (Lenski and May 1994; Bonhoeffer et al. 1996). Intuitively, it is unclear whether the resulting balance between horizontal and

vertical transmission would continue to favor the former in a dense population and the latter in a sparse population. Lipsitch et al. (1995, 1996) analyzed a similar situation mathematically and found that higher intrinsic rates of horizontal transfer may, at equilibrium, actually increase the importance of vertical transmission. To avoid this complication, we did not manipulate the total density of hosts in our experiment. Instead, we deliberately interrupted the epidemiological feedback by providing different levels of immigration to the experimental populations. These manipulations ensured that the density of uninfected hosts varied in a consistent manner across the three treatments.

Possibility of Multiple Infections.—This epidemiological model also assumes that there are no multiple infections; that is, an individual that is already infected cannot become secondarily infected. If individual hosts infected by one parasite can be subsequently infected by another, then the nature of selection acting on parasites may be fundamentally altered (Nowak and May 1994). Within-host competition becomes a major factor in their evolutionary dynamics, and one cannot obtain the optimal balance between horizontal and vertical transmission simply by maximizing the intrinsic rate of increase of a parasite in isolation. Multiple infections will generally favor more virulent parasites, provided that the benefit of reduced virulence (increased vertical transmission) is distributed to all parasites within a host, whereas the cost of reduced virulence (lower horizontal transmission) is borne only by those that practice restraint. In our experiments, however, half the plasmids evolved lower virulence; and in two cases they completely lost their conjugative ability, which would leave them vulnerable to secondary infections by more virulent plasmids. (However, a more virulent plasmid might mobilize the horizontal transfer of a less virulent one, further complicating the picture [Levin and Rice 1980].) Also, many plasmids encode proteins that make their hosts recalcitrant to secondary infections by the same or closely related plasmids (Lederberg et al. 1952; van der Hoeven 1985), although this has not been studied in pB15. Thus, it is unclear how multiple infections might explain our results; they would certainly complicate the evolutionary dynamics, and further studies are warranted.

Effect of Settling on Local Cell Densities.—Populations were cultured in tubes held in a nonshaking incubator. We employed these static conditions to prevent breakage of conjugative pili, but this may also have allowed the bacteria to settle, which would concentrate cells locally and thus violate the epidemiological model's assumption of mass action. In the worst case, the rate of conjugation on the bottom of the culture tubes might be independent of susceptible host density, thus undermining the differences between our treatments. In fact, however, this effect does not appear to be important. We ran a separate experiment in which we varied cell densities, and the number of transconjugants increased significantly with the product of donor and recipient densities ($r = 0.9608$, $n = 7$, $P = 0.0003$), as expected under mass action.

Reinfection of Segregants.—Another factor that could lessen the differences among our treatments would be if plasmid-free segregants were very common. In the extreme, they might be so common that horizontal transfer was as frequent in the absence of immigration by susceptible hosts as in its presence. In fact, this scenario is unlikely on three counts: antibiotic selection was imposed every third day in each treatment (Fig. 2), which prevented accumulation of plasmid-free cells; the ancestral segregation rate was low, less than 0.5% per generation; and the segregation rate evolved to become even lower, perhaps because of the imposed selection against segregants.

Confounding Effect of Treatments on the Plasmid's Effective Population Size.—To manipulate the level of uninfected immigrants, while keeping the total number of bacteria equal across treatments, it was necessary to vary the density of the resident plasmid-bearing cells. As a consequence, the effective population size for the plasmids must have varied among treatments. This effect was twofold between the L- and M-treatments, which received no immigrants and 50% immigrants every third day, respectively (Fig. 2). But the H-treatment had 99% immigrants every third day, so the plasmid-bearing population suffered a much more severe bottleneck. Even so, the bottleneck size was still very large, $\sim 10^6$ plasmid-bearing cells on immigration days in the H-treatment. Also, the extent of adaptive evolution in the bacteria-plasmid association was similar for all three treatments (Fig. 6), suggesting that effective population size was not a major factor in our results. Nonetheless, it was a confounding variable.

Small Differences in Selection between M- and H-Treatments.—The M- and H-treatments were designed to allow different levels of horizontal transfer. The former experienced a 1:1 ratio of susceptible immigrants to infected residents, whereas the latter had a 100:1 ratio, suggesting a vast difference in potential for horizontal transfer. However, this is misleading: we manipulated the relative *frequency* of susceptible and infected hosts, whereas it is the *density* of susceptible hosts that governs selection on the parasite's mode of transmission (Fig. 1). In M, the density of plasmid-free immigrants at the time of mixing was $\sim 5 \times 10^6$ mL^{-1} and that of infected residents was also $\sim 5 \times 10^6$ mL^{-1}. For H, the immigrant density was $\sim 1 \times 10^7$ mL^{-1}, whereas the resident density was $\sim 1 \times 10^5$ mL^{-1}. Hence, the susceptible host density was higher in the H- than M-treatment, but only by twofold. While the differences in selection between treatments might be smaller than they appear at first glance, this cannot explain why both more and less virulent plasmids evolved in replicates of the same treatment (Figs. 7, 8).

Low Proportion of Horizontal Transfer Even in M- and H-Treatments.—Perhaps the amount of horizontal transfer was very small relative to the amount of vertical transmission. If so, then selection might have favored gains in vertical transmission to a greater extent than improvements in horizontal transfer, even in the M- and H-treatments. Consistent with this possibility, we note that experiments performed to estimate the ancestral conjugation rate—which correspond closely to conditions in the M-treatment—yielded a final proportion of transconjugants (newly infected cells) among the total plasmid-bearing population of less than 0.1%. In other words, almost all the progeny plasmids were produced by vertical transmission. Therefore, a mutation in a plasmid that increased horizontal transfer by an order of magnitude would be disadvantageous overall if it reduced vertical transmission by only 1%. With the benefit of hindsight, this is the weakest

aspect of our experimental design. On the other hand, this hypothesis begs the question of how half the derived plasmids managed to evolve higher conjugation rates (Fig. 8) in the face of substantially increased costs of plasmid carriage (Fig. 7) relative to the ancestor.

Selection against Transconjugants.—Yet another scenario is that horizontal transfer was selected against because a plasmid would find itself in a host that was not adapted to the local environment. The fact that the immigrant pool evolved in the absence of antibiotic, whereas the residents were exposed to antibiotic every third day (Fig. 2), lends credibility to this hypothesis. However, competitions among plasmid-bearing strains gave identical results in the presence and absence of antibiotic (Fig. 7, Table 3). Moreover, this hypothesis suffers the same problem as the preceding one, insofar as it begs the question of why then did half the plasmids evolve higher conjugation rates despite substantially increased costs of plasmid carriage.

Selection for Transconjugants.—We can turn the last scenario on its head and, in so doing, perhaps explain this conundrum. The plasmid-free controls, which provided the immigrant pool, began to have a substantial fitness advantage relative to the plasmid-bearing controls during the latter half of the experimental evolution (Fig. 4). At this time, plasmids began to be seen on the immigrant background in the M- and H-treatments (Fig. 5). Perhaps there was not selection for higher conjugation rates per se, but rather mutant plasmids with higher conjugation rates would sooner colonize the more fit immigrant background, where upon they would "hitchhike" to high frequency. These highly conjugative plasmids would be maintained at some frequency, despite having lower fitness, by recurrent mutation. They would have had a disproportionate chance of being the first to colonize the immigrants by virtue of their higher conjugation rate. Once on the immigrant background, they would be carried to high frequency, provided that the higher cost of their carriage was less than the fitness advantage of the immigrant relative to the resident hosts. This hypothesis is similar to explanations for the high prevalence of mutator genotypes in asexual populations (Sniegowski et al. 1997; Taddei et al. 1997), except that the more infectious parasite becomes associated with the more fit host by finding it sooner rather than by causing it (as does a mutator). We do not claim to have proven this scenario, but it is intriguing and may have been a factor in our experiment. More generally, as a parasite evolves from horizontal to vertical modes of transmission, it loses the ability to move between host genotypes (in the case of asexual hosts) or species (for sexual hosts). The parasite is then unable to colonize a more successful host type, one which might exclude the present host and the parasite with it. In the same way that sexual reproduction can be viewed as an adaptation to cope with changing environments (Williams 1975; Bell 1982), so too might horizontal transmission be seen as an adaptation that allows a parasite to move onto superior hosts that emerge. Both hypotheses rely on breaking apart associations and forming new ones, so that individual elements (genes in one case, parasites in the other) can avoid the fate imposed by fixed associations. We suggest that this possible benefit of horizontal transfer is worthy of further analysis.

Findings Compared with Earlier Studies

Our experiment builds on two previous studies that examined the interactions between bacteria and their parasitic elements. Levin (1980) compared the cost of carriage of plasmid R1 and a mutant (R1-*drd*-19) that constitutively expresses the genes involved in conjugation. The mutant had a higher conjugation rate than its progenitor, but the mutant also imposed a greater cost of carriage on its bacterial host. Our study extends this tradeoff to another plasmid, where it was seen in many independently derived genotypes. For bacterial plasmids, at least, the tradeoff between horizontal and vertical transmission appears to be a general phenomenon.

Bull et al. (1991) performed an evolution experiment to test essentially the same model that we tested. Using bacteria as hosts and filamentous viruses as parasites, they manipulated the opportunities for vertical and horizontal transmission. The parasites became less virulent (more "benevolent") when the authors eliminated the necessity for horizontal transmission, whereas virulence was maintained when the parasites required horizontal transmission to uninfected hosts for their evolutionary survival. Thus, the study by Bull et al. (1991) supports the main prediction of the epidemiological model, whereas our study does not. Both studies found that parasites that became less costly to their hosts also had lower rates of infectious transmission (see also Bull and Molineux 1992). Bull et al. did not observe the evolution of increased horizontal transfer (relative to the ancestral parasite), whereas we showed that the correlation between cost of carriage and rate of infectious transfer held in both directions. (It may seem obvious that correlated responses to selection in opposite directions should be reciprocal, but in fact asymmetrical responses are sometimes observed [Mongold et al. 1996; Travisano 1997].) Thus, there are tradeoffs between vertical and horizontal transmission in both plasmids and viruses that infect bacteria; but whether a particular parasite evolves toward one transmission mode or the other seems to depend on other factors in addition to the availability of susceptible hosts.

Association of Transmission and Resistance Phenotypes

We observed a very strong, but unexpected, association between transmission phenotypes and antibiotic resistance profiles of the evolved plasmids. Those that evolved higher conjugation rates retained their resistance to tetracycline, whereas those that evolved lower conjugation rates became sensitive to tetracycline. The mechanistic basis for this association is unknown. A simple hypothesis is that large-scale deletions had simultaneously eliminated adjacent genes encoding the tetracycline resistance and conjugal transfer functions. Turner (1995) tested this by performing restriction digests on the ancestral and derived plasmids, but he saw no significant changes in the size of any of the derived plasmids. Another possibility is that the genes encoding tetracycline resistance and conjugal transfer are transcribed from the same or overlapping promoters. In that case, even point mutations in the promoter region could simultaneously change the expression of both functions. This hypothesis requires detailed sequence analyses and has not yet been tested.

Concluding Remarks

With short generations, large populations, and general ease of culture, bacteria provide powerful experimental systems for testing evolutionary hypotheses. However, some "macrobiologists" may feel that bacteria lack the complexities and surprises of "real" organisms (i.e., multicellular plants and animals), in effect implying that experiments with bacteria are nothing more than cumbersome computer simulations. The results of this study indicate otherwise—sometimes to our profound frustration. For reasons that are not clear, our experiments failed to support the predicted effect of susceptible host density on the evolution of plasmid virulence, despite compelling evidence for a tradeoff between horizontal and vertical modes of transmission. And while this genetic tradeoff conforms to a widely accepted (but rarely tested) hypothesis, it was completely unexpected that the tradeoff should also involve a gene for antibiotic resistance, and yet it does. Evidently, despite their apparent simplicity, the evolution of bacteria and their associated parasites can be surprisingly complex.

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