ASSOCIATIONS BETWEEN BACTERIA AND CONJUGATIVE PLASMIDS: MODEL SYSTEMS FOR TESTING EVOLUTIONARY THEORY

By

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ABSTRACT

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Interactions between bacteria and conjugative plasmids were used to address two distinct questions. (1) The first question concerns the relationship between genetic variability and rates of adaptation. Can plasmid-mediated recombination be used to generate novel genotypes, and thereby accelerate the rate of adaptation in otherwise asexual populations of bacteria? To test this hypothesis, genetically distinct Hfr (high frequency recombination) cells were periodically introduced into experimental populations of *Escherichia coli*. Recombinant genotypes became numerous (or even fixed) in all treatment populations, but, surprisingly, these recombinants showed no significant increase in fitness relative to the recipient's ancestor, nor relative to non-recombining control populations. One possibility is that fitness measurements do not accurately reflect complex selection dynamics, such as frequency-dependent and even nontransitive interactions, brought on by recombinant genotypes. In at least one recombination treatment population, further experiments confirmed that frequency-dependent selection allows recombinant genotypes to coexist. This stable coexistence of two genotypes on a single limiting resource was promoted by a cross-feeding interaction, but some evidence of a demographic tradeoff was also found. (2) The second question concerns the evolution of virulence in pathogens and other infectiously transmitted elements. Does host density influence the evolution of plasmid virulence and mode of transmission? This hypothesis was tested by allowing associations of *E. coli* and plasmid pB15 to evolve in replicated environments with different inputs of susceptible hosts. The plasmids' effects on host

fitness and their conjugation rates were both observed to evolve, with a consistent tradeoff between rates of horizontal and vertical transmission. However, manipulations of host density had no effect on the evolution of plasmid virulence and mode of transmission. One possible explanation is that conjugation of pB15 does not behave according to mass-action, and some evidence in support of this possibility is presented.

Dedicated to
Sylvia Baskerville Turner
and
Eugene Turner

For their neverending love,
and confidence in my abilities.

ACKNOWLEDGMENTS

I thank my parents, Eugene and Sylvia, and my siblings, Lennie and Peter, for providing a loving environment in which to grow. I am extremely grateful to all those friends who helped me escape from the tedium of graduate school, especially the Boys of Summer: Keith Hartley, Matthew Fitzgibbons, and Dennis Verrette, without whom I would (literally) not be here today. I thank my fellow graduate students and colleagues at University of California, Irvine, and Michigan State University for the discussion of ideas that led to this dissertation. These pillars of thought include (but are not limited to) Kevin Bailey, Tom Clark, Ananias Escalante, Jorge Santo Domingo, and the members of the Lenski lab (who, unfortunately, are too numerous to mention). I thank my advisory committee at MSU (Guy Bush, Dennis Fulbright, Donald Hall, and Andrew Jarosz) for the discussion of my written work and difficult (but insightful) exam questions. A special thanks goes to Joseph Graves for encouraging me to pursue graduate studies at UCI. I owe a debt of gratitude to Albert Bennett and Richard Hudson for their patient provision of laboratory and office space while I finished my thesis. In addition, I am forever indebted to my friend and mentor Richard Lenski, for his demonstration of the qualities necessary to excel in scientific research, and for his tolerance of my correspondence-course style graduate career. Most of all, I would like to thank my dear friend Mary Beth Decker, because graduate school would have been unbearable without the happiness and good times we shared over the past few years.

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INTRODUCTION

Plasmid Biology

Plasmids are circular, extrachromosomal DNA molecules able to autonomously replicate within a bacterial cell. Many characteristics exhibited by bacteria that are of importance in medicine, agriculture, commerce, and the environment are, in fact, plasmid-determined. Such characteristics include resistance to antibiotics, the ability of nitrogen-fixing *Rhizobium* strains to nodulate roots of legumes, antibiotic production by *Streptomycetes*, and the biodegradation of certain herbicides (Hardy 1986; Kinashi et al. 1987). Although plasmids are a nearly ubiquitous feature of naturally-occurring species of bacteria, under most growth conditions they are dispensable to their host cells (Freifelder 1987).

All plasmids are able to control their own replication using the host's cellular machinery and are transferred vertically across generations of the host. In the absence of selection on the host for specific plasmid-encoded characters (such as antibiotic resistance), most plasmids reduce the fitness of their hosts relative to isogenic plasmid-free counterparts (Levin 1980; Dykhuizen and Hartl 1983; Lenski and Bouma 1987; Lenski and Nguyen 1988; Nguyen et al. 1989); hence, they can be regarded as parasites under these conditions (Levin and Lenski 1983). Many plasmids are also able to transfer horizontally from an infected host (donor) to an uninfected host (recipient) through a process called conjugation (Lederberg 1956). Although some of the details of the conjugation process are still poorly understood, conjugation is initiated by contact between donor and recipient cells via a plasmid-encoded protein appendage called a sex pilus. Thus, conjugative plasmids are transmitted by two distinct modes: horizontal

(infectious) transmission occurs by conjugation, while vertical (intergenerational) transmission occurs by host cell division (Figure 1).

Bacterial reproduction per se is strictly asexual. However, bacteria may undergo sex via recombination when a plasmid integrates into the host chromosome and retains its ability to transfer by conjugation. The best known example of chromosomal transfer is the integration of an F plasmid into an *Escherichia coli* host, which converts the host into a high frequency recombination (Hfr) cell. An Hfr cell (donor) has the ability to conjugate with a recipient cell lacking the F plasmid (F^-) and to donate copies of its chromosomal genes. During transfer of Hfr DNA to a recipient cell, the mating pair usually breaks apart before the entire chromosome is transferred, but on average several hundred genes are transferred before the cells separate (Freifelder 1987; Lloyd and Buckman 1995). Separation almost always occurs before the final segment of F is transferred; thus, the recipient usually remains F^-. During or after Hfr transfer, regions of the transferred DNA fragment are frequently exchanged with the recipient chromosome, thereby converting the recipient into a recombinant genotype.

Thesis Overview

In this dissertation, I describe experiments using conjugative plasmids and their bacterial hosts that test two distinct evolutionary hypotheses. First, I hypothesize that plasmid-mediated recombination will generate novel genotypes and, thereby, accelerate the rate of adaptation in otherwise asexual populations of *E. coli*. Second, I hypothesize that the evolution of plasmid transmission and virulence is determined by the density of uninfected bacterial hosts in the environment.

Chapter I examines the effects of plasmid-mediated recombination on rates of fitness increase in experimental populations of bacteria. Earlier studies have described the evolutionary dynamics in experimental populations of *E. coli* whose sole source of genetic variation was spontaneous mutation (Lenski et al. 1991; Lenski and Travisano 1995).

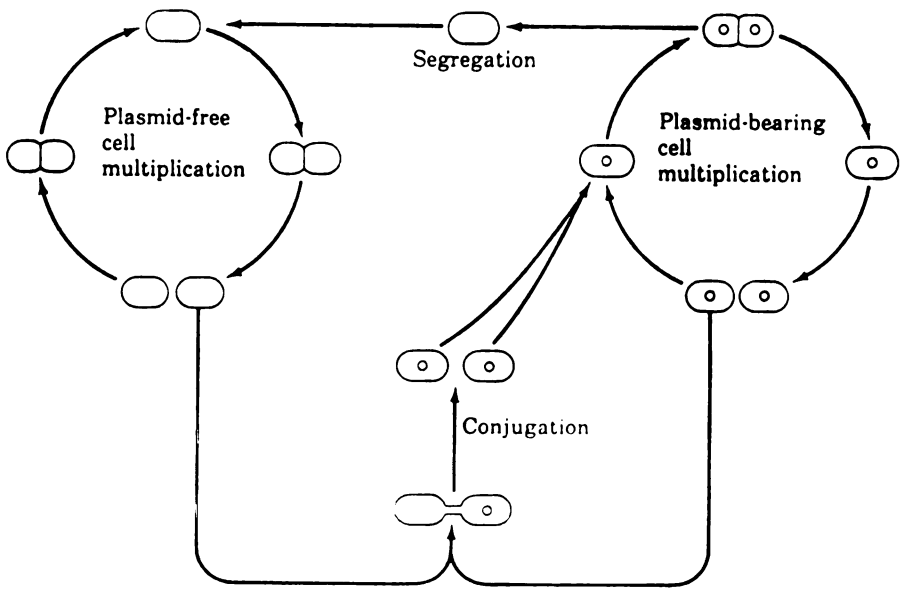

Figure 1. Schematic representation of the dynamics of a conjugative plasmid and its bacterial host. Conjugative plasmids (o) are transmitted in two ways: vertically through plasmid-bearing cell multiplication, and horizontally through conjugation. Modified from Levin and Lenski (1983).

Over the course of thousands of generations, the rate of fitness increase in these populations diminished substantially, apparently because the populations exhausted most of the beneficial mutations. Chapter I describes a series of experiments to determine whether the rate of fitness improvement in these populations could be re-accelerated by plasmid-mediated recombination, which would increase the available genetic variation.

In one of these treatment populations, I observed coexistence between two recombinant genotypes on a single limiting resource, glucose. This polymorphism does not conform to a simple model of competitive exclusion (Gause 1934; Hardin 1960). Chapter II demonstrates that this polymorphism is indeed stable, and then it describes a series of experiments to test two alternative hypotheses that might explain the stable polymorphism: (1) a demographic tradeoff, such that one genotype is competitively superior when glucose is abundant whereas the other genotype is the better competitor for sparse glucose; and (2) a cross-feeding interaction, whereby the superior competitor for glucose excretes a metabolite for which the other genotype is the better competitor.

Chapter III examines the impact of susceptible host density on the evolution of plasmid virulence and mode of transmission. When susceptible hosts are common, the opportunity for infectious transfer is great and selection is hypothesized to favor increased rates of horizontal transmission, even at the expense of reduced vertical transmission caused by increased virulence. When available hosts are rare, however, vertical transmission is the more frequent mode of transfer and selection should favor increased rates of vertical transmission by reducing virulence, even at the expense of reduced infectious transmission. I tested this hypothesis by allowing associations between *E. coli* and a plasmid to evolve in replicated environments supplemented with different densities of susceptible hosts.

A key assumption of the model for evolution of plasmid virulence is that bacterial conjugation behaves according to mass-action. That is, the opportunity for horizontal transfer by conjugative plasmids is supposed to increase in direct proportion to the density

of available hosts. In Chapter IV, I begin to explore the validity of this assumption in light of some unexpected results in the preceding experiment. To do so, I tested the ability of conjugative plasmids to invade bacterial populations at low, intermediate, and high densities of plasmid-free hosts, and I independently estimated their conjugation rates under each of these conditions.

CHAPTER I

EFFECTS OF RECOMBINATION WITH EXOGENOUS GENOTYPES ON THE RATE OF BACTERIAL EVOLUTION

INTRODUCTION

In contrast to most other organisms, reproduction and sexuality in bacteria are discrete and independent functions (Dykhuizen and Green 1991). Bacterial reproduction per se is strictly asexual, occurring by binary fission. But if sex is defined as the exchange of genetic material between organisms, then bacteria undergo sexual recombination through the processes of transformation, viral-mediated transduction, and plasmid-mediated conjugation (Levin 1988; Hopwood and Chater 1989; Maynard Smith 1990; Dykhuizen and Green 1991; Maynard Smith et al. 1993). For example, an F plasmid inserted into a bacterial chromosome converts the bacterium into an Hfr (high frequency recombination) donor strain.

Population-genetic studies of bacteria isolated from nature have demonstrated that some species, including *Escherichia coli*, have very high levels of linkage disequilibrium, indicating clonal population structures (Selander and Levin 1980; Whittam et al. 1983; Caugant et al. 1984; Maynard Smith et al. 1993; Whittam and Ake 1993). However, there is also molecular evidence for occasional chromosomal recombination in natural populations of even highly clonal bacteria such as *E. coli* (Milkman and McKane-Bridges 1990; Maynard Smith 1990; Bisercic et al. 1991; Dykhuizen and Green 1991; Maynard Smith et al. 1991; Whittam and Ake 1993; Guttman and Dykhuizen 1994a, 1994b). In addition, recent analyses of population genetic structure in *Bacillus subtilis* (Istock et al. 1992), *Rhizobium etli* (Souza et al. 1992), and *Neisseria gonorrhea* (Maynard-Smith et al. 1993) suggest that recombination is much more frequent in these species than in *E. coli*.

In all, the evolutionary significance of recombination in bacteria remains a contentious subject (Maynard Smith 1990; Maynard Smith et al. 1993; Lenski 1993).

The effect of recombination (along with mutation and migration) in basic models of population genetics is to increase genetic variation. Natural selection may then act on this variation to increase the mean fitness of an evolving population (Wright 1931, 1932; Fisher 1958; Roughgarden 1979). However, recent analyses have shown that the implications of increased genetic variation for mean fitness are not always so simple (see, e.g., Frank and Slatkin 1992).

Bacteria provide an excellent experimental model to study evolutionary processes such as mutation, natural selection, and adaptation (Luria and Delbruck 1943; Atwood et al. 1951; Helling et al. 1987; Dykhuizen 1990; Lenski et al. 1991; Bennett et al. 1992; Lenski 1992; Lenski and Travisano 1994; Travisano et al. 1995). Because of their large population sizes and short generation times, bacteria may be propagated in defined environments for hundreds and even thousands of generations, allowing evolutionary changes and processes to be observed in detail. While a few experimental evolution studies with bacteria have allowed recombination (Graham and Istock 1979, 1981), these have not been directly concerned with quantifying the effect of recombination on the rate of adaptive evolution. In this chapter, I present the results of a 1,000-generation experiment that was designed to examine the effects of recombination on the rate of adaptive evolution in otherwise asexual populations of *E. coli*.

Experimental overview. -- The bacterial strains used in this study were isolated from twelve populations of *E. coli* B maintained in the laboratory as part of a long-term evolution experiment (Lenski et al. 1991; Lenski and Travisano 1994). During 10,000 generations of evolution, the mean fitness of these populations relative to a common ancestor increased by about 50% on average (Figure 2). All twelve populations were descended from a single clone, which lacked plasmids or functional phage. Hence,

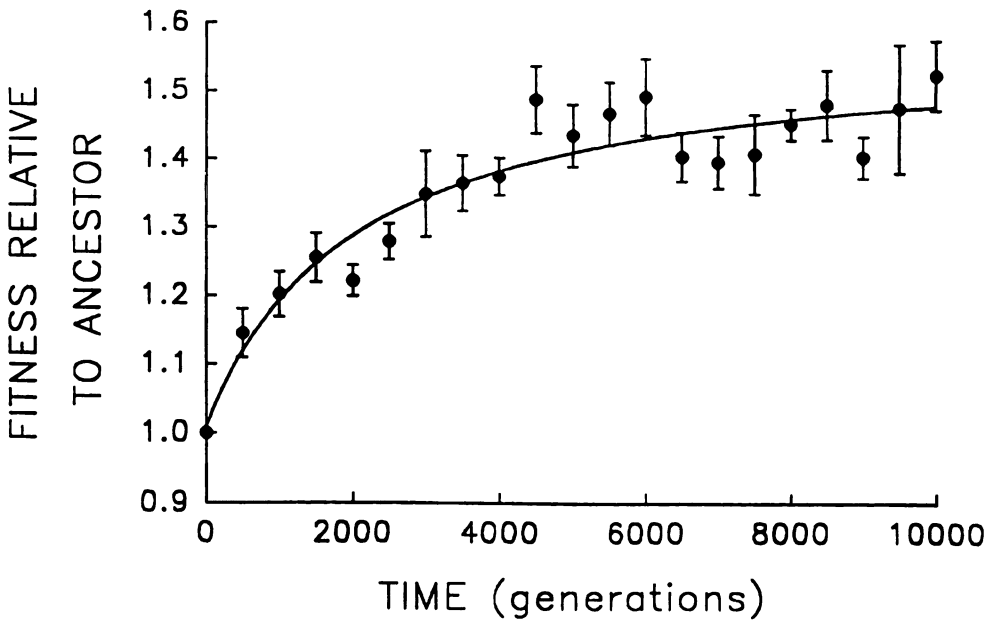

Figure 2. Mean fitness relative to the ancestor for the twelve *E. coli* populations described by Lenski and Travisano (1994). Symbols give grand mean and 95% confidence interval for the twelve populations. The curve gives the best fit of a hyperbolic model to the grand means, and it shows a significant deceleration in the rate of improvement over time. The populations used in the present study were derived from isolates obtained after 7,000 generations of this earlier study.

mutation was the only source of genetic variation for adaptation by natural selection. Most of the increase in mean fitness occurred during the first few thousands of generations of this experiment. Thus, by generation 5,000 or so, the rate of adaptation had slowed to such an extent that the approach to a selective plateau was apparent (Figure 2).

The central question that I sought to address in this study is whether the rate of adaptive evolution in these populations could be re-accelerated by providing an additional source of genetic variation, by means of sexual recombination with distantly related strains. To that end, a single clone was isolated from each of the twelve evolving populations after 7,000 generations, by which time their rates of increase in fitness had slowed substantially. Each clone was then used to found a new pair of populations, one of which would experience sexual recombination and the other of which would serve as an asexual control.

Recombination was achieved by periodically adding plasmid-bearing Hfr (high frequency recombination) *E. coli* K12 donor cells to the treatment populations. These donors are genetically quite distinct from the *E. coli* B recipients, thus providing an opportunity both to introduce substantial genetic variation by sexual recombination and to score the movement of several marker alleles. To prevent the spread of the donor genotypes by ecological competition -- in contrast to the spread of their genes by recombination and subsequent selection -- I chose to use donor genotypes that were deficient in growth in the experimental environment owing to mutations in critical metabolic genes. Thus, the donor genotypes could transmit their genes by conjugation, but they could not propagate themselves asexually.

The twelve pairs of recombination treatment and asexual control populations were then propagated for a further 1,000 generations, in the same environment in which their asexual progenitors had been propagated for the previous 7,000 generations. While their environments were identical, the control populations had only mutation as a source of genetic variation, whereas the treatment populations had recombination with the Hfr

donors as an additional source of variation. Thus, the experiment could address the following questions:

(i) Did genetic markers from the Hfr donors enter the recipient populations, indicating that the recombination treatment had the intended effect of increasing the available genetic diversity?

(ii) Did this additional diversity, in fact, allow more rapid adaptive evolution in the treatment populations than in the control populations? That is, did sexual recombination re-accelerate the rate of increase in mean fitness?

MATERIALS AND METHODS

Bacterial strains. -- A single clone was isolated at generation 7,000 from each of the twelve experimental populations described by Lenski and Travisano (1994). Six of the clones can grow on the sugar L-arabinose (Ara^+) and six cannot (Ara^-). Ara^+ and Ara^- strains form white and red colonies, respectively, on tetrazolium-arabinose (TA) indicator plates (Levin et al. 1977). Each clone was used to found one population in each of the recombination and control treatments described below. Each of these founder, or baseline, strains was characterized in terms of nine phenotypic traits and five electrophoretic loci (Table 1). There were no differences among the twelve baseline recipients, except for the Ara marker. All baseline strains were stored in a glycerol-based suspension at -80 ^{o}C to allow for direct comparisons between them and their evolved derivatives at a later time.

The donor strains used in this study were four Hfr strains of *E. coli* K12. Each of these donors has an F plasmid inserted at a different map position in its chromosome (Table 1). Thus, each has a different point of origin (OriT site) for conjugative transfer and subsequent recombination. These *E. coli* K12 donors are rather distantly related to

Table 1. Genetic differences between donor and recipient strains used in Chapter I.

	Phenotypic[1]									Electrophoretic[2]					
Strain	Ara	Lac	Tet	Str	Arg	Leu	Ilv	T1X	T6	IDH	6-PGD	ADH	MPI	PEP	OriT[3]
Recipients (*E.coli* B)[4]															
Baseline	+/-	+	s	r	+	+	+	s	r	1	2	2	2	1	--
Donors (*E.coli* K12)[5]															
REL288	+	+	r	s	+	+	-	s	s	2	1	1	1	2	67 ↑
REL291	-	-	r	s	-	+	+	r	r	2	1	1	1	2	2 ↓
REL296	+	-	r	s	-	-	+	r	s	2	1	1	1	2	12 ↓
REL298	+	-	r	s	-	-	+	r	r	2	1	1	1	2	84 ↓

[1] + and - indicate ability and inability, respectively, to grow on sugars L-arabinose (Ara) and lactose (Lac); s and r indicate sensitivity and resistance, respectively, to antibiotics tetracycline (Tet) and streptomycin (Str); + and - indicate prototrophy and auxotrophy,

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Recipients (*E.coli* B)[4]															
Baseline	+/-	+	s	r	+	+	+	s	r	1	2	2	2	1	--
Donors (*E.coli* K12)[5]															
REL288	+	+	r	s	+	+	-	s	s	2	1	1	1	2	67 ↑
REL291	-	-	r	s	-	+	+	r	r	2	1	1	1	2	2 ↓
REL296	+	-	r	s	-	-	+	r	s	2	1	1	1	2	12 ↓
REL298	+	-	r	s	-	-	+	r	r	2	1	1	1	2	84 ↓

[1] + and - indicate ability and inability, respectively, to grow on sugars L-arabinose (Ara) and lactose (Lac); s and r indicate sensitivity and resistance, respectively, to antibiotics tetracycline (Tet) and streptomycin (Str); + and - indicate prototrophy and auxotrophy,

Table 1 (cont'd).

respectively, for amino acids arginine (Arg), leucine (Leu) and isoleucine-valine (Ilv); s and r indicate sensitivity and resistance, respectively, to phages T1X and T6.

[2] Numbers indicate mobility classes for enzymes isocitrate dehydrogenase (IDH), 6-phosphogluconate dehydrogenase (6-PGD), alcohol dehydrogenase (ADH), mannose phosphate isomerase (MPI), and peptidase (PEP).

[3] Map position, in minutes, on the *E. coli* K12 chromosome for the origin of transfer of donor genes into recipient cells. ↑ or ↓ indicates transfer in order of ascending or descending map order, respectively.

[4] Except for the Ara phenotype, there were no differences in these traits among the twelve baseline recipient strains.

[5] The donors were obtained from B. Bachmann, curator of the *E. coli* Genetic Stock Center at Yale University. They were previously designated BW6159, BW6165, BW7261, and BW6169, respectively.

the *E. coli* B recipients and they differ at several genetic markers (Table 1; see also Selander and Levin 1980). Each donor has a tetracycline marker located approximately 10 minutes from its OriT site (Table 1). These genetic differences between donors and recipients allowed detection of recombinant genotypes generated over the course of the experiment, by means of selective-plating and isozyme-electrophoresis. In addition, each donor strain was auxotrophic for at least one amino acid (Table 1), so that it could not grow in the minimal medium employed in this experiment. Hence, these Hfr strains could donate genes via recombination but were themselves unable to proliferate and outcompete the prototrophic *E. coli* B recipients.

Culture conditions. -- The culture medium employed in all experiments was Davis Minimal (DM) broth (Carlton and Brown 1981) supplemented with $2x10^{-6}$ g thiamine hydrochloride and 25 μg glucose ml^{-1}. This medium supports a stationary-phase bacterial density of ~$5x10^7$ cells ml^{-1}. Culture volume was 10 ml, maintained in 50-ml Erlenmeyer flasks and placed in a shaking incubator at 37 °C and 120 rpm. All cultures were serially propagated each day by transferring 0.1 ml of each stationary-phase (24 h) culture into 9.9 ml of fresh medium. The resulting 100-fold re-growth represents ~6.64 generations of binary fission each day. The Hfr strains used in this experiment were grown up separately in Luria broth (LB), a rich medium which allows for a stationary-phase bacterial density of ~$2x10^9$ cells ml^{-1}.

Recombination treatment. -- A single clone of each baseline strain was used to initiate each of the twelve populations (six Ara^+ and six Ara^-) in the recombination treatment. Every day, for 150 days, 0.1 ml from the previous day's culture was transferred into 9.9 ml of fresh DM. On day 0, and every fifth day (or 33 generations) thereafter, I also added 0.01 ml of an equally-proportioned volumetric mixture of the four Hfr strains, which had been grown overnight in LB. This manipulation produced initial densities of

about $2x10^6$ and $5x10^5$ donor and recipient cells ml^{-1}, respectively, for a ratio of about 4:1. On those days when donors were added, the flasks were placed in a non-shaking incubator at 37 °C for one hour to allow uninterrupted mating between donors and recipients. Subsequently, the flasks were transferred to a shaking incubator at 37 °C for 23 hours. On all other days, the flasks were held in a shaking incubator for all 24 hours. During the next four days, the cultures were propagated using serial transfer. Every 15 days (100 generations), just prior to the addition of donor cells, 10% glycerol was added to a sample from each population, and the samples were placed into a freezer at -80 °C for future study.

Preliminary experiments were conducted to ensure that the protocol for the recombination treatment would work in two important respects. First, I wanted to make sure that recombinants (transconjugants) did, in fact, occur at measurable frequencies. Second, I wanted to make sure that the auxotrophic donors died out after their addition to experimental cultures. The dynamics occurring in the five-day cycle of the recombination treatment are shown in Figure 3. As desired, recombinants possessing the donor's tetracycline resistance and the recipient's streptomycin resistance were readily detected; and the number of auxotrophic donors fell below the limit of detection (< 10 cells ml^{-1}) after three days (Figure 3).

Control treatment. -- The same twelve clones (six Ara^+ and six Ara^-) used to found the populations in the recombination treatment were also used to initiate the control populations. On day 0, and every fifth day thereafter, each recipient population underwent a "sham" manipulation to duplicate the recombination treatment, but without allowing genetic recombination. In particular, the control populations received 0.01 ml of LB media in which the Hfr donor strains had been grown to stationary phase, but from which all cells had been removed by filtration. Also, for the first hour after receiving this "placebo", the control populations were placed in the non-shaking incubator prior to being

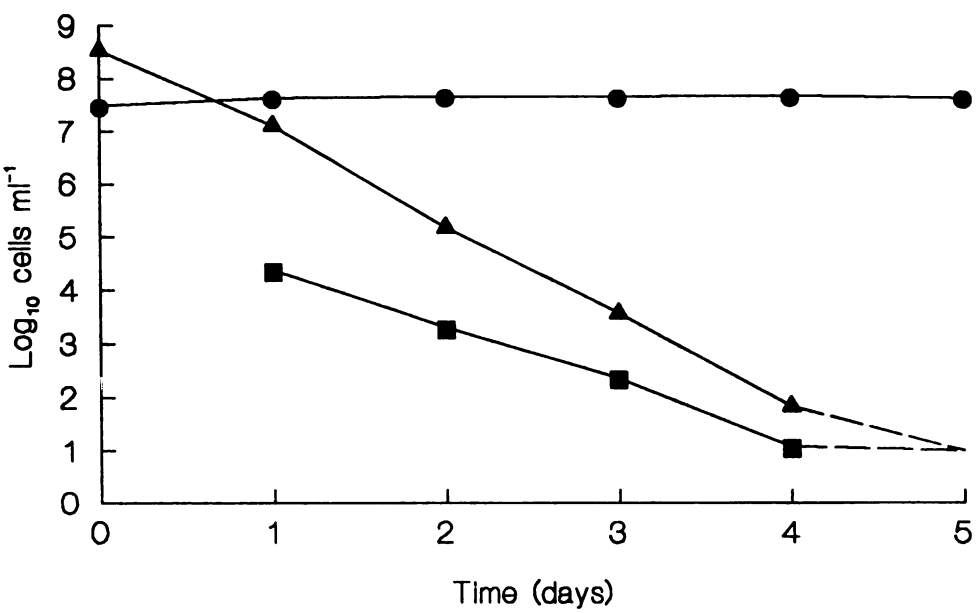


Figure 3. Results of a preliminary experiment to determine the survival of *E. coli* K12 donors (triangles), *E. coli* B recipients (circles), and recombinant genotypes (squares) during the five-day cycle of the recombination treatment.

moved to the shaking incubator. Thus, the control populations experienced the same selective environment as the recombination populations, except for the effects of adding donors.

Fitness assays. -- To assay relative fitness (W), two strains were placed in competition under the culture conditions described above, where one competitor was Ara^+ and the other was Ara^-. Each strain was grown separately for one day in DM, as a preconditioning step to ensure that both competitors were in comparable physiological states. The two competitors were mixed at a 1:1 ratio, then diluted 1:100 into fresh DM and allowed to grow and compete during a 24-hour growth cycle. Initial and final densities of each competitor were estimated by spreading them on TA plates, which permitted the competitors to be distinguished by colony color.

Let the initial densities of the Ara^+ and Ara^- competitors be $N_1(0)$ and $N_2(0)$, respectively; and let $N_1(1)$ and $N_2(1)$ be their corresponding densities after one day. The average rate of increase (or realized Malthusian parameter), m_i, for either competitor is then calculated as:

$$m_i = \ln[N_i(1)/N_i(0)] / (1 \text{ day}).$$

The fitness of one strain relative to another (W_{ij}) is estimated as the ratio of their Malthusian parameters (Lenski et al. 1991):

$$W_{ij} = m_i/m_j.$$

A fitness difference between the two competitors may reflect differences in their lag phase, maximum or submaximum growth rates, survival at stationary phase, or some combination thereof (e.g., Vasi et al. 1994).

Screening for recombinant genotypes

Phenotypic markers. -- Every 100 generations, ten clones were isolated at random from each population in the recombination and control groups. The phenotype of these randomly-chosen isolates was determined using nine genetic markers that could be scored on indicator plates: arabinose and lactose utilization, tetracycline and streptomycin resistance, resistance to the bacteriophages T1X and T6, and auxotrophy for the amino acids leucine, arginine and isoleucine-valine.

Allozyme-electrophoresis markers. -- Lysates were prepared for the ten clones chosen at random from each population at generation 1,000. Each clone was grown overnight in 10 ml of LB, and the resulting culture was sonicated using the method described by Pinero et al. (1988). These clones were scored for five allozyme markers using cellulose acetate (Hellena Laboratories) electrophoresis: alcohol dehydrogenase (ADH), isocitrate dehydrogenase (IDH), mannose phosphate isomerase (MPI), peptidase (PEP) and 6-phosphogluconate dehydrogenase (6-PGD). These five enzymes were chosen (from 16 in preliminary screens) because their mobilities were found to differ for the *E. coli* K12 donors and *E. coli* B recipients used in my experiment (Table 1). At least three independent electrophoretic assays were performed on each isolate for each allozyme tested.

RESULTS

Genetic changes. -- Figure 4 shows the genetic changes that were seen in one of the recombination treatment populations, based on scoring nine physiological traits for ten clones at 100-generation intervals. Three distinct recombinant genotypes were detected, and by generation 700 the ancestral genotype had fallen below the limit of detection.

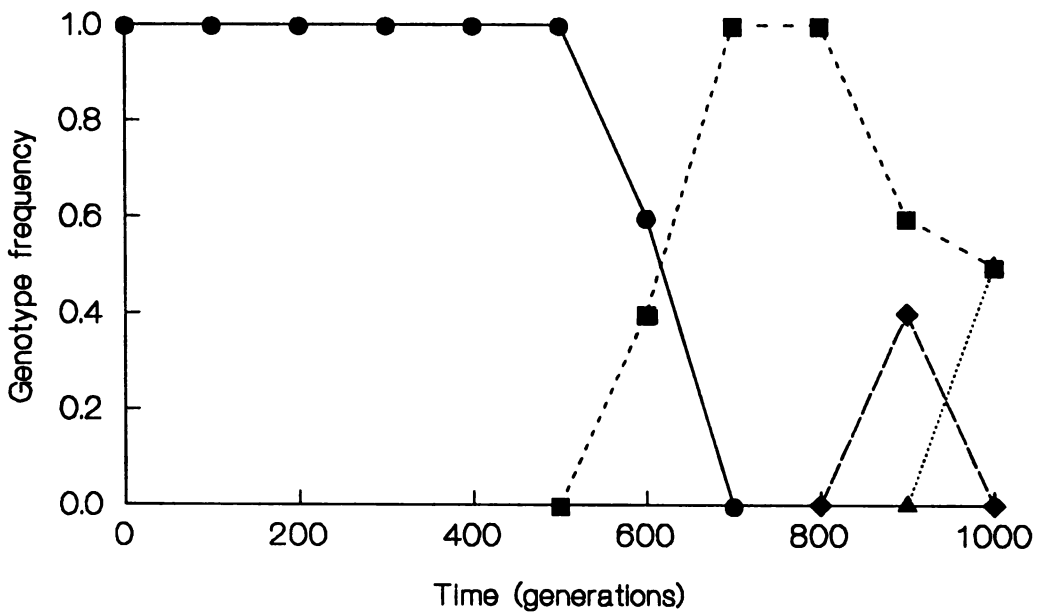


Figure 4. Evolutionary dynamics in population Ara⁻1 of the recombination treatment. Population samples were obtained every 100 generations, and changes in genotype frequency are based upon the nine phenotypic markers described in Table 1. The ancestral genotype (circles), and three distinct recombinant genotypes (squares, diamonds, triangles) were detected over the course of the study.

Qualitatively similar dynamics were seen in the other recombination treatment populations, as summarized in Table 2. New genotypes were first seen, on average, at about generation 550, with 2.67 distinct new genotypes seen during the 1,000-generation experiment. By contrast, using the same physiological traits and sampling scheme, absolutely no variants were detected in any of the control populations (Table 2).

The above data must severely underestimate the extent of genetic diversity, and the rate of genetic change, in the recombination treatment populations. Only nine physiological markers were scored; three of these markers (those for amino acid auxotrophy) were subject to strong selection for the ancestral state, and no variation was seen in those traits. To further evaluate the effect of the recombination treatment, I also scored ten clones taken from each population at generation 1,000 for the five electrophoretic markers that distinguished the donor and recipient populations (Table 1).

Table 3 summarizes the diversity revealed by combining the electrophoretic and physiological markers. On average, there were 6.08 distinct genotypes per recombination treatment population, which is especially remarkable given the fact that only ten clones were characterized for each population. The ancestral (recipient) genotype was absent from 6 of the treatment populations, and the frequency of the ancestral type was 20% or less in ten of the twelve populations. Again by contrast, none of the electrophoretic or physiological markers showed any variation in any of the twelve control populations. Table 19 (see Appendix) lists the various genotypes and their frequencies at 1,000 generations.

Evidently, the recombination treatment greatly increased the level of genetic variation available for natural selection, and it led to much faster rates of evolutionary change at the genetic level. In the next section, I examine the consequences of this additional variation for the rate and extent of adaptive evolution, as measured by gains in fitness.

Table 2. Summary of the temporal dynamics of genetic change in experimental populations.

Population	New genotype first seen	Ancestral type last seen	Number of distinct new genotypes
Recombination treatment			
Ara$^-$1	600 gen.	600 gen.	3
Ara$^-$2	600 gen.	1,000 gen.	1
Ara$^-$3	300 gen.	200 gen.	2
Ara$^-$4	200 gen.	1,000 gen.	1
Ara$^-$5	500 gen.	500 gen.	5
Ara$^-$6	200 gen.	1,000 gen.	3
Ara$^+$1	800 gen.	800 gen.	3
Ara$^+$2	1,000 gen.	1,000 gen.	1
Ara$^+$3	900 gen.	1,000 gen.	2
Ara$^+$4	200 gen.	1,000 gen.	5
Ara$^+$5	600 gen.	1,000 gen.	4
Ara$^+$6	700 gen.	1,000 gen.	2
Control			
All 12	Never	1,000 gen.	0

Note: Every 100 generations, ten clones were randomly chosen from each population and then scored for the nine phenotypic markers listed in Table 1. The dynamics for recombination treatment population Ara$^-$1 are shown in Figure 4.

Table 3. Summary of the genetic changes in experimental populations at the end of 1,000 generations.

Population	Proportion of ancestral type	Number of distinct genotypes, including ancestral type
Recombination treatment		
Ara$^-$1	0%	5
Ara$^-$2	20%	6
Ara$^-$3	0%	6
Ara$^-$4	80%	2
Ara$^-$5	0%	5
Ara$^-$6	0%	6
Ara$^+$1	0%	5
Ara$^+$2	10%	8
Ara$^+$3	10%	9
Ara$^+$4	40%	7
Ara$^+$5	0%	7
Ara$^+$6	20%	7
Control		
All 12	100%	1

Note: After 1,000 generations, ten clones were randomly chosen from each population and then scored for the five electrophoretic and nine phenotypic markers listed in Table 1.

Changes in fitness. -- At the end of the 1,000-generation experiment, the fitness of each population in the recombination treatment and control groups was measured relative to a common competitor. This common competitor (REL2543) was the ancestral genotype for recombination treatment and control populations Ara$^-$1 in this study, having been isolated at generation 7,000 in the experiment described by Lenski and Travisano (1994). A selectively neutral Ara$^+$ mutant (REL4190) of this clone was also obtained to allow competition experiments with the Ara$^-$ populations. The genetically heterogeneous populations in the control and recombination groups were competed against the common competitor bearing the opposite Ara marker state, after each competitor was preconditioned in DM for one day. I also estimated the fitness of all twelve ancestral genotypes relative to the common competitor. Fitness assays were replicated four-fold in blocks of 36 (corresponding to the twelve ancestral genotypes, and the twelve 1,000-generation populations in each treatment group). The mean fitnesses relative to the common competitor are shown in Table 4.

I first tested whether the variation provided by mutation alone had allowed the populations in the control group to gain in fitness relative to the ancestor. A one-tailed paired comparison between the means for each control population and the ancestor indicates that the ~4% improvement is statistically significant ($t_S = 2.349$, df = 11, $p = 0.039$).

I then tested whether the additional variation provided by sexual recombination allowed the twelve populations in the treatment group to gain fitness to a greater extent than their counterparts in the control group. A one-tailed paired comparison between the means for each treatment population and its control indicates no significant acceleration due to the recombination treatment ($t_S = -0.801$, df = 11, $p = 0.780$). Therefore, I must conclude that recombination did not allow the treatment populations to gain fitness more rapidly than the control populations, despite the fact that the recombination treatment clearly accelerated the rate of genetic change (Tables 2 and 3).

Table 4. Mean fitness for each experimental population, and for the ancestral genotypes.

		Treatment	
	Ancestor	Control	Recombination
Population			
Ara$^-$1	0.984	0.986	1.092
Ara$^-$2	1.017	1.043	1.087
Ara$^-$3	0.967	1.058	0.816
Ara$^-$4	1.000	1.015	0.984
Ara$^-$5	0.881	0.968	0.985
Ara$^-$6	0.954	1.005	1.032
Ara$^+$1	0.965	1.120	1.028
Ara$^+$2	1.016	1.064	1.050
Ara$^+$3	1.104	1.037	1.064
Ara$^+$4	1.049	1.082	1.031
Ara$^+$5	1.042	1.021	1.024
Ara$^+$6	1.009	1.051	1.039
Grand mean (± SE)	0.999 (0.016)	1.038 (0.012)	1.019 (0.021)

Note: Each population's fitness was assayed, with four-fold replication, relative to the common competitor bearing the alternative Ara marker state. Standard errors for the grand means are based on $n = 12$ replicate populations.

DISCUSSION

I sought to examine the effect of sexual recombination on the rate of evolution in a bacterial model system. The populations of *E. coli* that I studied had previously evolved under a constant environmental regime for 7,000 generations. Twelve populations had been founded by clones of an asexual strain, so that the populations depended entirely on mutation as a source of genetic variation for adaptation by natural selection. Over time, the rate of adaptive evolution in these populations slowed considerably from an initially rapid pace, based on changes in mean fitness (Figure 2).

For this study, I established two new sets of twelve populations each; one set served as controls while the other set underwent sexual recombination. The control populations were propagated for another 1,000 generations in the same environment as their ancestors, and they continued to depend on mutation as their sole source of genetic variability. The treatment populations were also propagated for 1,000 generations in the same environment, but they were also periodically subjected to matings with a pool of Hfr (high frequency recombination) donors. These donors were genetically distinct from the recipient populations, and the donors themselves could not grow in the experimental environment. However, the donors were able to transfer genes to the resident recipient populations by conjugation. The resulting recombination provided the recipient populations with an additional source of variation that might allow them to adapt more quickly.

My study can be summarized by two major results, which appear to be contradictory. On the one hand, the recombination treatment dramatically increased the genetic diversity present in the experimental populations and, indeed, accelerated the rate of their genetic change (Figure 4, Table 2). After 1,000 generations, ten individuals from each population were scored at 14 loci. On average, the twelve recombination treatment populations contained about six distinct genotypes, with the ancestral genotype

representing only 15% of the total (Table 3). But in the twelve control populations, every individual still had the ancestral allele at each locus scored.

On the other hand, the recombination treatment had no measurable effect on the rate of adaptive evolution, as measured by changes in mean fitness during 1,000 generations. The control populations improved, on average, by a few percent relative to their ancestors; the treatment populations improved by about the same amount or perhaps slightly less (Table 4). Thus, the dramatic increase in genetic diversity and rates of genetic change brought on by recombination did not produce any fitness advantage.

How can these two results be reconciled? I cannot offer a definitive answer, but I can suggest two possible explanations. One possibility is that my recombination treatment was, in some sense, far too effective. That is, rather than merely providing an additional source of potentially useful variation, the level of gene flow might have been so high that the recipient populations were faced with an onslaught of deleterious alleles from the donor strains. The other possibility is that complex selection dynamics may render invalid the estimates of fitness obtained relative to a common competitor. For example, if selection is frequency-dependent -- or, in the extreme, if competitive interactions are nontransitive -- then there is not necessarily the expectation that more rapid adaptive evolution would lead to higher "final" fitness values relative to an arbitrary competitor.

In the sections that follow, I consider in more detail the plausibility of these alternative explanations.

Potential for gene flow to have swamped adaptive evolution in the treatment populations. -- In general, recombination increases genetic variation in evolving populations. Natural selection may then use this increased variability to increase mean fitness (Wright 1932; Fisher, 1958). However, if recombination occurs at too high a rate, and involves a gene pool that is not well adapted to the local environment, then recombination pressure may actually decrease the mean fitness of a population because the

locally adapted resident genotypes are "swamped out" by gene flow. For example, plant populations that are locally adapted to living on soils contaminated by heavy metals from mining activities are subject to a high genetic load in the form of pollen flow from nearby populations of metal-intolerant plants (McNeilly 1968; Bradshaw 1971; Ford 1975). Interestingly, some plant populations living in such environments have evolved a high degree of selfing, apparently to avoid the problem of gene flow (Antonovics 1968; Macnair and Cumbes 1989). Thus, recombination pressure might, in principle, explain how recombinant genotypes spread through treatment populations in this study, without any concomitant increase in mean fitness.

The fact that, every fifth day, the recombination treatment populations were subject to four donors for every recipient might suggest extreme recombination pressure. However, bacterial conjugation is very different from regularized genetic exchange due to meiosis and fertilization, and it occurs at much lower rates than obligate outcrossing. To explore the quantitative extent of gene flow in my recombination treatment, I performed a series of mating experiments, with the four donors considered both individually and pooled. These one-day mating experiments were performed, with three-fold replication, under conditions identical to those used every fifth day in the evolution experiment proper. I monitored the accumulation during a standard mating cycle of transconjugants that carried both the Tet^r marker from the donor strains and the Str^r marker from the ancestral recipient REL2545. In each of the four donor strains, the Tet^r marker is located about 10 minutes from the origin of transfer (approximately one-tenth of the distance along the circular chromosome).

My data clearly show that two of the donor strains, REL288 and REL296, were responsible for most of the gene transfer (Table 5). More importantly, it is also clear that only a small proportion -- in no case greater than 0.1% -- of the recipient population received the Tet^r marker. However, the Tet^r marker is only one locus, and I am interested in estimating the fraction of recipients that might have received any donor genes

Table 5. Analysis of the proportion of recipients obtaining the Tet^r marker from the donor strains during a standard mating cycle.

	Population Densities (cells ml^{-1})					
	Initial		Final			Final Proportion Recombinants
	D	R	D	R	T	T / (R + T)
REL288	6.01×10^6	7.33×10^5	2.36×10^7	5.15×10^7	3.93×10^4	7.69×10^{-4}
REL291	1.63×10^6	8.13×10^5	3.90×10^6	5.13×10^7	2.00×10^1	3.92×10^{-7}
REL296	4.53×10^6	8.53×10^5	1.90×10^7	4.83×10^7	1.87×10^4	3.80×10^{-4}
REL298	4.57×10^6	6.07×10^5	1.04×10^7	5.44×10^7	6.67×10^2	1.21×10^{-5}
All four combined	3.78×10^6	1.12×10^6	1.87×10^7	5.53×10^7	2.20×10^4	3.95×10^{-4}

Note: Each mating assay was replicated three-fold, with the mean values shown above.

whatsoever (under the most extreme scenario of recombination pressure where almost all donor genes would be harmful to the recipient). A recent study has shown that, following an Hfr mating, the average fragment integrated into the recipient's chromosome is on the order of ten minutes, or about 10% of the chromosome (Lloyd and Buckman 1995). Therefore, one can estimate roughly that about 90% of all gene transfer events would *not* have included the Tet^r marker. In that case, the proportion of recipients receiving any genes from the Hfr donors would be about 10-fold higher than estimated from the Tet^r marker alone, but still less than 1%.

In fact, this number over-estimates the level of gene flow for three reasons. First, the mating cycle was imposed only every fifth day, so the effective rate of recombination per generation was much lower. Second, each Tet^r recombinant cell is not the product of an independent transfer event. For example, the transfer of a Tet^r marker that occurred two generations prior to the recipient population entering stationary phase would have left about four granddaughter cells. Third, the matings were not allowed to proceed for the full 100 minutes required to transfer the entire chromosome; instead, they were interrupted by moving the mating culture to a shaking incubator after one hour.

In any case, it does not seem likely that more than a small fraction of the cells in a recipient population actually received genes from the Hfr donors. Hence, I reject the hypothesis that an excessively high level of gene flow from maladapted donors may have overwhelmed the populations subjected to the recombination treatment. It seems more likely, therefore, that recombinants increased in frequency during the evolution experiment proper owing to their selective advantage.

Potential for complex selection dynamics to have obscured more rapid adaptive evolution in the treatment populations. -- In this study, I estimated the fitnesses of the derived populations relative to a common competitor (the ancestral genotype for one pair of populations). These data gave no indication that the recombination treatment

populations had adapted to any greater extent than the asexual control populations, despite much greater genetic diversity in the treatment populations. However, this method of fitness estimation, by using a common competitor as a yardstick, implicitly assumes that the dynamics of natural selection are frequency-independent and transitive. That is, it assumes that the fitnesses of all genotypes can be ranked relative to one another based on their ranking relative to a single competitor.

In previous experiments using the asexual progenitors of the populations in this study, this assumption was tested in two different ways; in both respects, it seemed to provide an accurate description of the selection dynamics. Fitnesses measured relative to the common ancestor increased monotonically in these experimental populations (Lenski et al. 1991; Lenski and Travisano 1994). Moreover, the magnitude of a derived genotype's advantage relative to another could be accurately predicted from each one's advantage relative to the ancestor (Lenski et al. 1991; Travisano et al. 1995).

However, more complex selection dynamics have been shown in some other systems. An experiment with evolving populations of the yeast *Saccharomyces cerevisiae*, provides an especially dramatic example of complex selection (Paquin and Adams 1983). In that study, each successive genotype that came to dominate a population was more fit than its immediate predecessor. However, the populations sometimes declined in fitness relative to the original ancestral genotype owing to nontransitive competitive interactions (in which B is more fit than A, C is more fit than B, but C is *less* fit than A). Other studies with bacteria have shown various kinds of frequency-dependent selection, including cases in which each of two genotypes has a selective advantage only when it is sufficiently rare (e.g., Rosenzweig et al. 1994) or common (e.g., Chao and Levin 1981). Thus, it is plausible that the unidimensional estimates of fitness in my study may have obscured important frequency-dependent effects on fitness, which (if fully understood) might show that adaptive evolution in the recombination treatment populations was more rapid than in the control populations.

Testing for nontransitive interactions and other frequency-dependent effects is a daunting task, given the large number of replicate populations and distinct genotypes in this study. I decided to focus on recombination treatment population Ara$^-$3. After 1,000 generations, the fitness of this population appeared to be *below* that of its ancestor (Table 4), suggesting nontransitivity may have been important.

Using genotypes from the samples stored at 100-generation intervals, I performed additional competition experiments to look more closely for nontransitivity. These experiments yielded results consistent with complex selection dynamics, but they did not provide an absolutely clear case of nontransitivity. As shown in Figure 5, the dominant genotype at generation 500 had a large disadvantage relative to the "usual" common competitor (an Ara$^+$ mutant of the ancestor for population Ara$^-$1). The same genotype had no discernible disadvantage (or advantage) relative to an Ara$^+$ mutant of its own direct ancestor. And the two ancestors themselves had very similar fitnesses, regardless of which one was marked by the Ara$^+$ mutation. Thus, fitness gains of certain genotypes from this population appear to have been underestimated relative to the common competitor.

I was unable, however, to identify a set of genotypes from *within* this population for which the interactions were clearly nontransitive. Even so, these experiments strongly suggest that fitnesses measured relative to a common competitor did not capture the full complexity of the selection dynamics in this experimental system. In the next chapter, I examine in some detail the ecological mechanisms that allow the stable frequency-dependent coexistence of two other recombinant genotypes that were also sampled from population Ara$^-$3.

Conclusions. -- The results of my study demonstrate a very substantial impact of Hfr plasmid-mediated recombination on the evolution of the *E. coli* chromosome. However, the consequences of this recombination for the rate of adaptive evolution were

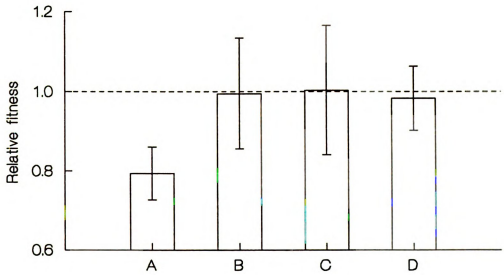


Figure 5. Complex selection dynamics revealed by pairwise interactions among three genotypes. REL4349 was the dominant genotype in recombination treatment population Ara^{-}3 after 500 generations. REL2545 is its ancestral genotype; REL4322 is identical except for the Ara^{+} marker. REL2543 is the ancestral genotype for another population; REL4190 is identical except for the Ara^{+} marker. REL2543 and REL4190 served as common competitors for the fitness experiments summarized in Table 4. (A) REL4349 is less fit than REL4190. (B) REL4349 is of equal fitness to REL4322. (C) REL2543 and REL4322 are equally fit, as are (D) REL2545 and REL4190. Error bars indicate 95% confidence intervals, based on five-fold replication of each fitness assay.

unclear. When fitnesses of derived genotypes were measured relative to a common competitor, there was no evidence that the increased genetic variation led to more rapid gains in fitness. However, supplementary experiments suggest that some genetic changes that occurred in the recombinant populations may have been adaptive only in the context of the particular milieu of genotypes in which they arose.

CHAPTER II

TESTS OF ECOLOGICAL MECHANISMS PROMOTING THE STABLE COEXISTENCE OF RECOMBINANT BACTERIAL GENOTYPES

INTRODUCTION

Frequency-dependent selection has long been hypothesized to maintain genetic polymorphisms in natural populations (Fisher 1958; Haldane and Jayakar 1963; Clarke 1964; Ayala and Campbell 1974; Levin 1988), and empirical evidence has been accumulated to support this notion (e.g., Cain and Sheppard 1954; Hori 1993). However, the complexity of the natural environment makes elucidation of the driving forces behind frequency-dependent selection extremely difficult. Also, the long generation times of most organisms often lead researchers to assume (rather than prove) that a stable polymorphism exists.

Conclusive evidence that the fitness of a genotype can be related to its relative frequency has come from laboratory studies involving species of *Drosophila* (e.g., Wright and Dobzhansky 1946; Kojima 1971; Van Delden et al. 1978). Due to controlled factors in the selective environment and shorter generation times, laboratory studies would seem to permit precise determination of the underlying causes of stable genetic polymorphisms. However, this is not necessarily the case: frequency-dependent selection involving the ADH locus in laboratory populations of *D. melanogaster* has been clearly demonstrated, but never fully explained (Van Delden 1982).

Because of their short generation times, very large population sizes, and general ease of propagation, bacteria provide an excellent model to study resource-based competition as well as other ecological and evolutionary processes (Levin 1972; Chao et al. 1977; Levin et al. 1977; Hansen and Hubbell 1980; Helling et al. 1987; Dykhuizen

1990; Lenski and Travisano 1994). But reproduction in bacteria is strictly asexual and laboratory environments typically provide only a single limiting resource. For these reasons, researchers have often assumed that populations of bacteria will conform to the classical model for the evolution of asexual organisms (Atwood et al. 1951; Moser 1958; Dykhuizen 1990). That is, experimental populations are subject to takeover by a single genotype that harbors a mutation conferring some selective advantage. Atwood et al. (1951) called this phenomenon "periodic selection" because an advantageous mutant periodically replaces its immediate predecessor. Thus, polymorphisms in bacterial populations are only expected to exist transiently, while an advantageous mutant is increasing in frequency relative to its ancestor. This notion is in accord with the competitive exclusion principle (or Gause's axiom), which states that two competitors cannot coexist on a single limiting resource (Gause 1934; Hardin 1960).

In apparent violation of this simple model leading to competitive exclusion, the evolution and persistence of stable polymorphisms in laboratory populations of bacteria has been clearly demonstrated. For instance, despite differences in maximum specific growth rates and glucose transport, three clones of *Escherichia coli* were reported to stably coexist in glucose-limited chemostat culture (Helling et al. 1987). It was later shown that these three strains had evolved a complex method of cross-feeding that involved differential patterns of secretion and uptake of two alternative metabolites, acetate and glycerol, in addition to glucose (Rosenzweig et al. 1994). Other studies have documented bacterial coexistence mediated by viruses (Chao et al. 1977), by detoxification of toxins (Lenski and Hattingh 1986) and by habitat structure (Korona et al. 1994).

In theory, a "demographic tradeoff" can also lead to stable coexistence between two bacterial strains growing in a serial culture environment that contains only a single limiting resource (Stewart and Levin 1973). Serial (batch) culture is analogous to a seasonal environment, where resources are abundant at the beginning of the bacterial

growth cycle but become scarce as the bacterial population approaches its carrying capacity. Levin (1972) observed that a "demographic tradeoff" led to coexistence between strains of *E. coli* B and K12 in serial culture. One genotype was able to grow better at high concentrations of glucose (apparently due to a shorter lag phase) while the other genotype grew better at low concentrations (due to its greater capacity for growth in transitional phase). This result provides an interesting example of contrasting life-history strategies for bacteria that is analogous to the proposed r-K tradeoff (MacArthur and Wilson 1967; Pianka 1970). The "r-selected" strain was able to reproduce rapidly in an uncrowded environment, while the "K-selected" strain made up for this early disadvantage by maximizing fitness when the population was near carrying capacity. Although Levin (1972) could not rule out the possibility that cross-feeding was also involved, demographic tradeoff alone is a viable mechanism for mediation of coexistence in serial culture (Stewart and Levin 1973).

In a study to examine the effect of recombination on the dynamics of bacterial evolution (Chapter I), I observed prolonged genotypic diversity when a population of *E. coli* was propagated serially in an environment in which glucose was provided as the limiting nutrient. Because the experimental environment was free of viruses and antibiotics, I propose that either a cross-feeding interaction or a demographic tradeoff in growth rates may explain coexistence. These two hypotheses are not mutually exclusive, so that coexistence might be explained by both mechanisms jointly.

Cross-feeding. -- Cross-feeding allows one strain to monopolize the primary resource, while excreting some metabolite into the environment that disproportionately enhances the growth of a second strain. Assuming the amount of metabolite produced is proportional to the density of primary competitor, then a cross-feeder benefits from being in the minority. Similarly, the primary competitor benefits when the population contains a high density of cross-feeders due to its inherent growth advantage on the primary

resource. Thus, the relative fitnesses of both strains will be decreasing functions of their own frequencies, so that each strain has a higher relative fitness when it is the minority competitor. Hence, competition is fiercest among competitors of the same genotype and a stable polymorphism may arise from the inability of either genotype to displace the other.

Demographic tradeoff. -- Coexistence mediated by a demographic tradeoff requires that one strain is competitively superior when the sole limiting resource is at high concentration, whereas the other strain is superior in competition when that resource is scarce. Under the simple case of the Monod model (1949), each strain's growth rate is given by:

$$dN/dt = N\,[V_{max}\,S\,/\,(S + K_S)],$$

where N is cell density, V_{max} is maximum growth rate, S is resource concentration, and K_S is the concentration required to support growth at half the maximum rate. The rate of resource depletion is given by:

$$dS/dt = -\,c\,(dN/dt),$$

where c is the conversion efficiency. A necessary (but insufficient) requirement for coexistence is that one strain has a higher V_{max}, while the other strain has a higher ratio of V_{max}/K_S. Each strain will have an advantage at a different stage of the growth cycle and the two strains may stably coexist, each having an advantage when rare (Figure 6).

Here I present the results of a series of experiments to determine whether a cross-feeding interaction or demographic tradeoff best explains coexistence between two recombinant strains of *E. coli*.

Figure 6. Numerical simulations showing stable coexistence of two strains on a single resource in a seasonal environment, mediated by a demographic tradeoff. One strain has V_{max} = 0.4 h^{-1} and K_S = 0.1 ug mL^{-1}, whereas the other strain has V_{max} = 0.59 h^{-1} and K_S = 7.5 ug mL^{-1}; $c = 5x10^{-7}$ ug for both. The vertical axis shows the frequency of the first strain. At the beginning of the simulation, and after every 24 h thereafter, the competing populations are diluted 1:100 into fresh medium that contains 25 ug mL^{-1} of the limiting resource. In each 24-h period, there are three phases during which (1) the resource concentration is sufficiently high that the strain with the higher V_{max} has an advantage, (2) the resource concentration has been reduced so much that the strain with the lower K_S has an advantage, and (3) the resource has been exhausted to the point that neither strain can grow. The three curves differ only in the initial frequency of the competing strains; each strain has an advantage when rare, and so their coexistence is stable. Simulations were run with a time step of 0.001 h using SOLVER.SWV (Blythe et al. 1990). This program employs the fourth-order Runge-Kutta method, with a modification that allows for a switch to be thrown (here, periodic dilution into fresh medium).

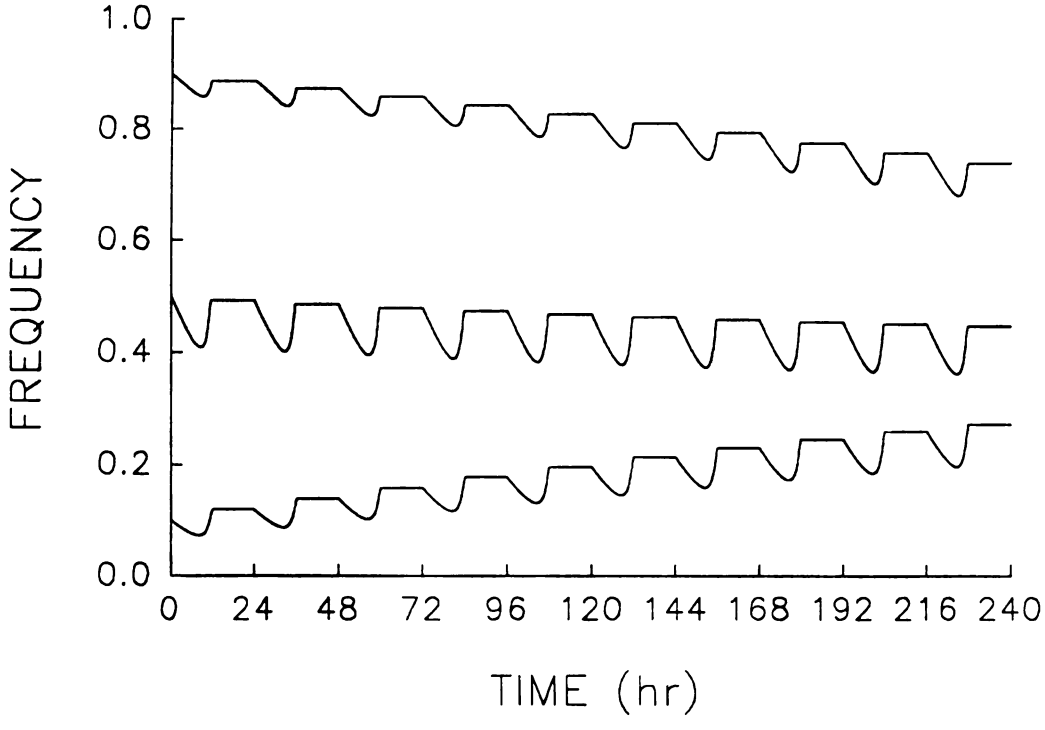

1.0
0.8
0.6
0.4
0.2
0.0
FREQUENCY
0
24
48
72
96
120
144
168
192
216
240
TIME (hr)

Figure 6.

MATERIALS AND METHODS

Bacterial strains. -- Chapter I describes the experiment from which the strains used in this study were obtained. Briefly, twelve treatment populations were founded from independently derived clones of *E. coli* B that had been serially propagated in the selective environment for 7,000 generations (Lenski and Travisano 1994). These populations (recipients) were serially propagated for another 1,000 generations (150 days), during which time they were allowed to undergo recombination with donor strains approximately every fifth day. The donors were four Hfr (high frequency of recombination) strains of *E. coli* K12, which are rather distantly related to *E. coli* B (Table 5; see also Selander and Levin 1980). These donors were all auxotrophic for at least one amino acid. Thus, the Hfr donors were able to donate genes via recombination but were unable to survive and out-compete the prototrophic *E. coli* B recipients.

For the purpose of this study, I isolated two recombinant genotypes (REL4397 and REL4398) at generation 1,000 from one of the treatment populations. Using nine physiological and five electrophoretic markers, it is evident that both recombinant genotypes contain a mixture of the markers present in *E. coli* B and K12 (Table 5). The two recombinants also differ from one another at a number of loci, including in their ability to utilize the sugar lactose. Thus, REL4397 (Lac^+) and REL4398 (Lac^-) form white and red colonies, respectively, when they are spread on tetrazolium-lactose (TL) indicator plates (Levin et al. 1977). Both recombinants are prototrophic and so can grow without supplemental amino acids. From here on, I refer to REL4397 and REL4398 simply as Lac^+ and Lac^-, respectively.

Media and culture conditions. -- Unless otherwise noted, the culture medium employed was Davis Minimal (DM) broth (Carlton and Brown 1981) supplemented with 2×10^{-6} g thiamine hydrochloride mL^{-1} and glucose at a specified concentration. For

Table 6. Genetic markers for the two recombinant strains of *E. coli* used in Chapter II, as well as their parental donors and recipient.

	Phenotypic[1]									Electrophoretic[2]					
Strain	Ara	Lac	Tet	Str	Arg	Leu	Ilv	T1X	T6	IDH	6-PGD	ADH	MPI	PEP	OriT[3]
Recombinants															
REL4397	-	+	r	r	+	+	+	s	r	2	2	1	2	2	--
REL4398	-	-	r	s	+	+	+	r	s	1	2	2	2	1	--
Recipient															
REL2545	-	+	s	r	+	+	+	s	r	1	2	2	2	1	--
Donors															
REL288	+	+	r	s	+	+	-	s	s	2	1	1	1	2	67 ↑
REL291	-	-	r	s	-	+	+	r	r	2	1	1	1	2	2 ↓
REL296	+	-	r	s	-	-	+	r	s	2	1	1	1	2	12 ↓
REL298	+	-	r	s	-	-	+	r	r	2	1	1	1	2	84 ↓

Table 6 (cont'd).

[1] + and - indicate ability and inability, respectively, to grow on sugars L-arabinose (Ara) and lactose (Lac); s and r indicate sensitivity and resistance, respectively, to antibiotics tetracycline (Tet) and streptomycin (Str); + and - indicate prototrophy and auxotrophy, respectively, amino acids arginine (Arg), leucine (Leu) and isoleucine-valine (Ilv); s and r indicate sensitivity and resistance, respectively, to phages T1X and T6.

[2] Numbers indicate mobility classes for enzymes isocitrate dehydrogenase (IDH), 6-phosphogluconate dehydrogenase (6-PGD), alcohol dehydrogenase (ADH), mannose phosphate isomerase (MPI), and peptidase (PEP).

[3] Map position, in minutes, on the *E. coli* K12 chromosome for the origin of transfer of donor genes into recipient cells. ↑ or ↓ indicates transfer in order of ascending or descending map order, respectively.

example, DM25 indicates DM supplemented with 25 ug glucose mL^{-1}, which yields ~ $5x10^7$ cells mL^{-1} at stationary phase. In all experiments, culture volume was 10 mL maintained in 50-mL Erlenmeyer flasks; these flasks were placed in a shaking incubator at 37 °C and 120 rpm. As indicated, populations were propagated daily by transferring 0.1 mL of each stationary-phase (24 h) culture into 9.9 mL of fresh medium. The resulting 100-fold daily growth of each population represents ~6.64 generations of binary fission.

Fitness assay and definitions. -- Relative fitness was assayed by allowing Lac^+ and Lac^- to compete under the culture conditions described above, except where other culture conditions are specifically noted. Prior to competition assays, each strain was grown separately for (at least) one day in the experimental medium as a preconditioning step to ensure both competitors were in comparable physiological states. The two competitors were then mixed at a 1:1 or other defined volumetric ratio, then diluted 1:100 into fresh medium and allowed to grow and compete during a standard one-day growth cycle. Initial and final densities of each competitor were estimated by spreading cells on TL plates, which distinguish the strains by colony color.

Let the initial densities of the Lac^+ and Lac^- competitors be $N_1(0)$ and $N_2(0)$, respectively; and let $N_1(1)$ and $N_2(1)$ be their corresponding densities after one day. The average rate of increase (or realized Malthusian parameter), m_i, for either competitor is then calculated as:

$$m_i = \ln[N_i(1)/N_i(0)] / (1 \text{ day}).$$

The fitness of one strain relative to another (W_{ij}) is estimated as the ratio of their Malthusian parameters (Lenski et al. 1991):

$$W_{ij} = m_i/m_j.$$

A fitness difference between the two competitors may reflect differences in their lag phase, maximum or submaximum growth rate, survival at stationary phase, or some combination thereof (e.g., Vasi et al. 1994).

RESULTS

Demonstration of the stable equilibrium

Evidence for frequency-dependence. -- I first sought to establish whether coexistence between recombinant strains Lac^+ and Lac^- was stable, such that each strain could increase in frequency when it was initially rare. To that end, I performed fitness assays in DM25 in which the two strains were mixed at thirteen different initial frequencies of Lac^+: 0.99, 0.95, 0.9, 0.8, 0.75, 0.6, 0.5, 0.4, 0.25, 0.2, 0.1, 0.05, and 0.01. Assays were replicated twice for each initial frequency.

Two aspects of the results (shown in Figure 7) are of particular interest. First, there is compelling evidence that the relative fitness of each strain is a decreasing function of its own initial frequency (slope = -0.338, t_S = -7.855, df = 24, $p < 0.001$). Second, these data allow me to predict the equilibrium frequency of Lac^+ and Lac^- in DM25. By definition, equilibrium will occur when the strains achieve frequencies in the population where they are equally fit. Thus, the point where the regression line intersects a relative fitness of 1.0 corresponds to a predicted equilibrium frequency for Lac^+ of about 0.45.

Evidence for stable equilibrium. -- To further prove the existence of a stable polymorphic equilibrium, Lac^+ and Lac^- were allowed to compete during daily serial transfers in DM25 for 200 generations (30 days). Each strain was separately preconditioned and then they were mixed at five initial frequencies of Lac^+ (0.9, 0.75, 0.5,

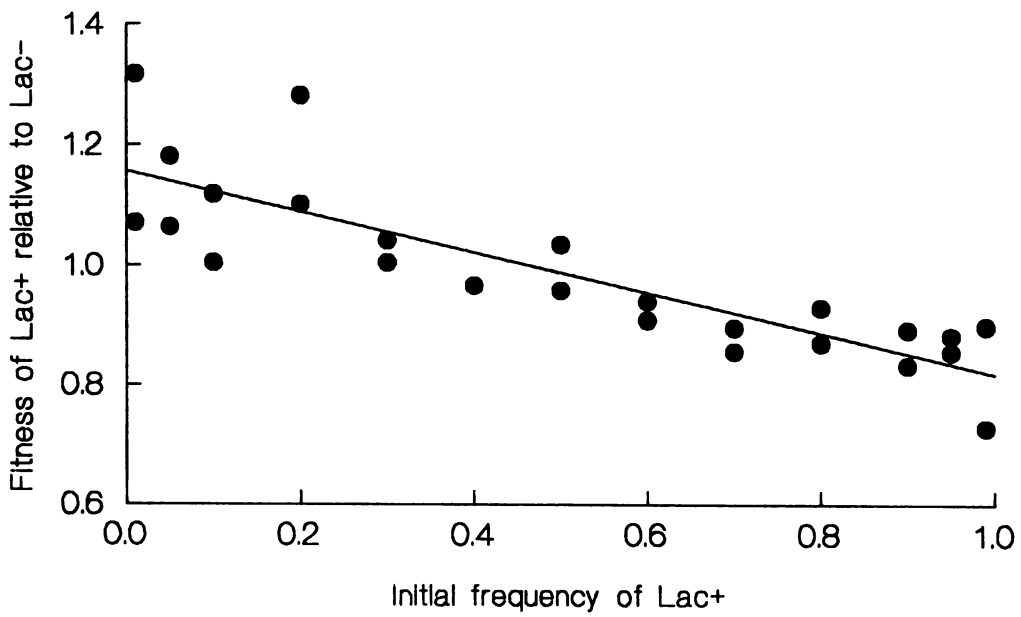


Figure 7. The fitness of recombinant *E. coli* strain Lac^+, relative to strain Lac^-, is a decreasing function of its own frequency. Relative fitness was calculated as the ratio of Malthusian parameters estimated during competition over one-day growth cycles in DM25, starting with initial frequencies of Lac^+ that ranged from 0.01 to 0.99. The line indicates the least-squares regression. These data imply a stable equilibrium frequency for Lac^+ of about 0.45, at which point relative fitness equals one.

0.25, 0.1), with three-fold replication. After 16 days, I temporarily suspended this experiment by adding 3 mL glycerol to each culture flask, and storing the cultures at -80 °C. I restarted this experiment two weeks later by inoculating from each thawed freezer sample into Luria broth for one day. Cultures were then diluted into DM25 for one day of re-acclimation before continuing the experiment proper for another 14 days in DM25. (Two replicates were terminated at days 19 and 27, due to contamination.) During the entire 30-day competition experiment, I spread samples taken from stationary-phase cultures (i.e., at the end of the 24 hour growth-cycle) onto TL plates to determine the density of Lac^+ and Lac^- strains.

As shown in Figure 8, the frequency of Lac^+ converged upon an equilibrium value, regardless of its initial frequency and despite the fact that the experiment was perturbed midway by freezing and restarting the competition. Interestingly, the perturbation resulted in a systematic benefit to the Lac^+ competitor, but this effect was transient and disappeared as the experiment continued. By day 30 of the competition experiment, the mean frequency of Lac^+ was 0.509 ($\pm$ 0.012 SE), which agrees well with the equilibrium predicted from the regression of relative fitness on initial frequency (Figure 7). I conclude that frequency-dependent selection is strong enough to drive the strains toward a stable equilibrium ratio, at which point the two strains are of equal fitness.

Evaluation of the demographic tradeoff hypothesis

Estimation of maximum growth rates. -- For each strain, I measured the maximal growth rate, V_{max}, in DM1000, wherein glucose is well above the concentration at which it limits growth rate. V_{max} was estimated by regressing ln N versus time during the period of exponential-phase growth. Cell densities were estimated by counts obtained using a Coulter electronic particle counter (model ZM and channelyzer model 256), and experiments were performed with five-fold replication for each strain.

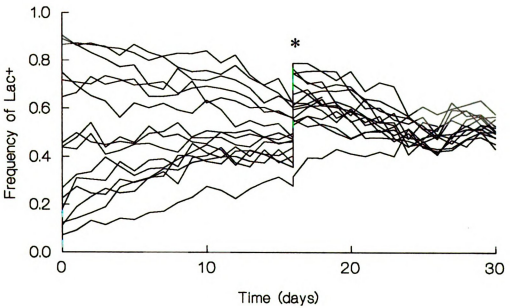


Figure 8. Starting from different initial frequencies, strains Lac^+ and Lac^- establish a stable polymorphism in DM25. After 30 days, observed frequencies agree rather well with the equilibrium predicted from one-day competition experiments (Figure 7). The asterisk (*) indicates a perturbation when the experiment was put in a freezer for temporary storage.

Lac^+ and Lac^- gave mean estimates for V_{max} of 0.9225 h^{-1} ($\pm$ 0.0137 SD) and 0.9770 h^{-1} ($\pm$ 0.0081 SD), respectively. This difference in V_{max} was statistically significant ($t_S = 7.651$, 8 df, $p < 0.001$).

Estimation of affinity for limiting resource. -- K_S is the concentration of resource at which a strain can grow at half of its maximum rate (i.e., $V_{max}/2$), according to the Monod (1949) model. As such, $1/K_S$ provides a measure of a strain's affinity for a limiting resource. To determine whether Lac^+ and Lac^- differ in their affinity for glucose, I sought to estimate K_S for each strain.

I followed the estimation procedure of Vasi et al. (1994), which uses the range of concentrations over which a population can replace itself, given a set dilution rate, to estimate K_S. Three replicates of each strain were removed from the freezer and propagated in DM25 to acclimate them to growth on glucose. I then transferred inocula into DM containing 0.01, 0.025, 0.1, 0.25, or 1 ug mL^{-1} glucose, with the inocula adjusted proportionately to account for expected differences in final density. As a control for growth on possible contaminating resources, I also inoculated cells into DM without any glucose. Cultures were propagated by 1:100 serial dilution for seven days. Immediately prior to each dilution, samples were spread onto TL plates to estimate cell densities. I then regressed ln N against time to determine the lowest glucose concentration at which each strain could maintain a constant population size.

Lac^+ was found to be able to replace itself in the face of serial dilution at glucose concentrations of 0.1 ug mL^{-1} and higher. Let $V = (1/N)(dN/dt)$ be some submaximal rate of increase. The 100-fold daily increase (to offset dilution) implies that $V > \ln(100)/24\ h = 0.19\ h^{-1}$. From the Monod (1949) model, and using V_{max} for Lac^+ as estimated above, it follows that $V > 0.19\ h^{-1}$ can be sustained in DM0.1 only if $K_S < 0.39$ ug mL^{-1}. Similarly, the inability of Lac^+ to persist in DM0.025 implies that $K_S > 0.10$ ug mL^{-1}. In contrast, Lac^- was found to be able to sustain itself in the face of 1:100 daily serial dilution only at

glucose concentrations of 0.25 ug mL^{-1} and higher. Using V_{max} for Lac$^-$ as estimated above, it follows similarly that K_S for that strain lies between 1.04 and 0.41 ug mL^{-1}.

My results are consistent with a demographic tradeoff between V_{max} and K_S. Lac$^-$ has a higher maximum growth rate than Lac$^+$, whereas Lac$^+$ has the greater affinity for glucose and can evidently grow faster at very low glucose concentrations. However, the existence of such a tradeoff is not sufficient to explain stable coexistence, which relies on the input concenration of limiting resource and the dilution factor (Stewart and Levin 1973).

Thus, it was necessary to explore the parameter space (within the limits of experimental uncertainty for each strain's V_{max} and K_S) to evaluate if the input resource concentration (25 ug mL^{-1}) and dilution factor (1:100 d^{-1}) that were imposed would allow stable coexistence. To that end, numerical simulations using SOLVER (Blythe et al. 1990) were run. The criterion for coexistence was that each strain must be able to increase in frequency when it was initially rare.

A summary of the results from many numerical simulations follows. First, the mean estimates for each strain's V_{max} were used to consider the effects of different K_S values within the bounds of uncertainty. Lac$^-$ was found to exclude Lac$^+$ competitively unless the difference in the K_S values for the strains was rather close to the maximum allowed by the experimental uncertainty. But if the difference in their K_S values was too large, then Lac$^+$ would competitively exclude Lac$^-$. However, there was a small region of stable coexistence in between these two extremes. For instance, coexistence resulted if Lac$^+$ had V_{max} = 0.9225 h^{-1} and K_S = 0.12 ug mL^{-1} while Lac$^-$ had V_{max} = 0.9770 h^{-1} and K_S = 0.9 ug mL^{-1} (Figure 9). Next, the effect of uncertainty in the estimate of the difference in V_{max} between the two strains was considered. If the size of this difference was increased, then the advantage shifted towards Lac$^-$; if it was decreased, then the advantage shifted towards Lac$^+$. In all cases, however, there was at most a small region of coexistence in terms of K_S values. More importantly, in those cases where stable

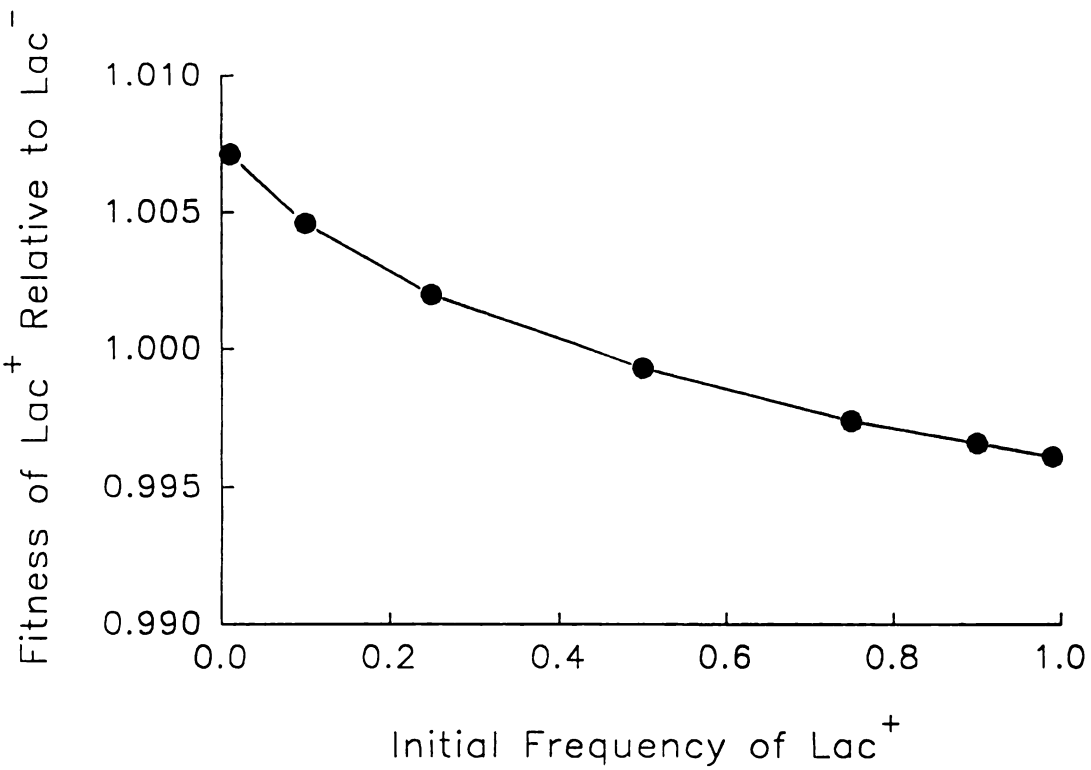


Figure 9. Numerical simulations of the fitness of Lac^+ relative to Lac^-, as a function of the initial frequency of Lac^+, assuming only a demographic tradeoff between growth rates at high and low glucose concentrations. For Lac^+, $V_{max} = 0.9225\ h^{-1}$ and $K_S = 0.12\ ug\ mL^{-1}$. For Lac^-, $V_{max} = 0.977\ h^{-1}$ and $K_S = 0.9\ ug\ mL^{-1}$. For both strains, $c = 5x10^{-7}$ ug. Simulations assume combined population is diluted 1:100 into medium containing 25 ug mL^{-1} of the limiting resource. Simulations were run using SOLVER (Blythe et al. 1990) with a time-step of 0.001 h. Relative fitness was calculated as the ratio of Malthusian parameters, exactly as in the experiments. A demographic tradeoff that is consistent with the estimates for each strain's V_{max} and K_S (see text) allows stable coexistence. However, the predicted frequency-dependence is much weaker than was observed in experiments (see Figure 7).

coexistence was observed, the strength of the resulting frequency-dependence was very weak, with fitness advantages for the rare strain on the order of only 1% (Figure 9). This result contrasts with the much stronger frequency-dependence observed in the actual experiments, where each strain had a fitness advantage in excess of 10% when it was rare (Figure 7).

These results strongly suggest that a demographic tradeoff between growth rates at high and low glucose concentrations contributes only slightly to the observed stable coexistence between Lac^+ and Lac^-. Thus, I also sought to evaluate the possible importance of cross-feeding interactions involving the differential secretion and utilization of metabolic by-products.

Evaluation of the cross-feeding hypothesis

Effect of resource concentration on frequency-dependence. -- If cross-feeding is an important factor promoting frequency-dependence, then one might expect the stable coexistence of Lac^+ and Lac^- to break down at low glucose concentrations. In other words, at low glucose concentrations, the population density of the metabolite reducing strain would be reduced, with a concomitant reduction on the concentration of metabolite and therefore a diminished opportunity for cross-feeding (see also Rosenzweig et al. 1994). To examine this possibility, I looked for an influence of resource concentration on the frequency-dependence of relative fitness. I performed fitness assays in which the two strains were mixed at five initial frequencies of Lac^+ (0.9, 0.75, 0.5, 0.25, 0.1), and allowed to compete in media supplemented with five different concentrations of glucose (DM1, DM2.5, DM25, DM250, DM1000). In all cases, both strains were removed from the freezer into DM1000, acclimated for two days in the competition medium, and then competed for one day. Each treatment combination was replicated five-fold, but two replicates were excluded due to contamination.

An ANOVA indicates that the interaction between glucose concentration and initial frequency has a highly significant effect on relative fitness (Table 7). As shown in Figure 10A, the fitness of each strain is a decreaseing function of its own frequency when strains are competed at glucose concentrations of 25, 250, and 1000 ug mL^{-1}. At all three

Table 7. ANOVA of effects of initial frequency and glucose concentration on fitness of Lac^+ relative to Lac^-.

Source	SS	df	MS	*F*	*P*
Initial frequency	0.230	4	0.058	11.22	<0.001
Glucose concentration	0.211	4	0.053	10.28	<0.001
Interaction	0.297	16	0.019	3.62	<0.001
Error	0.503	98*	0.005		

* Treatment combinations were replicated five-fold, but there were two missing values.

of these concentrations, the one-tailed regression of relative fitness on initial frequency is highly significant (DM25: slope = -0.300, t_S = -10.349, df = 23, $p < 0.001$; DM250: slope = -0.254, t_S = -5.097, df = 23, $p < 0.001$; DM1000: slope = -0.171, t_S = -3.992, df = 23, $p < 0.001$). However, at the lower glucose concentrations of 1 and 2.5 ug mL^{-1}, the dependence of relative fitness on initial frequency breaks down, with Lac^+ having a small advantage over Lac^- regardless of frequency (Figure 10B). In contrast to the conditions necessary for stable coexistence, there is no significant negative regression of fitness on

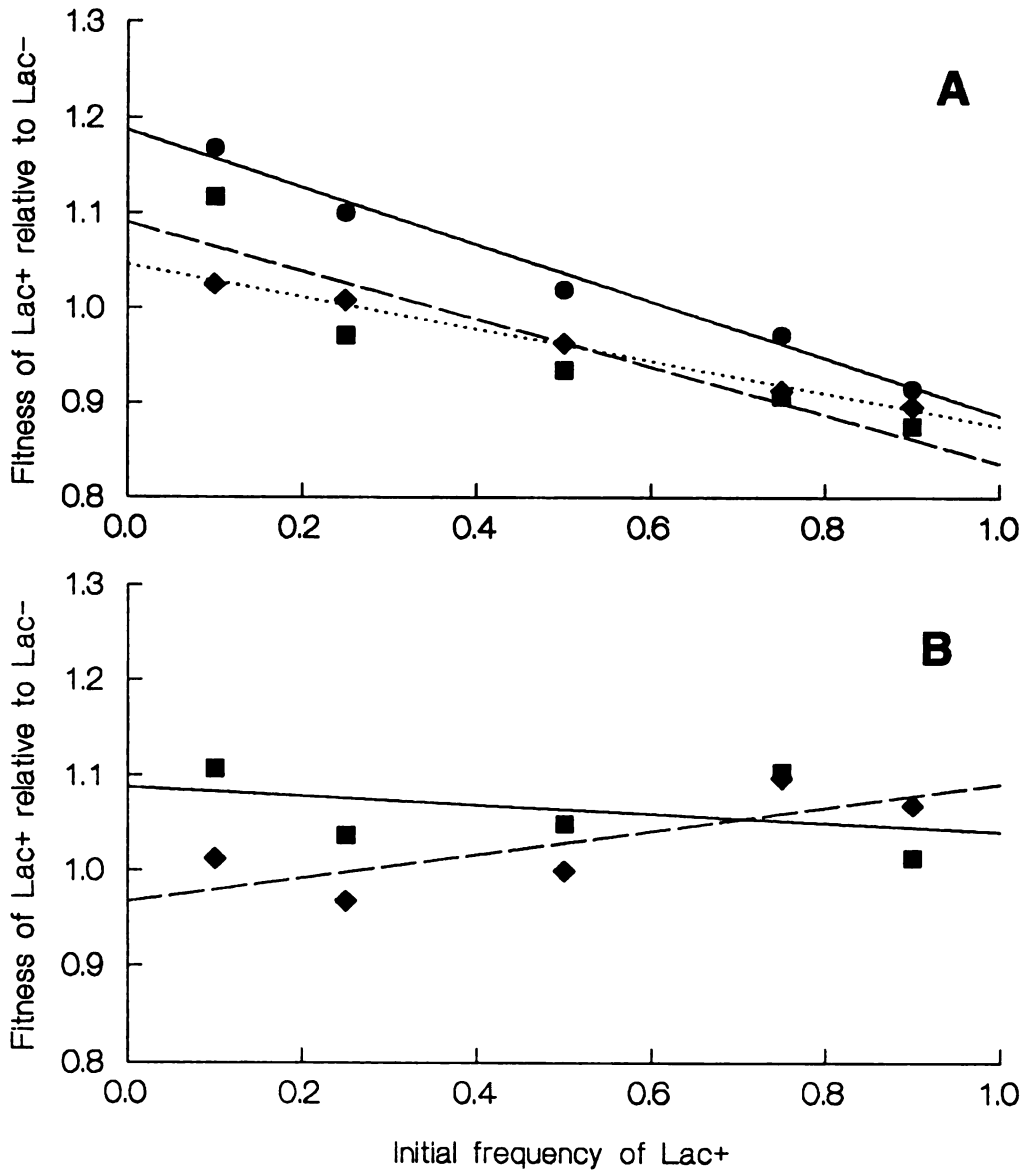


Figure 10. Fitness of strain Lac$^+$, relative to strain Lac$^-$, as a function of its initial frequency, in DM media containing five different glucose concentrations. (A) In medium containing glucose at concentrations of 25 (circles), 250 (squares), or 1000 (diamonds) ug mL^{-1}, each strain has an advantage when rare such that there exists a stable polymorphism. (B) In medium containing glucose at concentrations of 1 (squares) or 2.5 (diamonds) ug mL^{-1}, there is no evidence that the rarer strain has an advantage. Each point is the mean of five replicates. Lines indicate least-squares regressions; see the text for statistical analyses.

frequency (DM1: slope = -0.048, t_S = -0.776, df = 21, p = 0.448; DM2.5: slope = 0.124, t_S = 2.219, df = 23, p = 0.982). I also performed all pairwise comparisons using the slopes of relative fitness on initial frequency obtained at the different glucose concentrations (5x4/2 = 10 comparisons in all). I employed the sequential Bonferroni criterion (Rice 1989) to compute significance levels. None of the three higher concentrations (DM25, DM250, DM1000) yielded significantly different slopes from one another, nor were the slopes from the two lower concentrations (DM1, DM2.5) significantly different. Yet the slopes for all other pairs were significantly different at $p < 0.05$, with the marginally nonsignificant exception of DM1 and DM1000 (p = 0.084). Evidently, frequency-dependence favoring the rarer strain is manifest at glucose concentrations of 25 ug mL^{-1} and higher, but it breaks down at much lower concentrations.

The observation that Lac^+ prevails in competition at low glucose concentrations is consistent with the demographic tradeoff documented above. Averaging over all initial frequencies, Lac^+ has a fitness of 1.060 ($\pm$ 0.038 95% C.L.) relative to Lac^- when the two strains compete in medium with glucose at 1 ug mL^{-1}. This relatively small fitness differential, even at so low a concentration, implies a difference in K_S values between the two strains that is much smaller than was used in numerical simulations (Figure 9) to obtain stable coexistence based on a demographic tradeoff between V_{max} and K_S. This discrepancy adds further support to my earlier claim that a demographic tradeoff alone is unable to explain the observed stable coexistence. Moreover, the finding that the conditions for stable coexistence break down at low glucose concentrations supports the hypothesis that cross-feeding is responsible for the stable coexistence of Lac^- and Lac^+.

Evidence for cross-feeding after glucose has been depleted. -- In the absence of frequency-dependent forces, it was observed that Lac^- has an inherent growth advantage over Lac^+ owing to its higher V_{max}. I therefore sought to determine in what phase of the population growth cycle Lac^+ makes up for this deficiency. To that end, I performed

fitness assays in which the two strains were mixed at three initial frequencies of Lac^+ (0.9, 0.5, 0.1), with eleven-fold replication, in DM25. Treatments comprising all Lac^+ and all Lac^- cells with three-fold replication were also included. Samples were spread on TL plates at 0, 12, and 24 h to determine the densities of Lac^+ and Lac^-.

Figure 11 shows the fitness of Lac^+ relative to Lac^- in DM25 calculated first using the 0 and 12 h data and then using the 0 and 24 h data. Once again, the relative fitnesses of these two strains are shown to be frequency-dependent. But Figure 11 also shows that

Table 8. ANOVA of effects of initial frequency and final sample time (12 or 24 h) on fitness of Lac^+ relative to Lac^-.

Source	SS	df	MS	*F*	*P*
Initial frequency	0.154	2	0.077	13.644	<0.001
Final sample time	0.091	1	0.091	16.113	<0.001
Interaction	0.003	2	0.002	0.286	0.752
Error	0.338	60	0.006		

Note: The experiment was carried out in medium containing glucose at 25 ug mL^{-1}.

Lac^+ has a systematic advantage relative to Lac^- between 12 and 24 h. An ANOVA reveals that the effects of initial frequency and sampling interval are highly significant (Table 8).

According to numerical simulations using the values of V_{max} and K_S estimated for Lac$^-$ and Lac$^+$, as well as the conversion efficiency c (5x10^{-7} ug), glucose should have been thoroughly depleted from the culture medium within the first 10 h, even allowing for a lag phase prior to growth of 1 or 2 h (see Vasi et al. 1994). Thus, the finding that Lac$^+$ gains a significant advantage between 12 and 24 h suggests either that Lac$^+$ is growing on some metabolite (and at a faster rate than Lac$^-$) or that Lac$^-$ is dieing (and at a faster rate than Lac$^+$), or perhaps both.

To evaluate these two alternative explanations, I computed the absolute rate of change in population density between 12 and 24 h for each strain. A positive or negative value indicates net growth or death, respectively. Figure 12 shows that, in the absence of the other strain (i.e., the initial frequency of Lac$^+$ equals either 0 or 1), each strain is subject to some cell death, although Lac$^+$ and Lac$^-$ do not differ significantly in their death rates ($t_S = 1.161$, df = 4, $p = 0.310$). However, in the presence of Lac$^-$, Lac$^+$ experiences net growth between 12 and 24 h (Figure 12). Lac$^-$ also benefits from the presence of Lac$^+$, in that Lac$^-$ shows no net decrease due to death as it does when it is alone. Therefore, between 12 and 24 h, each strain benefits in absolute terms from the presence of the other strain (Figure 12), although Lac$^+$ has the advantage in relative terms (Figure 11).

Based on these results, I conclude that cross-feeding interactions occur after glucose has been depleted from the culture medium. These interactions favor Lac$^+$, but the fact that each strain benefits from the other's presence between 12 and 24 h suggests that two (or more) metabolic by-products may be involved in the dynamics of this stable polymorphism. Although the primary aims of this study were to distinguish between the demographic-tradeoff and cross-feeding hypotheses, in the next section I report some preliminary experiments in which two potential metabolites were experimentally manipulated.

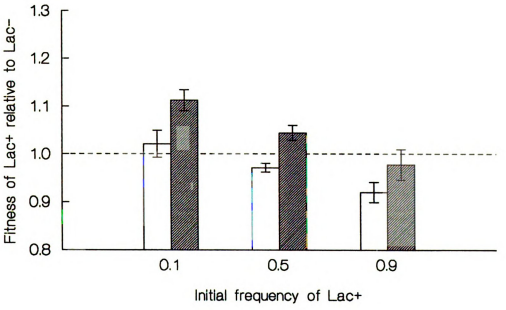


Figure 11. Fitness of strain Lac$^+$, relative to strain Lac$^-$, as a function of its initial frequency, in DM25, calculated between 0 and 12 h (open bars) and between 0 and 24 h (filled bars). The fitness of Lac$^+$ relative to Lac$^-$ increases between 12 and 24 hours, long after glucose has been exhausted (see text), implying either differential mortality or growth on metabolic by-products. Each bar represents the mean ($\pm$ SE) of 11 replicates; the ANOVA is given in Table 8.

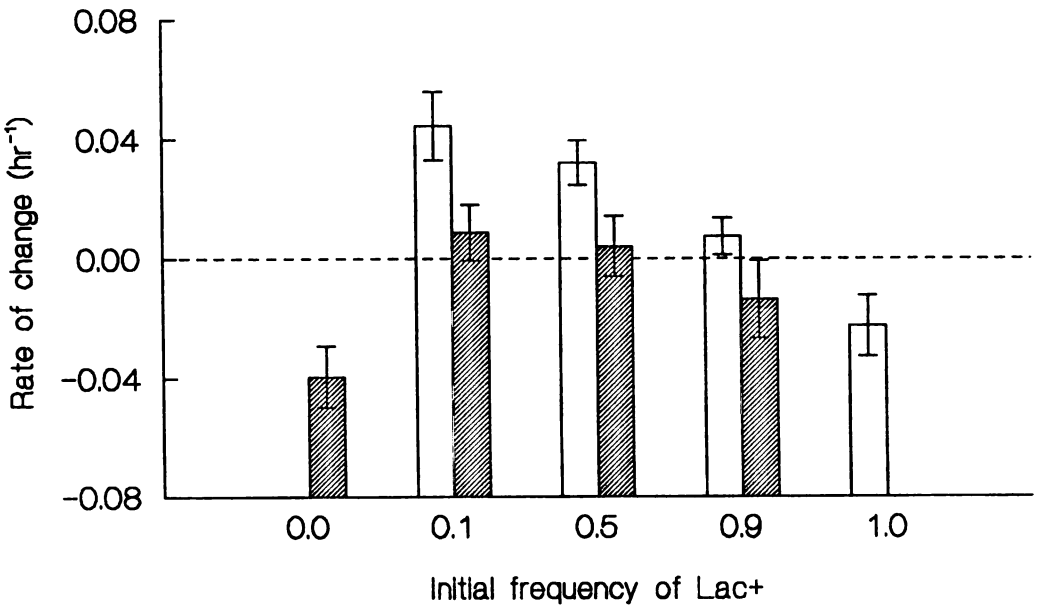


Figure 12. Net rate of change in viable cell density for Lac^+ (open bars) and Lac^- (filled bars) in DM25 between 12 and 24 h, after glucose has been exhausted from the medium. In the absence of the other strain, each strain declines due to death. However, Lac^+ experiences net growth between 12 and 24 h when Lac^- is present. Lac^- also benefits from the presence of Lac^+ between 12 and 24 h, although Lac^+ has the advantage in relative terms (see also Figure 11). Each bar represents the mean (± SE) rate of change in viable cell density (h^{-1}) between 12 and 24 h with 11-fold replication for initial frequencies of 0.1, 0.5, and 0.9 and with 3-fold replication for initial frequencies of 0 and 1.

Effect of potential metabolites on relative fitness. -- During either aerobic or anaerobic growth on glucose, *E. coli* generates a complex mixture of metabolic by-products (Neidhardt et al. 1990). A strain that has an enhanced ability to utilize one of these metabolites might be able to persist, even if it was at a disadvantage in acquiring glucose. In fact, Rosenzweig et al. (1994) recently demonstrated the stable coexistence of *E. coli* strains in chemostat culture (in which the demographic-tradeoff hypothesis is not applicable, because the environment is temporally constant). Their physiological analyses implicated acetate and glycerol as the relevant metabolites promoting coexistence, and these are the two metabolites that I will also consider.

In particular, I sought to determine if the addition of either acetate or glycerol would alter the outcome of competition between Lac^+ and Lac^- and might even allow these two strains to stably coexist in a medium where otherwise one strain excluded the other. I showed previously that stable coexistence broke down in DM containing only 2.5 ug mL^{-1} (Figure 10B). Performing experiments at this low glucose concentration has the further advantage that the concentration of metabolites produced by the bacteria should be proportionately less, so that the effect of an added metabolite might be more clearly elucidated.

I performed an experiment in which Lac^+ and Lac^- were allowed to compete at two initial frequencies of Lac^+ (0.1 and 0.9) in DM2.5 supplemented with the following concentrations of acetate: 0, 1, 2.5, and 10 ug mL^{-1}. These assays were replicated five-fold. I also performed an identical experiment, except using glycerol instead of acetate. In all cases, competitors were removed from the freezer into DM25, preconditioned for one day in the competition medium, and then allowed to compete for one day.

An ANOVA (Table 9) shows no effect of either acetate concentration or initial frequency, nor any interaction between them, on relative fitness. It seems unlikely, therefore, that acetate plays any important role in the stable coexistence between Lac^- and Lac^+ that I have observed.

Table 9. ANOVA of effects of initial frequency and supplemental acetate concentration on fitness of Lac^+ relative to Lac^-.

Source	SS	df	MS	*F*	*P*
Initial frequency	0.002	1	0.002	0.348	0.559
Acetate concentration	0.038	3	0.013	2.341	0.092
Interaction	0.025	3	0.008	1.557	0.219
Error	0.174	32	0.005		

Note: Experiment was carried out in medium also containing 2.5 ug mL^{-1} glucose.

Table 10. ANOVA of effects of initial frequency and supplemental glycerol concentration on fitness of Lac^+ relative to Lac^-.

Source	SS	df	MS	*F*	*P*
Initial frequency	0.006	1	0.006	1.987	0.168
Glycerol concentration	0.036	3	0.012	4.171	0.013
Interaction	0.008	3	0.003	0.929	0.438
Error	0.092	32	0.003		

Note: Experiment was carried out in medium also containing 2.5 ug mL^{-1} glucose.

By contrast, glycerol concentration has a significant effect on the outcome of competition between Lac^+ and Lac^-, although this effect is independent of initial frequency (Table 10). The effect of glycerol is complex, however, as shown in Figure 13. Increasing the glycerol concentration from 0 to 1 ug mL^{-1} shifts the advantage towards Lac^+, while increasing the concentration from 1 to 10 ug mL^{-1} shifts the advantage back towards Lac^-. This finding suggests that glycerol could be an important metabolite in the stable coexistence between Lac^- and Lac^+. That is, the addition of glycerol provides an advantage to Lac^+, which may offset its slower growth at high glucose concentrations, provided that the concentration of glycerol is not too high.

DISCUSSION

In a study intended to examine the effects of genetic recombination on the dynamics of bacterial evolution (Chapter I), I found that at least two distinct genotypes of *E. coli* coexisted in one of the experimental populations. This observation was unexpected since the populations were provided with only a single resource (glucose) which limited population density. In this chapter, I sought to determine the ecological mechanisms responsible for the coexistence of two of these recombinant strains. To that end, I first demonstrated that the coexistence was dynamically stable by showing that each strain possessed a competitive advantage when it was in the minority (Figures 7 and 8).

I then considered two mechanistic hypotheses to explain the stable coexistence. One hypothesis relies on the seasonal nature of the experimental environment, such that the glucose concentration changed temporally due to periodic transfers of the bacteria into fresh medium. In a seasonal environment, two genotypes may stably coexist on a single limiting resource (Figure 6) if one of them has an advantage when the resource is abundant and the other has a sufficiently large opposing advantage when the resource has become

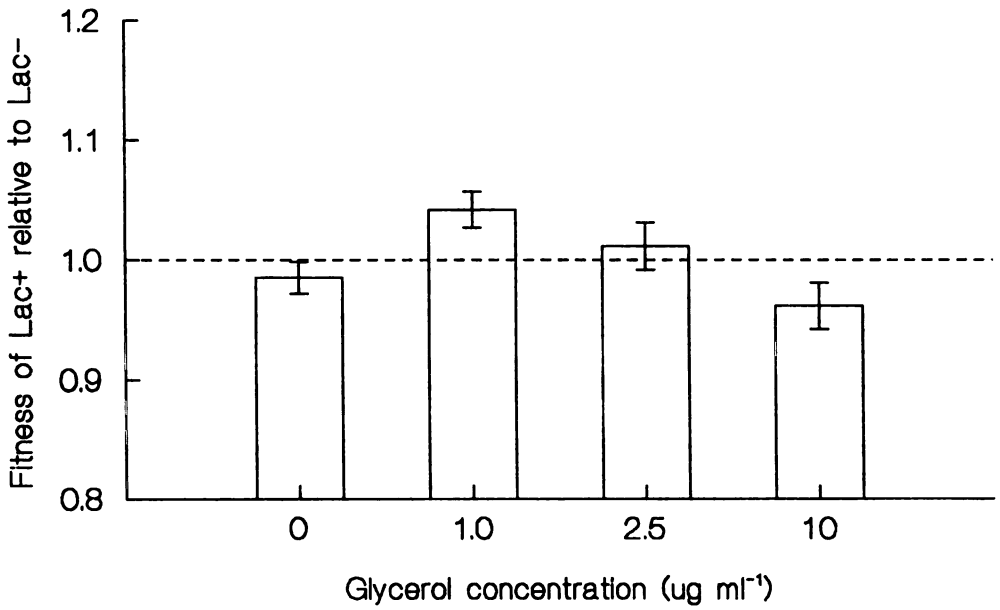


Figure 13. Effect of supplemental glycerol concentration on the fitness of Lac^+ relative to Lac^-, in medium also containing glucose at 2.5 ug mL^{-1}. Bars show the means (± SE) of ten replicates, five each with Lac^+ at initial frequencies of 0.1 and 0.9. Data were pooled across initial frequencies, which had no significant effect in this experiment (see Table 10). The addition of small amounts of glycerol shifts the competitive advantage to strain Lac^+, but this advantage disappears at higher glycerol concentrations.

scarce (Stewart and Levin 1974; Tilman 1982). According to the other hypothesis, one or more additional resources are introduced into the environment by the metabolic activities of the genotypes themselves. Here, two or more genotypes may stably coexist if one of them has an advantage on the exogenously supplied resource whereas the other has a sufficiently large opposing advantage in acquiring the metabolic by-product.

I demonstrated a demographic tradeoff between growth rates at high and low glucose concentrations. That is, strain Lac^- was the better competitor for abundant glucose whereas strain Lac^+ was superior in competition for sparse glucose. But the expected magnitude of each strain's advantage when rare, based on this tradeoff, was insufficient to explain the observed strength of frequency-dependence (Figure 9 versus Figure 7) and, in fact, may even have been too weak to allow stable coexistence. Thus, although a tradeoff between growth rates at high and low glucose concentrations exists, it cannot be primarily responsible for the stable polymorphism that led me to look for the hidden ecological mechanism.

I also demonstrated cross-feeding interactions between the two strains. After glucose was exhausted by cell growth, each strain, when grown alone, experienced a net decrease in population density due to death (Figure 12). However, when the two strains were grown together, each strain fared better (after the glucose was depleted) than when it was alone. In fact, strain Lac^+ actually increased in density, indicating net population growth, when Lac^- was abundant. Thus, Lac^+ can offset a disadvantage relative to Lac^- in competition for glucose by its greater ability to acquire and assimilate one or more metabolic by-products of glucose utilization. However, the polymorphism becomes unstable at low concentrations of glucose (Figure 10B), because population density is reduced with a corresponding reduction in the concentration of metabolites. Evidently, the stable coexistence between these two recombinant strains was mediated primarily by a cross-feeding interaction.

In addition to the inherent interest in determining the ecological mechanisms responsible for the stable coexistence of competitors in this simple model system, I believe that my results have several other more general implications. First, this system shows how organisms can, through their own biological activities, alter a simple environment into one that is more complex. In turn, this environmental complexity allows for diversity to be stably maintained where it could not otherwise persist. As eloquently stated by Rosenzweig et al. (1994, p. 915), "It seems clear that ... even starting with the simplest possible genetic and environmental conditions, complexity is generated from uniformity, allowing biodiversity to build upon itself."

Second, because I studied recombinant strains bearing many easily discernable genetic markers (Table 5), I had the opportunity to observe polymorphisms that might otherwise have gone undiscovered. In a related study with populations that are evolving in a strictly asexual fashion, and which lacked readily distinguished genetic markers, Lenski et al. (1991) found no obvious evidence for complex ecological interactions of the sort discerned in this study. It would be interesting to examine those asexual populations more thoroughly to determine whether some stable polymorphisms might have been overlooked. If such polymorphisms were not observed in those strictly asexual populations, it raises the interesting question of whether the likelihood for stable polymorphisms to evolve are greater in a system that allows for recombination between strains that are already genetically divergent. Of possible relevance is Levin's (1972) observed stable coexistence between *E. coli* B and K12 strains in environmental conditions similar to those that I employed, since the recombinants used in this study were hybrids of *E. coli* B recipients and K12 donors (Table 5). Even though Levin did not conclusively establish the ecological mechanism that mediated coexistence between *E. coli* B and K12, it seems plausible that it might be the same mechanism observed here.

Third, many evolutionary ecologists seem to believe it is enough to show that there exists a tradeoff between two ecological traits (e.g., r versus K) in order to explain stable

coexistence of the corresponding genotypes or species. But while such tradeoffs are generally *necessary* for stable coexistence, they may not be *sufficient*. In this study, I observed a demographic tradeoff between growth rates at high and low glucose concentrations. Such a tradeoff may in principle promote stable coexistence in a temporally fluctuating environment (Stewart and Levin 1973; Tilman 1982), but whether it does so depends on the size of the tradeoff as well as the extent of fluctuations in population density and resource concentration. In fact, a concentration-mediated tradeoff for the two recombinant strains in this study was not an adequate explanation for the observed strength of the frequency-dependent advantage that each strain had when rare (Figures 7 and 9). Therefore, when possible, experimental studies of the ecological mechanisms that promote stable coexistence should move beyond merely demonstrating that a required tradeoff exists and look to establish the quantitative agreement between observed and predicted dynamics. While able to fulfill this objective for the demographic-tradeoff model, I could not perform such an analysis for the cross-feeding hypothesis because the identity of the relevant metabolites and the parameters governing their rates of production and consumption were unknown.

Lastly, ecologists have long been concerned with the factors responsible for maintaining greater or lesser diversity of species within communities (Connell and Orias 1964; MacArthur and Wilson 1967; Connell 1978). One hypothesis suggests that communities with greater primary production are more diverse than less productive communities, which might contribute to the latitudinal cline in ecological diversity (Fischer 1960; Pianka 1966). Several possible mechanistic bases for such a relationship are imaginable. For instance, more productive communities should support longer food chains, thereby directly increasing diversity. Another possible mechanism is the production by one species of a metabolite (or other secondary resource) that supports another species, which is inferior in competition for the primary resource and would otherwise be excluded. When the abundance of primary resource is diminished, then the

density of the producer species may be reduced to a level where the secondary resource is not abundant enough to maintain the other species. In fact, the stable coexistence of recombinant bacterial strains that I observed in more productive environments (i.e., with higher inputs of glucose) was eliminated when productivity was reduced. Although this is but one example in a highly simplified experimental system, it illustrates the value of understanding the specific dynamical mechanisms responsible for maintaining ecological diversity.

CHAPTER III

TRADEOFF BETWEEN HORIZONTAL AND VERTICAL MODES OF TRANSMISSION IN BACTERIAL PLASMIDS

INTRODUCTION

Horizontal transmission occurs whenever a parasite is transmitted from an infected individual to an uninfected individual, whether by direct contact or via an infectious particle. Vertical transmission occurs when an infected individual reproduces (either sexually or asexually), giving rise to progeny which also harbor the infectious agent. For certain parasites there exists a fundamental conflict between these two modes of genetic transmission. Many activities of a parasite that increase its rate of infectious transmission (e.g., greater intra-host production of infectious particles) are likely to lower host fitness. This reduction in host fitness reduces the potential for vertical transmission of the parasite. Thus, a tradeoff between vertical and horizontal transmission is likely to exist.

Parasite virulence is a relative term, and it may be usefully defined in terms of 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). Group-selectionist arguments once led to widespread acceptance of the notion that parasites always evolve toward a state of attenuated virulence (Ewald 1994). However, recent theoretical and empirical studies have contradicted 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 implicate the availability of uninfected hosts in the environment as a key factor in determining the evolution of parasite virulence and, in those parasites that can also be transmitted vertically, which mode of transmission is selectively favored. The basic

argument is as follows. Consider the case of a parasite that is transmitted by direct contact between infected and uninfected (susceptible) hosts. When the density of uninfected hosts is high, the expected time to transmission of the parasite from an infected host to an uninfected host is short. Consequently, it is relatively advantageous for the parasite to maximize its transmission to new hosts, regardless of the effect on its current host's fitness. Thus, selection favors more virulent forms of the parasite. However, when the density of uninfected hosts is low, the time to transmission will be much greater. Here, a parasite benefits from doing little damage to its present host because the rate of transmission to new hosts is small, and a parasite that reduces the fitness of its present host too much may actually reduce its own likelihood of infectious transmission. By the same logic, if a parasite can be transmitted vertically, this mode of transmission becomes increasingly important and selectively favored when uninfected hosts are rare.

Experimental system. -- All plasmids are able to control their own replication using the host machinery and are transferred vertically across generations of the host cell. In the absence of selection on the host for specific plasmid-encoded characters (such as antibiotic resistance), most plasmids reduce the fitness of their hosts relative to isogenic plasmid-free counterparts (Levin 1980; Dykhuizen and Hartl 1983; Lenski and Bouma 1987; Lenski and Nguyen 1988; Nguyen et al. 1989); hence, they can be regarded as parasites under these circumstances (Levin and Lenski 1983; Bouma and Lenski 1988). Many 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 of the details of the conjugation process are still poorly understood, conjugation is initiated by contact between donor and recipient cells via a plasmid-encoded protein appendage known as a sex pilus. Thus, conjugative plasmids can be transmitted by two distinct modes: horizontal (infectious) transmission occurs by conjugation, whereas vertical (intergenerational) transmission occurs by host cell division.

Theoretical predictions. -- Here I define plasmid "virulence" as the magnitude of the reduction in host fitness due to plasmid carriage, which concomitantly lowers the plasmid's ability to be transmitted across generations of the host. Activities of a plasmid that increase its horizontal transmission (such as production of more sex pili) should generally increase virulence (i.e., lower the host's fitness), thereby reducing the plasmid's own rate of vertical transmission. Therefore, there exists a fundamental conflict between the two modes of transmission available to conjugative plasmids. When susceptible hosts are abundant, horizontal transmission is more important and selection should favor increased rates of horizontal transfer (increased virulence). When susceptible hosts are scarce, vertical transmission is the more frequent mode of transmission and selection should favor increased rates of vertical transmission (reduced virulence).

A simple model describes how different modes of plasmid spread should be favored under different densities of susceptible hosts. Four assumptions are implicit in the model: (i) no dynamic feedbacks affect the density of susceptible hosts (i.e., host density is treated as a constant); (ii) plasmid-bearing cells cannot be reinfected; (iii) horizontal transfer depends on mass-action kinetics; and (iv) there is a genetically-determined tradeoff between the rates of horizontal and vertical plasmid transmission. Let H be the density of plasmid-free hosts (volume^{-1}) and P be the density of plasmid-bearing hosts (volume^{-1}). If γ is the rate at which a plasmid is able to conjugatively transfer (volume time^{-1}) and m is the intrinsic growth rate of plasmid-bearing cells (time^{-1}), then the rate of change in plasmid-bearing cells is

$$dP/dt = m P + \gamma H P,$$

and the per capita rate of change is

$$r = dP/Pdt = m + \gamma H.$$

The individual components of r reveal that the vertical component of plasmid spread (m) is independent of host density, while the horizontal component (γH) is directly proportional to the density of potential recipients. In theory, a conjugative plasmid can be stably maintained in a bacterial population if the rate of horizontal transfer is sufficiently high to offset losses due to its harmful effects on host fitness (Stewart and Levin 1977).

To further illustrate the tradeoff between horizontal and vertical transmission, consider two plasmid genotypes A and B. Plasmid A conjugates at a rate of $\gamma_A = 1$ and allows its host to grow at the rate $m_A = 1$. Plasmid B conjugates at a rate of $\gamma_B = 1.5$ but reduces its host's growth rate to $m_B = 0.5$, and it is therefore more virulent than A. Figure 14 depicts the horizontal, vertical and net rates of transfer for plasmids A and B under different densities of susceptible hosts, as predicted by the model. When $H < 1$, $r_A > r_B$ so that the less virulent plasmid A prevails by virtue of its greater vertical transmission. But when $H > 1$, $r_A < r_B$ and the more virulent plasmid B prevails by virtue of its superior horizontal transmission. Thus, whether a more or less virulent plasmid is favored depends on the abundance of uninfected (susceptible) hosts.

Experimental overview. -- Bacterial populations provide an excellent means to study evolutionary theory for several reasons. Bacteria have short generation times and large population sizes, making relatively long-term evolutionary studies possible. Entire populations of bacteria can be stored in a suspended state, so that direct comparisons between ancestors and evolutionary descendants are possible. Lastly, bacteria such as *Escherichia coli* have been widely used as an experimental model in genetics, molecular biology and microbial physiology. Therefore, much is already known about the biology of

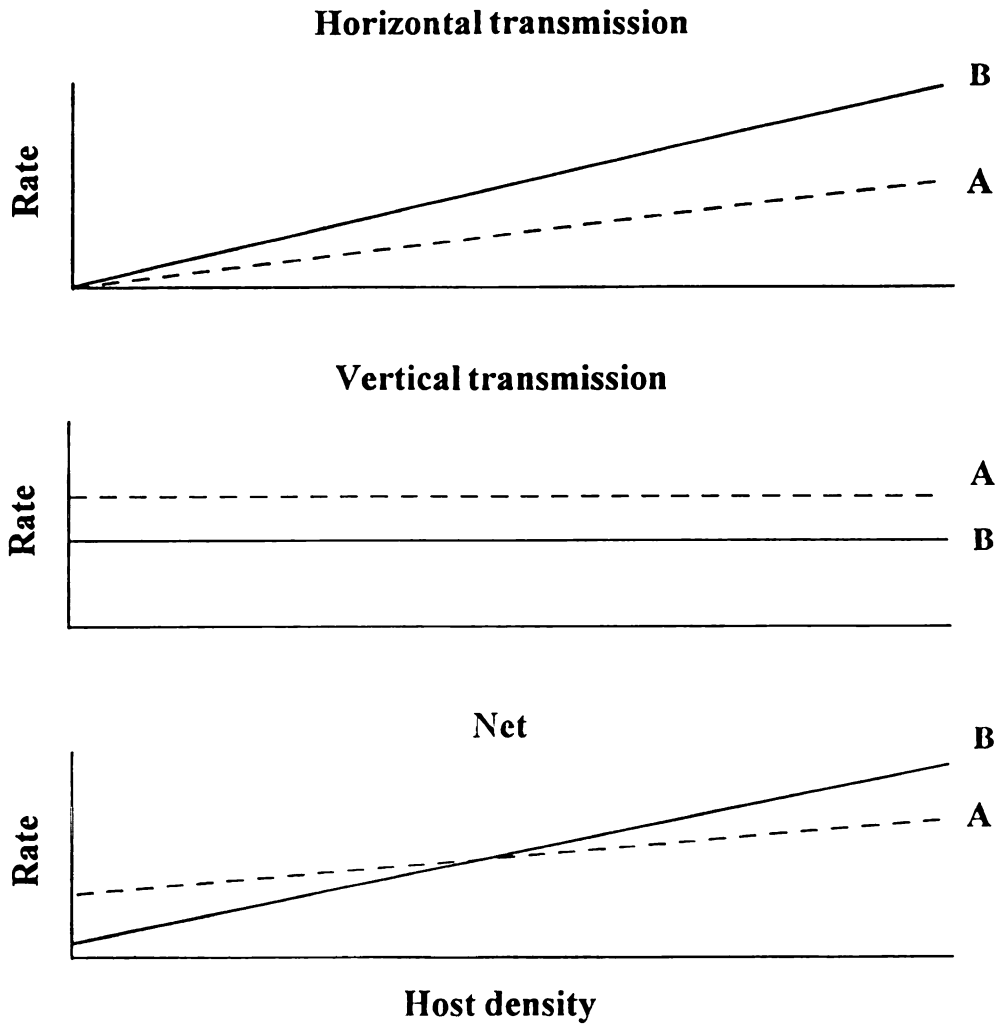


Figure 14. Horizontal, vertical, and net rates of increase for two plasmid genotypes, A and B, as a function of susceptible (plasmid-free) host density. The more virulent plasmid (B) is favored only when susceptible hosts are sufficiently abundant. See text for details.

E. coli and its interactions with plasmids and other infectious elements. For these reasons, evolution experiments with bacteria and their plasmids provide a powerful system to study host-parasite interactions and the evolution of virulence (Levin and Lenski 1983; Bouma and Lenski 1988; Bull et al. 1991).

I conducted a 500-generation (75-day) experiment to look at the influence of susceptible host density on the evolution of plasmid virulence and mode of transmission. For this study, I used plasmid pB15, a naturally occurring plasmid previously reported to persist by a high rate of horizontal transmission in chemostat culture (Lundquist and Levin 1986). I subjected three replicated treatments of the *E. coli*/pB15 association to batch culture environments that contained different input densities of susceptible (plasmid-free) hosts. Two of the three treatments allowed a fixed immigration of plasmid-free hosts into resident plasmid-bearing populations at periodic intervals (every 20 generations), creating immigrant-to-resident ratios in these populations of either 1:1 or 100:1. In contrast, the third treatment never received any plasmid-free immigrants. Therefore, the last treatment was expected to favor vertical transmission exclusively, while the other two treatments should favor horizontal transmission to different degrees. To ensure that the bacterial populations retained their plasmids, all three treatment groups were periodically placed in an antibiotic environment, to preclude over-growth by plasmid-free immigrants or segregants.

Two control groups were created for the experiment as well. One control contained plasmid-free *E. coli* populations that served as an immigrant pool for the treatments described above. The other control contained replicated *E. coli*/pB15 associations and was designed to study the effects of spontaneous plasmid segregation (loss) in the absence of antibiotic. All five experimental groups are summarized in Table 11.

To evaluate whether host density affected the plasmid's evolution, I looked for changes in plasmid conjugation rate and the cost of plasmid carriage to the host. As

Table 11. Summary of experimental and control treatment groups for Chapter III.

Group	Description	Antibiotic Selection	Approximate Immigrant to Resident Ratio
F	plasmid-free immigrant pool	no	0:1
B	plasmid-bearing control to examine effects of spontaneous plasmid segregation (loss)	no	0:1
L	plasmid-bearing with low opportunity for horizontal transfer	yes	0:1
M	plasmid-bearing with medium opportunity for horizontal transfer	yes	1:1
H	plasmid-bearing with high opportunity for horizontal transfer	yes	100:1

Note: There were three replicate populations in each group.

explained earlier, I expect both the cost of carriage and the conjugation rate to be higher under conditions of high host density than under conditions of low host density (Table 12). Cost of carriage and conjugation rate are both easily measured (Bouma and Lenski 1988; Simonsen et al. 1990).

Table 12. Expected evolutionary changes in traits pertaining to plasmid virulence.

	Susceptible host density	
Trait	Low	High
Cost of carriage	-	+
Conjugation rate	-	+

MATERIALS AND METHODS

Bacterial strains. -- Table 13 describes the pertinent features of the bacteria used in this study. All strains were derived from a single clone of *E. coli* B (REL1206), previously evolved for 2,000 generations in a glucose-limited environment (Lenski et al. 1991). Although this strain is prototrophic, it is unable to utilize the sugar L-arabinose as a nutrient (Ara^-). A spontaneous arabinose-utilizing (Ara^+) mutant of the strain (REL1207) was obtained in a previous study (Bennett et al. 1992). The Ara^+_0 and Ara^-_0

Table 13. Key bacterial strains used in Chapter III.

Strain	Relevant characteristics[1]	Abbreviation
REL1206	plasmid-free *Escherichia coli* B	Ara^-_0
REL1207	spontaneous Ara^+ mutant of REL1206; ancestral immigrant	Ara^+_0
REL5382	transconjugant of REL1206 using plasmid pB15; ancestral resident	Ara^-_0/p_0
REL5384	transconjugant of REL1207 using plasmid 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	$Ara^+_0/Nal^r/p_0$

[1] Ara^+ indicates ability to use arabinose; all strains are Ara^- unless otherwise indicated. Nal^r indicates resistance to nalidixic acid; all strains are Nal^s unless otherwise indicated.

ancestors in this study form white and red colonies, respectively, on tetrazolium-arabinose (TA) indicator plates (Levin et al. 1977).

The conjugative plasmid used in this study, pB15, was obtained from Prof. B. R. Levin (Emory University, Atlanta, Georgia). This plasmid was originally isolated from an *E. coli* strain sampled from a human under antibiotic treatment (B. R. Levin, personal communication). It is a large (~80 kb), low-copy number plasmid, that confers resistance to the antibiotics kanamycin (Kan^r) and tetracycline (Tet^r) (Lundquist and Levin 1986). Kanamycin binds to the 70S ribosomal subunit and is lethal to sensitive cells (bactericidal). Tetracycline inhibits protein synthesis and stops sensitive cells from dividing (bacteriostatic). I confirmed resistance encoded by pB15 to these antibiotics by spreading plasmid-bearing and plasmid-free cells onto TA plates supplemented with 25 ug mL^{-1} Kan and 1 ug mL^{-1} Tet.

Plasmid pB15 was transferred to the REL1206 and REL1207 backgrounds by mixing each strain with a donor, and then selecting for transconjugants generated in overnight mating cultures. In this way, I obtained ancestral strains REL5382 (Ara^-_0/p_0) and REL5384 (Ara^+_0/p_0), which harbored pB15 but were otherwise isogenic to REL1206 and REL1207, respectively (Table 13).

Media and culture conditions. -- The culture medium employed in all experiments was Davis minimal broth (Carlton and Brown 1981) supplemented with 2 mg L^{-1} thiamine hydrochloride and 1000 mg L^{-1} glucose (DM1000). A plasmid-selective medium of DM1000 supplemented with 25 ug mL^{-1} Kan (DM1000+Kan) was also used. Either medium allows a stationary-phase bacterial density of ~10^9 cells mL^{-1}. Culture volume was 10 mL, maintained in 18x150 mm borosilicate glass tubes and placed in a non-shaking incubator at 37 ^oC. All cultures were propagated daily by vortexing and then transferring 0.1 mL of each culture into 9.9 mL of fresh medium (serial transfer). During this 24-hour

cycle, bacterial populations attained stationary-phase densities. The resulting 100-fold daily growth of each bacterial population represents ~6.64 generations of binary fission.

Plasmid electrophoresis and restriction. -- Plasmid DNA was extracted using the method of Birnboim and Doly (1979). The relative sizes of restriction-endonuclease-digested plasmid fragments were examined by electrophoresis on 0.6% agarose gels. Restriction endonucleases *Eco*RI and *Bam*HI were used (New England Biolabs). Restriction digests were prepared using the procedure described in Sambrook et al. (1989).

Experimental treatments: control populations. -- Three clones of Ara^{+}_{0} were used to initiate the three populations in the plasmid-free (F) control. Each population underwent serial transfer into DM1000 for 75 days. Every three days, each population also served as an immigrant pool to manipulate susceptible host density (see below). Three clones of Ara^{-}_{0}/p_{0} were similarly used to initiate the three populations in the plasmid-bearing (B) control. Each population also underwent serial transfer into DM1000 for 75 d. B control populations did not experience antibiotic selection for plasmid maintenance, and therefore they were potentially subject to takeover by spontaneous plasmid-free segregants.

Experimental treatments: host density manipulations. -- Three clones of Ara^{-}_{0}/p_{0} were used to found the three replicate populations in each experimental treatment. At the start of the experiment (day 0), each population in the low opportunity for horizontal transfer (L) treatment underwent serial transfer into DM1000. On day 1, each of these populations underwent serial transfer into DM1000+Kan to ensure plasmid-maintenance (i.e., to select against any plasmid-free segregants). On day 2, each population was serially-transferred into DM1000. This three-day cycle was repeated for 75 d.

On day 0, each resident population in the medium opportunity for horizontal transfer (M) treatment and a paired immigrant population from the F control were mixed at a 1:1 volumetric ratio and diluted 1:100 into DM1000. The populations in the high opportunity for horizontal transfer (H) treatment were initiated in the same way, but at a 100:1 immigrant-to-resident ratio. All populations were allowed to grow and conjugate during a standard daily growth cycle. On day 1, all populations underwent serial transfer into DM1000+Kan to remove any plasmid-free immigrants or spontaneous segregants. On day 2, all populations were serially-transferred into DM1000. On day 3, each population experienced an identical immigration event, involving the same paired immigrant population, as on day 0. This three-day cycle was repeated for 75 d.

Aside from differences in immigrant-to-resident ratios, all aspects of the environment (including the frequency of antibiotic selection) were kept constant between the L, M, and H treatments. All five experimental treatment and control groups are summarized in Table 11.

Every three days for the first 100 generations and periodically during the subsequent 400 generations, a sample from each experimental population was spread on TA and TA+Kan plates. Plate counts were used to determine the density of total cells and of plasmid-bearing cells, respectively; the density of plasmid-free cells was obtained by subtraction. Every 15 days (100 generations), a sample from each population was also spread on a TA+Tet plate to determine whether plasmid-bearing lines still retained their resistance to tetracycline. Every 15 days, after serial transfer had taken place, glycerol was added to a sample from each population and the sample was stored in a freezer at -80 ^{o}C for future study. Single colonies were also chosen at random from each population at generation 500 and stored at -80 ^{o}C.

Fitness assay. -- To assay relative fitness (*W*), two strains were placed in competition under the culture conditions described above, where one competitor was

Ara^+ and the other was Ara^-. Each strain was grown separately for one day in the experimental medium, as a preconditioning step to ensure that both competitors were in comparable physiological states. The two competitors were mixed at a 1:1 ratio, then diluted 1:100 into fresh medium and allowed to grow and compete during a standard 24-hour growth cycle. Initial and final densities of each competitor were estimated by spreading samples on TA plates, which permitted the competitors to be distinguished by colony color.

Let the initial densities of the Ara^+ and Ara^- competitors be $N_1(0)$ and $N_2(0)$, respectively; and let $N_1(1)$ and $N_2(1)$ be their corresponding densities after one day. The average rate of increase (or realized Malthusian parameter), m_i, for either competitor is then calculated as:

$$m_i = \ln[N_i(1)/N_i(0)] / (1 \text{ day}).$$

The fitness of one strain relative to another (W_{ij}) is estimated as the ratio of their Malthusian parameters (Lenski et al. 1991):

$$W_{ij} = m_i/m_j.$$

A fitness difference between the two competitors may reflect differences in their lag phase, maximum or submaximum growth rates, survival at stationary phase, or some combination thereof (e.g., Vasi et al. 1994).

Conjugation rate. -- In order to assay rates of plasmid transfer, I obtained mutants of Ara^+_0 and Ara^-_0 that were resistant to nalidixic acid (Nal^r). Thus, I obtained strains PET318 and PET319 that were Nal^r, but otherwise isogenic to Ara^+_0 and Ara^-_0, respectively (Table 13).

Conjugation rate (γ) was assayed under the DM1000 culture conditions described above by mating a plasmid-bearing donor with a plasmid-free Nal^r recipient that bore the opposite arabinose marker. Donors and recipients were grown to stationary phase in DM1000+Kan and DM1000, respectively. Each strain was then preconditioned for one day in DM1000. Donors and recipients were mixed at a 1:1 volumetric ratio, then diluted 1:100 into fresh medium and allowed to grow and mate during a standard daily growth cycle. After 0 and 24 hours, the densities of donors (D), recipients (R) and transconjugants (T) were determined by colonies formed on appropriate selective and nonselective plates. I also estimated the growth rate (hr^{-1}) in exponential phase (ψ) of mating cultures by regressing ln [total cell density] versus time during the period of exponential-phase growth. Total cell densities were estimated by counts obtained using a Coulter electronic particle counter (model ZM and channelyzer model 256).

I estimated the rate of transfer (mL hr^{-1}) for matings between donor and recipient cells in batch culture using the formula of Simonsen et al. (1990):

$$\gamma = \psi \ln[1 + (T/R)(N/D)]/(N - N_0),$$

where $N = T + R + D$ and N_0 is initial population size.

Cost of plasmid carriage. -- Cost of carriage (c) was assayed by competing a plasmid-bearing strain with a plasmid-free strain that differed only by a neutral marker. The plasmid-bearing and plasmid-free competitors were grown separately in DM1000+Kan and DM1000, respectively. The two competitors were then preconditioned for one day in DM1000. The strains were then mixed at a 1:1 ratio and allowed to compete during a standard daily growth cycle, as previously described. The fitness of the plasmid-bearing strain relative to the plasmid-free strain over that cycle was then calculated from plate counts obtained after 0 and 24 hours.

The *change* in cost of plasmid carriage (Δc) was assayed by allowing a strain bearing the ancestral plasmid to compete against a neutrally marked isogenic strain harboring an evolved plasmid. To do so, I moved p_0 onto the Ara^+_0/Nal^r background to create strain PET354, $Ara^+_0/Nal^r/p_0$ (Table 13). An evolved plasmid of interest was moved to an Ara^-_0/Nal^r background, and the resulting strain was allowed to compete against PET354. In all assays, competitors were grown separately in DM1000+Kan, and then preconditioned for one day in the competitive environment. The strains were then mixed at a 1:1 ratio and allowed to compete during a standard daily growth cycle. The fitness of the strain bearing the evolved plasmid relative to the strain bearing the ancestral plasmid was estimated, and Δc was calculated by subtracting this fitness estimate from 1.0.

RESULTS

Ancestral Plasmid Traits

Ancestral cost of plasmid carriage. -- Experiments were performed to determine the fitness of the ancestral plasmid-bearing strain (Ara^-_0/p_0) relative to the plasmid-free ancestor (Ara^+_0). To examine whether segregation and conjugation might complicate estimation of this quantity, I sampled from competition cultures to determine the proportion of segregants and transconjugants generated. I then computed relative fitness with and without adjusting for segregants and transconjugants. In ten replicate fitness assays, the estimated mean fitness of Ara^-_0/p_0 relative to Ara^+_0, adjusting for plasmid losses and gains, was $W = 0.981$; mean fitness estimated without such adjustments was $W = 0.979$. These two estimates were not significantly different ($t_S = 0.077$, 18 df, $p = 0.939$). Because monitoring plasmid losses and gains provided no better estimate of relative fitness in these one-day competition experiments, I performed two more blocks of fitness assays without such monitoring.

The combined results from three blocks of fitness assays showed that the mean fitness of Ara^-_0/p_0 relative to Ara^+_0 was $W = 0.989$. There was no significant effect of block on fitness ($p = 0.655$), and mean fitness did not differ significantly from 1.0 ($t_S = 1.226$, 29 df, $p = 0.230$). $1 - W = 0.011$ provides an estimate of the cost of carriage for pB15 in the ancestral background. I conclude that pB15 imposes only a slight (and statistically insignificant) fitness cost in Ara^-_0.

Table 14. Ancestral plasmid traits.

	Mean Estimate	95% Confidence Interval
Cost of plasmid carriage, c	0.011	$\pm$ 0.019
Log_{10} conjugation rate, γ	-12.161	$\pm$ 0.085

Fitness assays with ten-fold replication also showed that the plasmid-bearing resident (Ara^-_0/p_0) and its plasmid-bearing Ara^+ counterpart (Ara^+_0/p_0) were equally competitive in both the presence of kanamycin ($W = 1.003 \pm 0.025$ SE; $t_S = 0.113$, 9 df, $p = 0.912$) and in its absence ($W = 0.996 \pm 0.014$ SE; $t_S = 0.256$, 9 df, $p = 0.804$). Thus, the arabinose marker has no significant effects on fitness under my experimental conditions.

Ancestral conjugation rate. -- Twenty replicate mating assays between the ancestral plasmid-bearing strain, Ara^-_0/p_0, and an Ara^+_0/Nal^r recipient were conducted in

the antibiotic-free evolutionary environment (DM1000). The conjugation rate coefficient, γ, was estimated using the endpoint method of Simonsen et al. (1990), as summarized in the Materials and Methods. The mean estimate of $\log_{10} \gamma$ for the ancestral plasmid was -12.161 ($\pm$ 0.040 SE) mL hr^{-1}. Means and 95% confidence intervals for the ancestral plasmid traits are given in Table 14.

Control Populations

Evolutionary dynamics. -- Plasmid-bearing (B) control populations were potentially subject to takeover by spontaneous plasmid-free segregants. I observed that, on average, segregants never reached an average frequency of more than about one-third of the total population (Figure 15). This result suggests that horizontal transfer occurred at a high enough rate to reinfect segregants, so that they always remained in the minority.

Fitness changes. -- The ancestral plasmid-free resident (Ara^-_0) and the ancestral immigrant (Ara^+_0) were competitively equivalent in the antibiotic-free evolutionary environment (DM1000). Twenty replicate fitness assays yielded a mean fitness of Ara^-_0 relative to Ara^+_0 of $W = 1.008$, which did not differ significantly from 1.0 ($t_S = 1.068$, 19 df, $p = 0.299$). Using these two strains as equivalent baseline competitors, I sought to determine whether the plasmid-bearing controls and plasmid-free controls differed in their rates of adaptation to the antibiotic-free environment (DM1000). To do so, I measured the fitness, relative to either Ara^-_0 or Ara^+_0, at 100 generation intervals for each population in the F and B controls, respectively. Fitness measurements were replicated twice for each population, and the grand mean fitness over the three replicate populations in each control group was calculated at each time point. The F and B controls underwent nearly parallel fitness improvements during the first half of the experiment, but the B controls later fell behind in their rate of adaptation (Figure 16). The relevance of this result will become apparent in the next section.

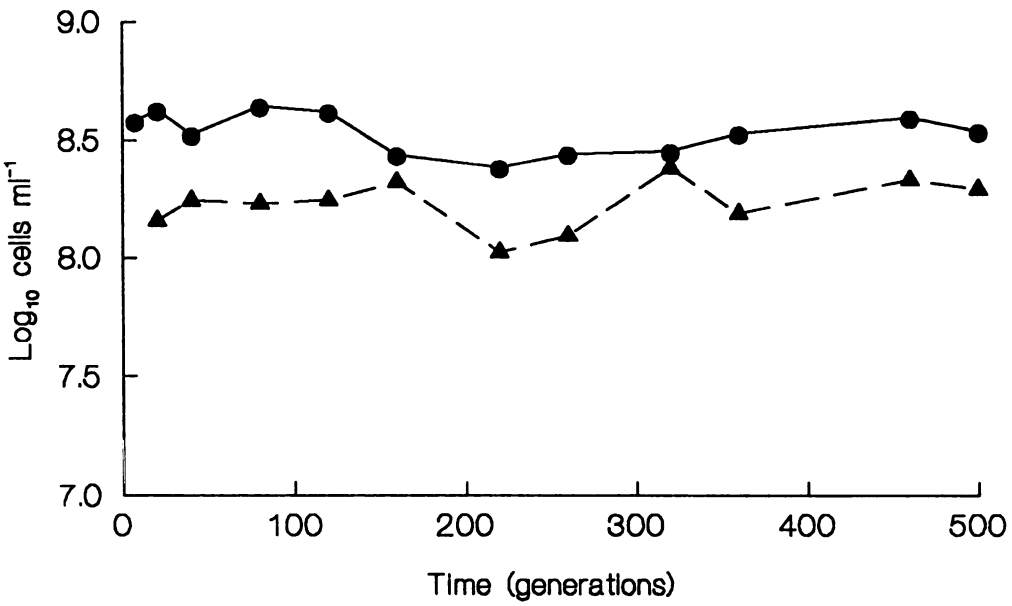


Figure 15. Evolutionary dynamics in the B control populations (see Table 11), in which the plasmid-bearing resident populations received neither plasmid-free immigrants nor antibiotics. Each point represents the mean of three replicate populations. Plasmid-free segregants (triangles) were detected as a minority population, but they were unable to replace the plasmid-bearing genotypes (circles). Points are averages based on the three replicate B populations.

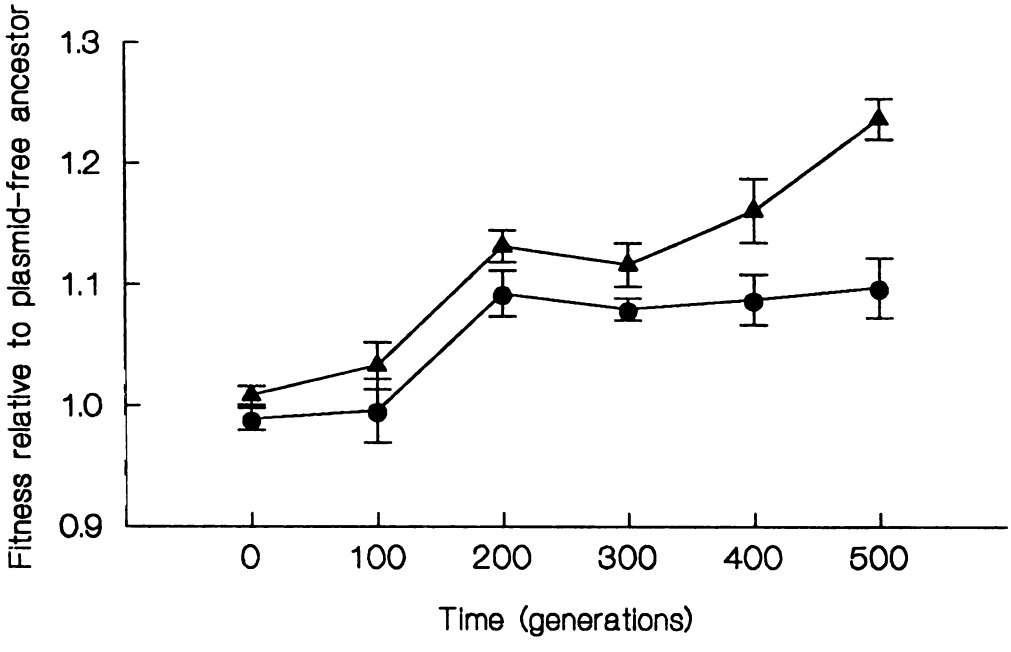


Figure 16. Fitness trajectories for plasmid-bearing and plasmid-free control populations during evolution in the antibiotic-free environment. Plasmid-free (F) control populations (triangles) continued to adapt to the environment throughout the 500 generations, whereas plasmid-bearing (B) control populations (circles) did not. Fitness was measured relative to the plasmid-free ancestor. Each point represents the grand mean (± SE) of three populations.

Host Density Manipulations

Evolutionary dynamics. -- Plasmid-bearing populations in the medium (M) and high (H) opportunity for horizontal transfer treatments were allowed to conjugate with, and be transmitted to, immigrant genotypes at regular intervals. The immigrants could not become established, however, unless they acquired a plasmid from the resident population, owing to my imposition of antibiotic selection every third day. Every 100 generations, I sampled from these plasmid-bearing populations to determine the frequency of Ara^+ immigrants. My results showed that novel associations between immigrant hosts and resident plasmids were rare until relatively late in the experiment (Figure 17). This result may coincide with the fitness trajectories for the F and B controls, which diverged late in the experiment (Figure 16). Apparently, there was strong selection for the resident plasmids to reach the immigrant genetic background only after the fitness gains for the plasmid-free immigrants had begun to outpace the gains for the plasmid-bearing lines. I also observed that the higher immigration rates in the H treatment did *not* lead to a higher final proportion of immigrant hosts. Rather, all three M populations contained nearly 100% immigrant hosts, whereas the three H populations showed roughly 0%, 50% and 100% immigrant hosts (Figure 17).

All populations in the low (L), medium (M), and high (H) opportunity for horizontal transfer treatments were founded by an ancestral plasmid that conferred resistance to kanamycin and tetracycline (Kan^r Tet^r). Every 100 generations, I sampled from treatment populations to determine whether plasmid-bearing cells had become sensitive to tetracycline (Tet^s), an unselected marker. I observed that the first novel genotype to be detected in each treatment population was an ancestral-type (resident) host bearing a Tet^s plasmid; the final frequency of Tet^s plasmids in each population was highly variable within treatments (Figure 18). Interestingly, Tet^s plasmids were never detected in the B controls, even though they were founded by the same ancestral plasmid used to

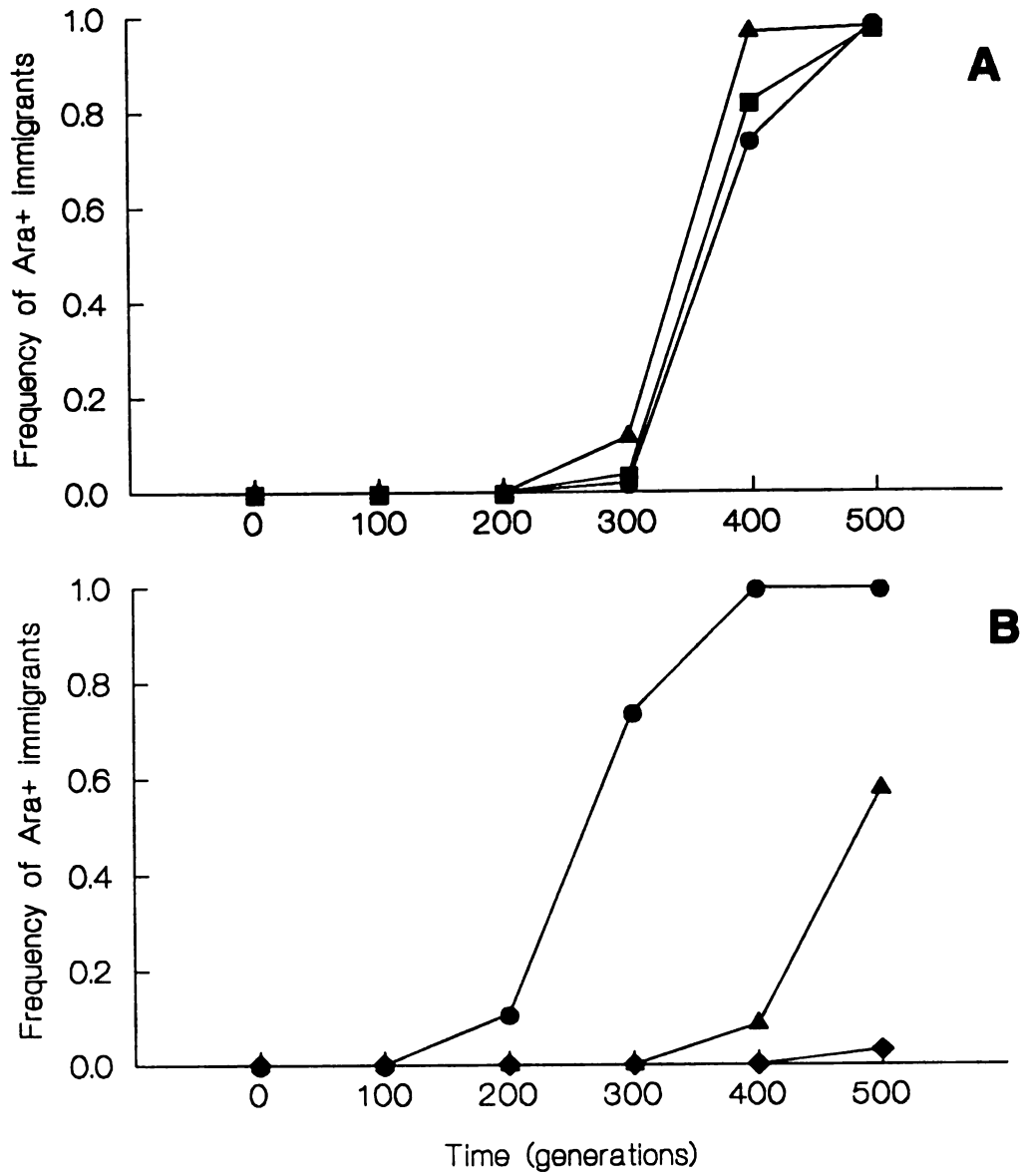


Figure 17. Changes in the frequency of Ara^+ immigrant backgrounds in plasmid-bearing treatment populations subjected to medium (M: panel A) or high (H: panel B) levels of immigration by plasmid-free cells. The final frequency of Ara^+ immigrants showed no significant association with treatment (Mann-Whitney test, $p > 0.2$). Each curve represents an independent replicate population.

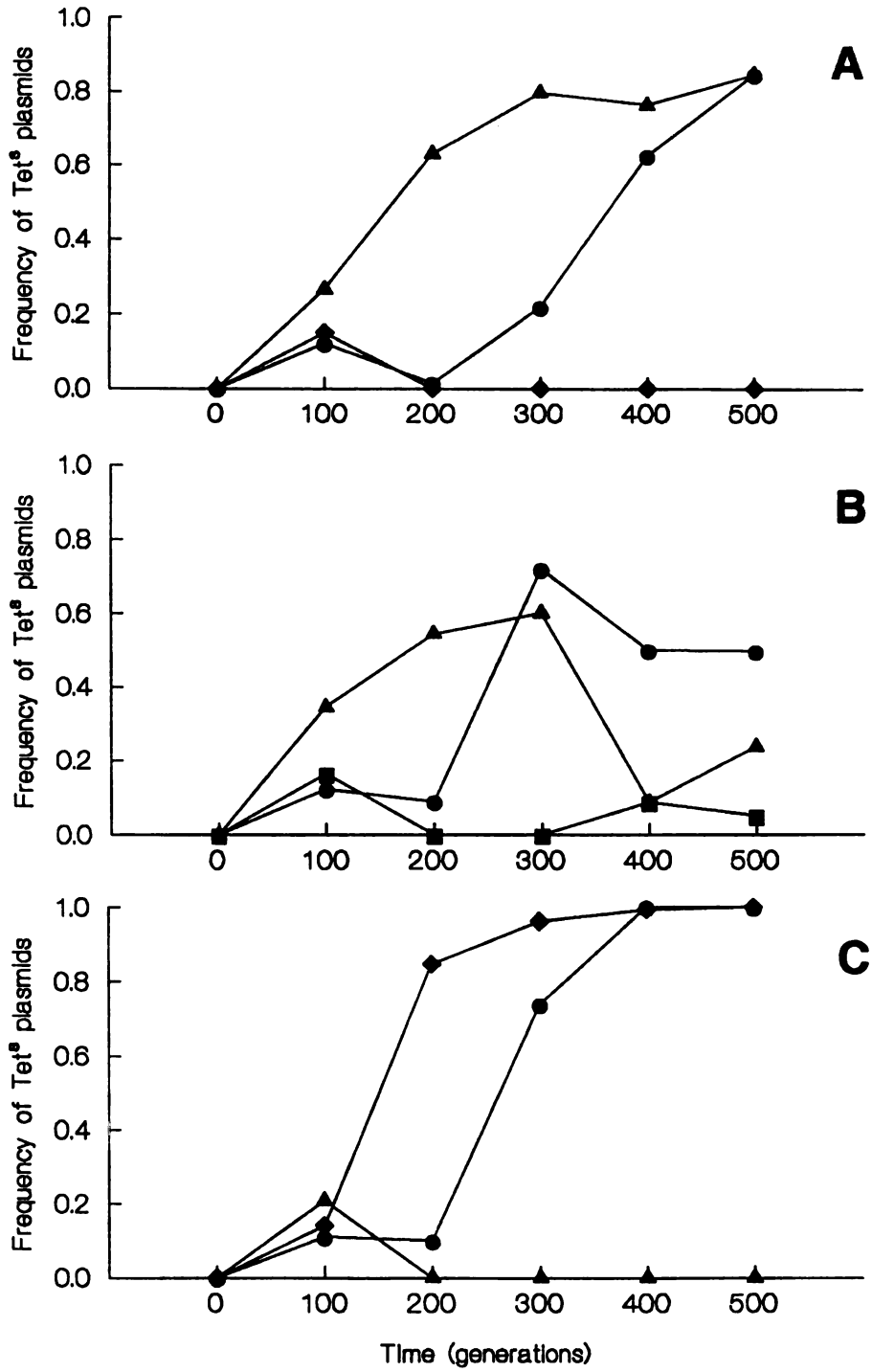


Figure 18. Changes in the frequency of Tet^S plasmid variants in treatment populations subjected to low (L: panel A), medium (M: panel B), or high (H: panel C) levels of immigration by plasmid-free cells. The final frequency of Tet^S plasmids showed no significant association with treatment (Kruskal-Wallis test, $p > 0.5$). Each curve represents an independent replicate population.

found treatment populations. Why was the loss of tetracycline resistance common in all three treatment groups but not in the controls?

During the experiment, treatment populations, but not controls, were periodically subjected to kanamycin in order to ensure plasmid maintenance (see Materials and Methods). Although the original plasmid encoded resistance to both kanamycin and tetracycline, kanamycin was chosen as the selective agent because it is lethal to plasmid-free cells, whereas tetracycline merely prevents these cells from growing. Only resistance to kanamycin determined survival in the treatment environments, and my data suggest that the loss of tetracycline resistance may have been a response to kanamycin selection. More importantly, there was no overall pattern in the final frequency of Tet^S plasmids in relation to the susceptible-host-density treatments (e.g., the final frequency of Tet^S plasmids ranged from near 0% to $\geq$ 50% in L, M, and H treatments).

Fitness Changes. -- I sought to determine whether host density manipulations in treatment lines led to differential improvements in fitness. To do so, I allowed a heterogeneous sample from each of the nine evolved populations to compete against the reciprocally marked plasmid-free ancestor, Ara^+_0 or Ara^-_0, in DM1000. To ensure accurate estimates of relative fitness, I randomly sampled from competition cultures to track the possible movement of plasmids. The proportion of segregants and transconjugants generated in each competition was used to adjust my estimates of the number of plasmid-bearing and plasmid-free competitors. Similarly, in competitions involving an evolved population that was heterogeneous for the Ara marker, the proportion of Ara^+ and Ara^- plasmid-bearing cells was used to adjust the number of evolved competitors. I then computed the fitness of each mixed population relative to the plasmid-free ancestor. The mean of five fitness assays was obtained for each final treatment population, and the grand mean for the three populations in each experimental group was calculated. The grand mean fitness relative to the plasmid-free ancestor

increased in all three treatment lines (Figure 19), indicating adaptation to the antibiotic-free environment. I performed a nested ANOVA to examine the variation in relative fitness within and between treatment groups (Table 15). There was no significant variation in mean fitness either within treatments or between treatments.

Table 15. Nested ANOVA to examine the effects of susceptible-host-density treatment, and population within treatment, on fitness of evolved populations relative to 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		

I also performed fitness assays by allowing single-colony isolates (instead of heterogeneous samples) from each L, M, and H treatment population to compete against the plasmid-free ancestor. In close accord with the heterogeneous samples, the single-colony isolates had relative fitnesses averaging 1.167 ($\pm$ 0.012 SE) and there was no significant effect of treatment (data not shown). I conclude that differences in susceptible host density did not lead to differential increases in fitness relative to the plasmid-free ancestor.

The preceding fitness measurements reflect a composite of effects due to evolutionary changes in both the host and plasmid. A more sensitive measure of changes

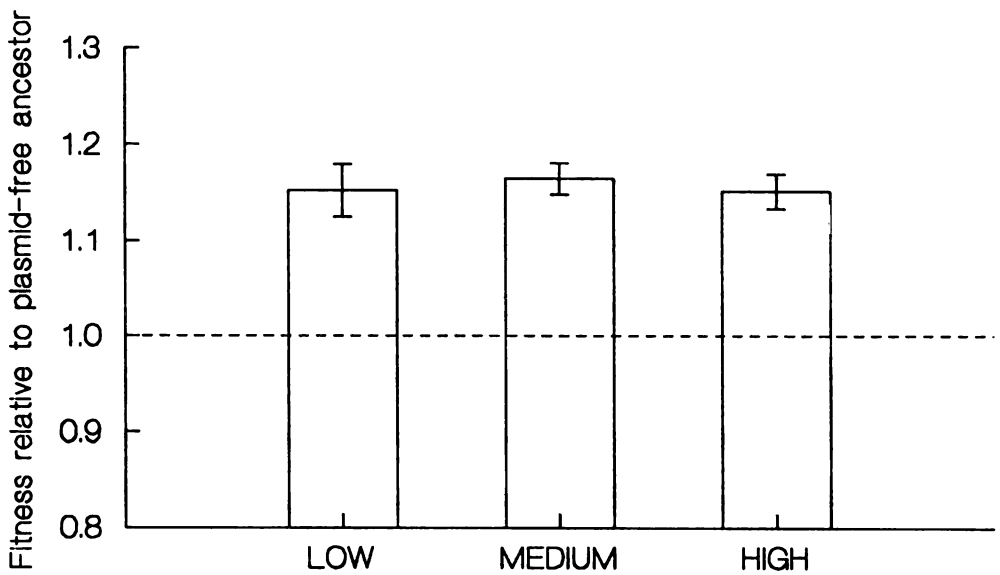


Figure 19. Mean fitness in treatment populations subjected to low, medium, and high levels of immigration by plasmid-free cells. Each bar represents the grand mean (± SE) of three replicate populations. Fitnesses were measured relative to the plasmid-free ancestor in an antibiotic-free environment. See Table 15 for statistical analysis.

in plasmid effects would be to place each evolved plasmid on the same -- ancestral -- host background, and then estimate the cost of carriage for each evolved plasmid (see Bouma and Lenski 1988). To do so requires plasmid transfer and, hence, competitions involving clonal isolates instead of heterogeneous populations. In fact, I can allow an ancestral host carrying an evolved plasmid to compete against a marked but otherwise isogenic ancestral host carrying the original plasmid, and thereby estimate directly the *change* in cost of plasmid carriage. Such competitions may be carried out in each of the two environments (DM1000 and DM1000+Kan) that plasmids experienced during their evolution (see Materials and Methods). I may then ask: Do the changes in cost of plasmid carriage (if any) differ among susceptible-host-density treatments? Alternatively, do any such changes correlate with the loss or retention of plasmid-encoded tetracycline resistance function?

Changes in Plasmid Traits

For each population in the L, M, and H treatments, I isolated the majority genotype present at generation 500 and stored it in the freezer. Population M1 was polymorphic for two equally common genotypes that differed in their resistance to tetracycline (Figure 18B), and so two genotypes were used for that population. Thus, a total of ten clonal isolates were obtained for the nine populations. From here on, I refer to the evolved plasmid present in each majority genotype by the population from which it was obtained (with the Tet^r and Tet^s plasmids from population M1 referred to as M1r and M1s, respectively).

Cost of plasmid carriage. -- I sought to determine whether evolved plasmids had undergone changes in the cost of their carriage to the ancestral host. To do so, I moved each evolved plasmid into strain Ara^-_0/Nal^r to create eight new host-plasmid associations. As shown in the next section, two of the ten evolved plasmids were unable to conjugate and so could not be transferred to a new host. Then, I allowed each of these eight

constructs to compete against $Ara^{+}_{0}/Nal^{r}/p_{0}$ in antibiotic (DM1000+Kan) and in antibiotic-free (DM1000) environments. Fitness assays were replicated three-fold, and the fitness of the evolved association relative to the ancestral association was determined. The difference of this fitness value from 1.0 gives a direct estimate of the *change* in cost of carriage, Δc. I observed that, in both environments, cost of carriage had decreased for the

Table 16. Mixed-model two-way ANOVA to examine the effects of plasmid genotype and assay environment on the change in cost of plasmid carriage.

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		

Note: Plasmid genotype is a random effect and assay environment is a fixed effect.

evolved plasmids which were Tet^{s}, but had typically increased for those that were Tet^{r} (Figure 20). I performed a two-way ANOVA to evaluate the effects of plasmid genotype and assay environment on Δc (Table 16). This test confirmed that Δc was indeed heterogeneous among the evolved plasmids, whereas neither the environment nor the genotype-environment interaction had any significant effect on this trait. The lack of correspondence between susceptible-host-density treatment and Δc is illustrated by the

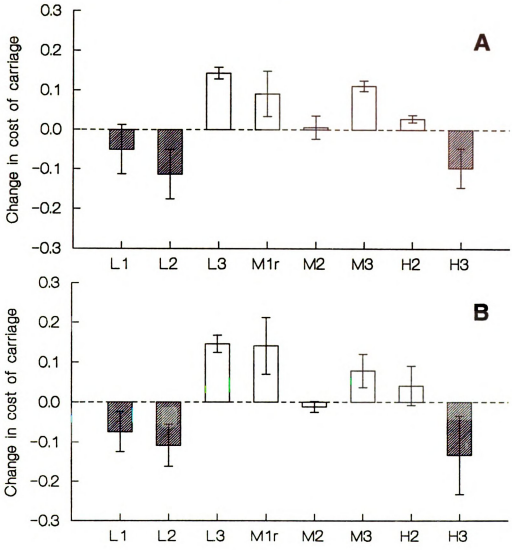


Figure 20. Change in cost of carriage to the host (Δc) for the eight evolved plasmids that retained their ability to conjugate. To estimate Δc, each evolved plasmid was transferred to the ancestral host background, which was then allowed to compete against a neutrally marked, isogenic host carrying the ancestral plasmid. A $\Delta c < 0$ indicates that an evolved plasmid was less costly than the ancestral plasmid, and may even have become beneficial. Open and filled bars represent Tet^r and Tet^s evolved plasmids, respectively. (A) Competition assays performed in an antibiotic-free environment. (B) Assays performed in medium containing kanamycin. Error bars show SE. See Table 16 for statistical analysis.

fact that treatment L plasmids, for example, show both significant increments and decrements in cost (Figure 20). I also computed the grand mean Δc in each assay environment for the three evolved Tet^S plasmids and the five evolved Tet^r plasmids. These data indicate a statistically significant relationship between the tetracycline marker and Δc (DM1000: $t_S = 4.382$, 6 df, $p = 0.005$; DM1000+Kan; $t_S = 4.414$, 6 df, $p = 0.005$). I conclude that the evolved Tet^S plasmids substantially reduced the cost of their carriage in relation to the ancestral plasmid, whereas the evolved Tet^r plasmids are actually more costly than the ancestral plasmid.

Conjugation rate. -- I measured the conjugation rate (γ) for each of the ten evolved plasmids. Mating assays were performed, with three-fold replication, between each single-colony isolate and a recipient bearing the opposite arabinose marker (Ara^+_0/Nal^r or Ara^-_0/Nal^r). All five evolved plasmids that still expressed the tetracycline-resistance function (Tet^r) conjugated at rates slightly higher than that of the ancestral plasmid (Figure 21). Conversely, all five of the evolved plasmids that lost resistance to tetracycline (Tet^S) conjugated at rates much lower than the ancestral plasmid; in fact, two showed a complete inability to conjugate. The lack of any correspondence between susceptible-host-density treatment and conjugation rate of the evolved plasmids is illustrated by the tremendous variation in γ for plasmids within each treatment. Treatment M, for example, yielded one plasmid with γ greater than the ancestor, one with γ reduced somewhat, and one in which γ equals 0. Although the treatment effect was not significant, a Mann-Whitney test indicates that the difference in conjugative rates between the Tet^S and Tet^r derived plasmids is highly significant ($U_S = 0.0$, $n_1 = 5$, $n_2 = 5$, $p = 0.008$). I conclude that the evolved Tet^S plasmids show a deficiency in their ability to conjugate, or an inability to transfer at all, in comparison to the ancestor and the evolved Tet^r plasmids.

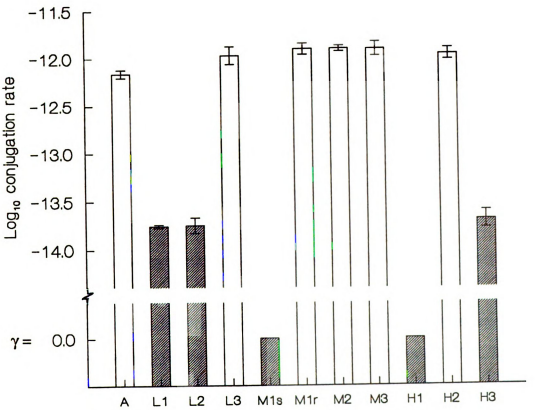


Figure 21. Conjugation rates (γ) for the ancestral (A) and ten evolved plasmids. Each bar represents the mean (± SE) of three measurements, except for the ancestor, which is based on 20 assays. Two plasmids (M1s and H1) had conjugation rates at or near 0, for which transconjugants were not detected. Open and filled bars represent Tet^r and Tet^s plasmids, respectively. γ shows no significant association with immigration treatments L, M, and H (Kruskal-Wallis test, $p > 0.2$).

Tradeoff in modes of transmission. -- Figure 22 shows the correlation between $\log_{10} \gamma$ and Δc (in DM1000) for the eight evolved plasmids that could be transferred to the common background. The correlation is highly significant ($r = 0.867$, $p = 0.005$), which clearly shows that a plasmid's conjugation rate is tightly coupled with the cost of its carriage to the host bacterium. In other words, I observed the predicted genetic tradeoff between opportunities for horizontal and vertical transmission of the plasmid. In addition, I observed that retention versus loss of the tetracycline resistance function served (for an unknown reason) as a proxy phenotypic marker for greater or lesser plasmid virulence, respectively.

DISCUSSION

Many parasites can be transmitted either horizontally (infectiously) or vertically (via host reproduction). In such cases, it is commonly assumed that there exists a genetically determined tradeoff between a parasite's potential to be transmitted horizontally and vertically (Levin and Lenski 1983, May and Anderson 1983, Bull 1994, Ewald 1994). This tradeoff presumably occurs because activities of a parasite that increase its infectiousness will be generally harmful to its host. If one assumes also that hosts can only be infected by a single genotype of the parasite, then within-host competition between parasites can be ignored. With these two assumptions, a simple model predicts that the density of uninfected (susceptible) hosts should determine whether a parasite evolves to become more or less infectious and, concomitantly, more or less virulent in terms of its effect on host fitness (Figure 14). That is, when uninfected hosts are common, the opportunity for infectious transfer is large and selection should favor increased rates of horizontal transmission (increased virulence). But when uninfected hosts are scarce, then vertical transmission is more frequent and selection should favor

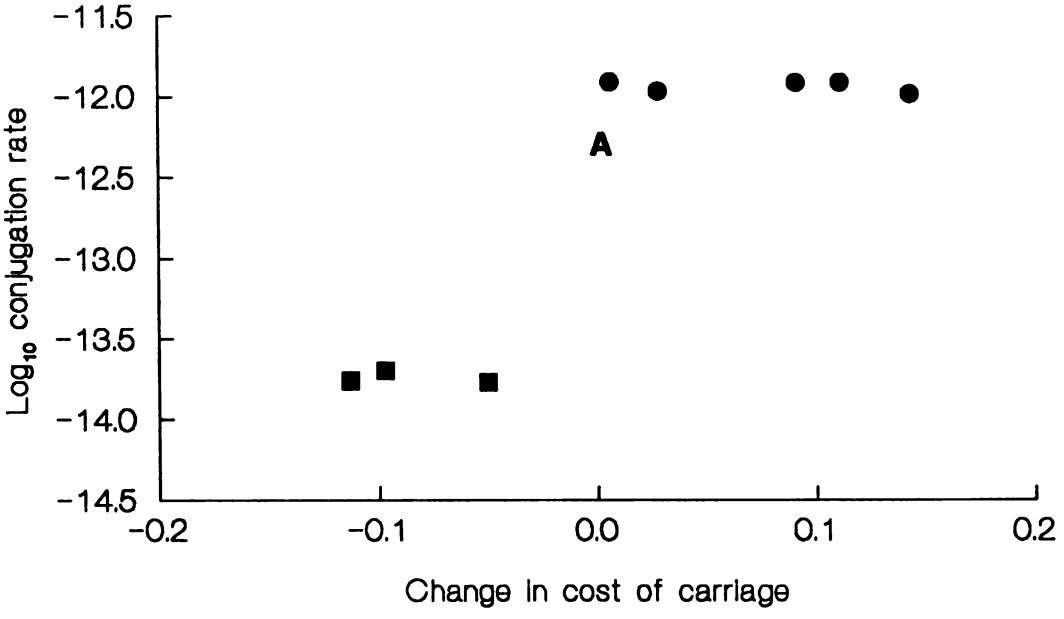


Figure 22. Genetic correlation between rate parameters governing horizontal and vertical modes of plasmid transmission in pB15 and its evolved derivatives. "A" is the ancestral plasmid. The circles show five evolved plasmids that retained the ancestral expression of tetracycline resistance. The squares show three evolved plasmids that became tetracycline sensitive. The correlation between conjugation rate (see Figure 21) and change in cost of carriage (see Figure 20A) for the eight evolved plasmids is highly significant ($r = 0.867$, $p = 0.005$).

reduced virulence. I tested these predictions by allowing a conjugative plasmid that infects the bacterium *E. coli* to evolve for 500 generations in replicated environments in which I manipulated the densities of uninfected hosts.

I can summarize the main findings of my study as follows. (1) I observed a clear tradeoff between evolved plasmids' conjugation rates and their effects on host fitness, demonstrating an inability for these plasmids to simultaneously maximize both horizontal and vertical modes of transmission. (2) However, the susceptible-host-density treatments had no systematic effect on the evolution of plasmid transmission and virulence, contrary to the predictions of the epidemiological model. Instead, I observed that more and less virulent plasmid genotypes took over the treatment populations with almost equal success, irrespective of differences in susceptible-host-density treatments. Several successful genotypes have a high conjugation rate coupled with a high cost of plasmid carriage, while several others have a low conjugation rate and low cost of carriage. Taken together, these two main findings present something of a puzzle. The epidemiological model's key assumption -- that there exists a fundamental tradeoff between the two modes of plasmid transmission -- was fulfilled, but the prediction that susceptible host density would mediate the evolution of virulence failed to materialize.

Bull et al. (1991) have also performed experiments to test essentially the same evolutionary model that I tested. Using bacteria as hosts and filamentous viruses as parasites, they manipulated the opportunity for vertical versus horizontal transmission. Consistent with theory, Bull et al. (1991) saw that the parasites became less virulent (more "benevolent") when there was no opportunity for horizontal transmission to uninfected hosts. Thus, this earlier study supported the evolutionary predictions of the epidemiological model, whereas my study did not. What might be the explanation for this difference in evolutionary outcomes?

The two studies obviously differ in the bacterial parasites that were studied. Perhaps the simplest explanation would be if the assumed genetic tradeoff between rates

of horizontal and vertical transmission did not hold for the plasmid that I looked at. But this is not the case. Although I found no evidence for an effect of susceptible host density, I did observe substantial genetic variation in both cost of carriage and conjugation rate and, moreover, these two plasmid traits were highly correlated as predicted by theory (Figure 22). Another simple explanation might be that the duration of my experiment (500 generations) was too short to observe the predicted evolution. But this also seems unlikely, since I observed evolutionary changes with large effects on the relevant phenotypes; these changes were simply not associated with my susceptible-host-density treatments.

Presumably then, the theory which describes the evolution of plasmid virulence in my system is wrong, or at least incomplete in some respect. Another key assumption of the model shown in Figure 14 is that transmission dynamics behave according to mass-action. That is, the opportunity for horizontal transfer by conjugative plasmids is supposed to increase in direct proportion to the density of susceptible hosts (H). In Chapter IV of this dissertation, I begin to examine the validity of this assumption. I have observed that, contrary to the mass-action assumption, the conjugation rate parameter, γ, is not constant but instead declines with increasing H. Thus, the product γH, which reflects the opportunity for horizontal transmission does not scale proportionately with H. In some experiments, the rate of horizontal transmission appeared to be highest at intermediate host densities. This failure to fulfill the assumption of mass-action dynamics might explain the failure to observe the predicted evolutionary effect of host density, despite the clear genetic tradeoff between horizontal and vertical modes of transmission.

The ancestral plasmid in this study, pB15, is resistant to the antibiotics kanamycin and tetracycline. An interesting finding was the unexpected association between the tetracycline-resistance function and the tradeoff between cost of carriage and conjugation rate in the evolved plasmids. In particular, every evolved plasmid that retained its resistance to tetracycline was both more virulent and more transmissible than every

plasmid that had become tetracycline sensitive. One possible explanation for this association might be that the functions for tetracycline resistance and conjugal transfer are located adjacent to one another on the plasmid. Thus, deletion mutations might simultaneously affect expression of both functions. At present, there is no genetic map for pB15. However, I explored this possibility of deletions by running restriction digests on the ancestral and derived plasmids using *Eco*RI and *Bam*HI endonucleases. I saw no obvious changes in plasmid size in any of the derived plasmids, either Tet^r or Tet^s (data not shown). Another possibility might be that the tetracycline resistance and conjugal transfer functions are transcribed from the same or overlapping promoters, in which case point mutations in the promoter region could simultaneously increase or decrease expression of both functions. However, I have not yet tested this hypothesis.

Because of their short generation times, large population sizes, and general ease of culture, bacterial populations provide powerful experimental systems to test evolutionary hypotheses. However, some individuals might worry that bacteria behave too much like computer simulations and are not full of complications and surprises like "real" organisms. My results show otherwise. For reasons that are not obvious, my study failed to support the predicted effect of susceptible host density on the evolution of virulence, despite a clear demonstration of an underlying genetic tradeoff between horizontal and vertical modes of transmission. And while the observed tradeoff conformed to theoretical expectations, it is not at all clear how or why this tradeoff should involve the gene for tetracycline resistance, and yet it does. Evidently, despite their seeming simplicity, plasmid-bacterium interactions are sometimes unexpectedly complex.

CHAPTER IV

UNEXPECTED EFFECT OF HOST DENSITY ON CONJUGATION RATE AND INVASION OF PLASMID pB15

INTRODUCTION

In the absence of selection on the host bacterium for specific plasmid-encoded characters such as antibiotic resistance, most plasmids reduce the fitness of their hosts relative to isogenic plasmid-free counterparts and so can be regarded as parasites (Levin 1980; Dykhuizen and Hartl 1983; Lenski and Bouma 1987; Lenski and Nguyen 1988; Nguyen et al. 1989). However, a conjugative plasmid can invade a plasmid-free population of bacteria (and be stably maintained) if the rate of horizontal transfer by conjugation is sufficiently high to offset losses due to harmful effects on host fitness (Stewart and Levin 1977). The opportunity for a conjugative plasmid to invade a population of susceptible hosts is expected to increase in proportion to the density of the hosts (Levin et al. 1979; Simonsen et al. 1990). This expectation is based on the assumption that the kinetics of plasmid transfer behave according to mass-action; i.e., the rate at which newly infected hosts are formed depends on the product of plasmid-bearing and plasmid-free host densities (cell ml^{-1}) and on the rate constant of conjugation, γ (ml $cell^{-1}$ hr^{-1}).

Plasmid pB15 is a naturally occurring plasmid, and it has been previously reported to persist by horizontal transmission in chemostat culture (Lundquist and Levin 1986). In chapter III of this dissertation, pB15 was used to test the hypothesis that higher densities of susceptible hosts would favor plasmids with higher conjugation rates and greater virulence (i.e., more deleterious effects on host fitness). The evolved plasmid variants fulfilled the assumption of a genetic correlation between conjugation rate and virulence.

However, I found no evidence for the predicted effect of susceptible-host-density treatments on the direction of plasmid evolution. One possible explanation for this failure to confirm the evolutionary prediction, despite fulfilling the key genetic assumption, is that plasmid transfer may not have obeyed mass-action kinetics. Therefore, in this chapter, I examine the effect of host density on the conjugation rate of pB15 and on its ability to invade bacterial populations growing in batch culture.

MATERIALS AND METHODS

The *Escherichia coli* B host strain used in this study had evolved for 2,000 generations in a glucose-limited environment (Lenski et al. 1991). Plasmid pB15 is a large (~80 kb), low-copy-number plasmid that confers resistance to kanamycin and tetracycline (Kan^r Tet^r). Bacterial populations were cultured by serial transfer in Davis minimal (DM) broth (Carlton and Brown 1981) supplemented with $2x10^{-6}$ ug ml^{-1} thiamine hydrochloride and glucose at a specified concentration. For example, DM25 indicates DM with 25 ug glucose ml^{-1}, which yields ~$5x10^7$ cells ml^{-1} at stationary phase. Culture volume was 10 ml, maintained in 50-ml Erlenmeyer flasks placed in a non-shaking incubator at 37 ^oC.

To assay conjugation rate (γ), donor and recipient strains were mixed at a 1:100 ratio, then diluted 1:100 into fresh medium and allowed to grow and mate during a standard 24-hour growth cycle in a non-shaking incubator at 37 ^oC. After 24 h, the final densities of donors (D), recipients (R), and transconjugants (T) were determined by the number of colonies formed on selective and nonselective plates. I also estimated the growth rate (h^{-1}) in exponential phase (ψ) of mating cultures by regressing ln [total cell density] versus time during the period of exponential-phase growth. The rate of conjugal

plasmid transfer (ml cell^{-1} h^{-1}) for matings in batch culture may be estimated using the formula of Simonsen et al. (1990):

$$\gamma = \psi \ln[1 + (T/R)(N/D)]/(N - N_0),$$

where $N = T + R + D$ and N_0 is initial population size.

RESULTS

I first performed a series of experiments to examine the influence of susceptible host density on the ability of plasmid pB15 to invade a population of plasmid-free hosts. Plasmid-bearing (donor) and plasmid-free (recipient) cells were mixed at a ~1:500 ratio, in each of seven glucose concentrations: DM12.5, DM25, DM50, DM100, DM200, DM400, and DM800. Donors and recipients in this study differ in their ability to utilize the sugar L-arabinose as a nutrient; thus the Ara$^+$ donors and Ara$^-$ recipients form white and red colonies, respectively, on tetrazolium-arabinose (TA) indicator plates (Levin et al. 1977). Populations were propagated by serial transfer for ten days. Samples from experimental populations were plated daily on TA and TA containing 25 ug kanamycin ml^{-1} to determine total cell density and density of plasmid-bearing cells, respectively. The density of plasmid-free cells was determined by subtraction. [At the end of the experiment, population samples were also tested on agar containing 1 ug tetracycline ml^{-1}. No dissociation between the two antibiotic resistance markers was observed.] The expectation was that the rate of increase for pB15-bearing cells would increase in direct proportion to the density of plasmid-free hosts (manipulated by changing the glucose concentration). As expected, the population of plasmid-bearing donors and transconjugants declined at the lowest density of susceptible hosts, ~3.05 x 10^7 cell ml^{-1}

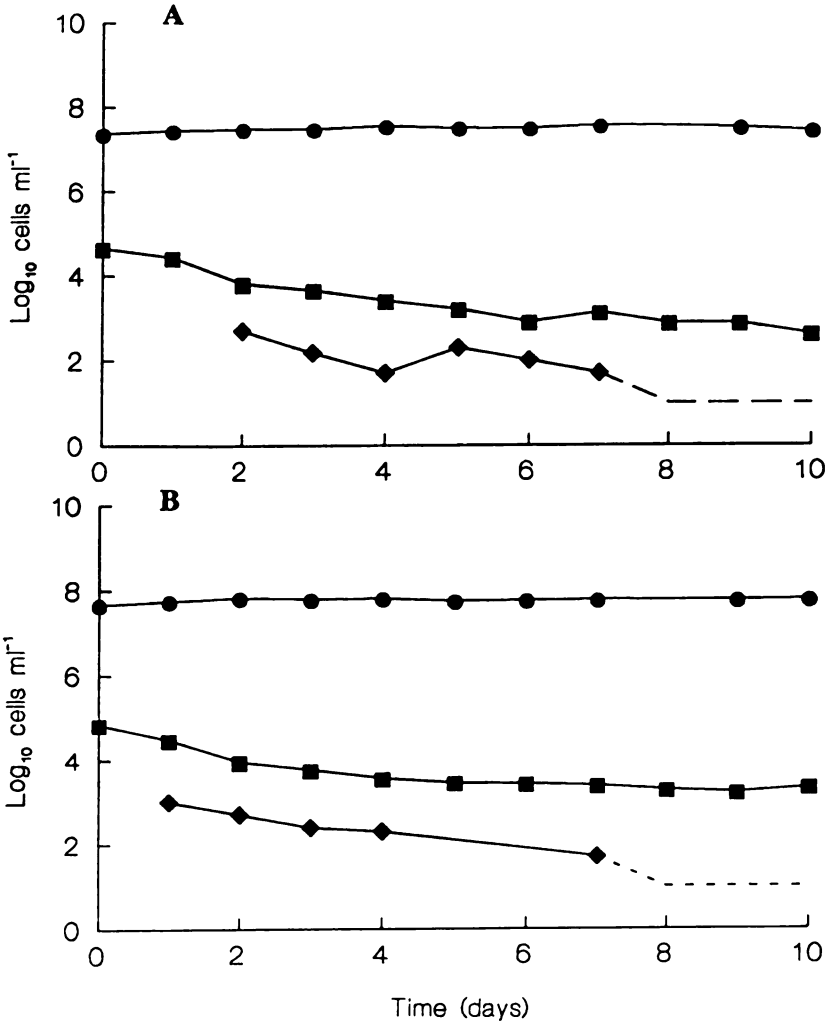
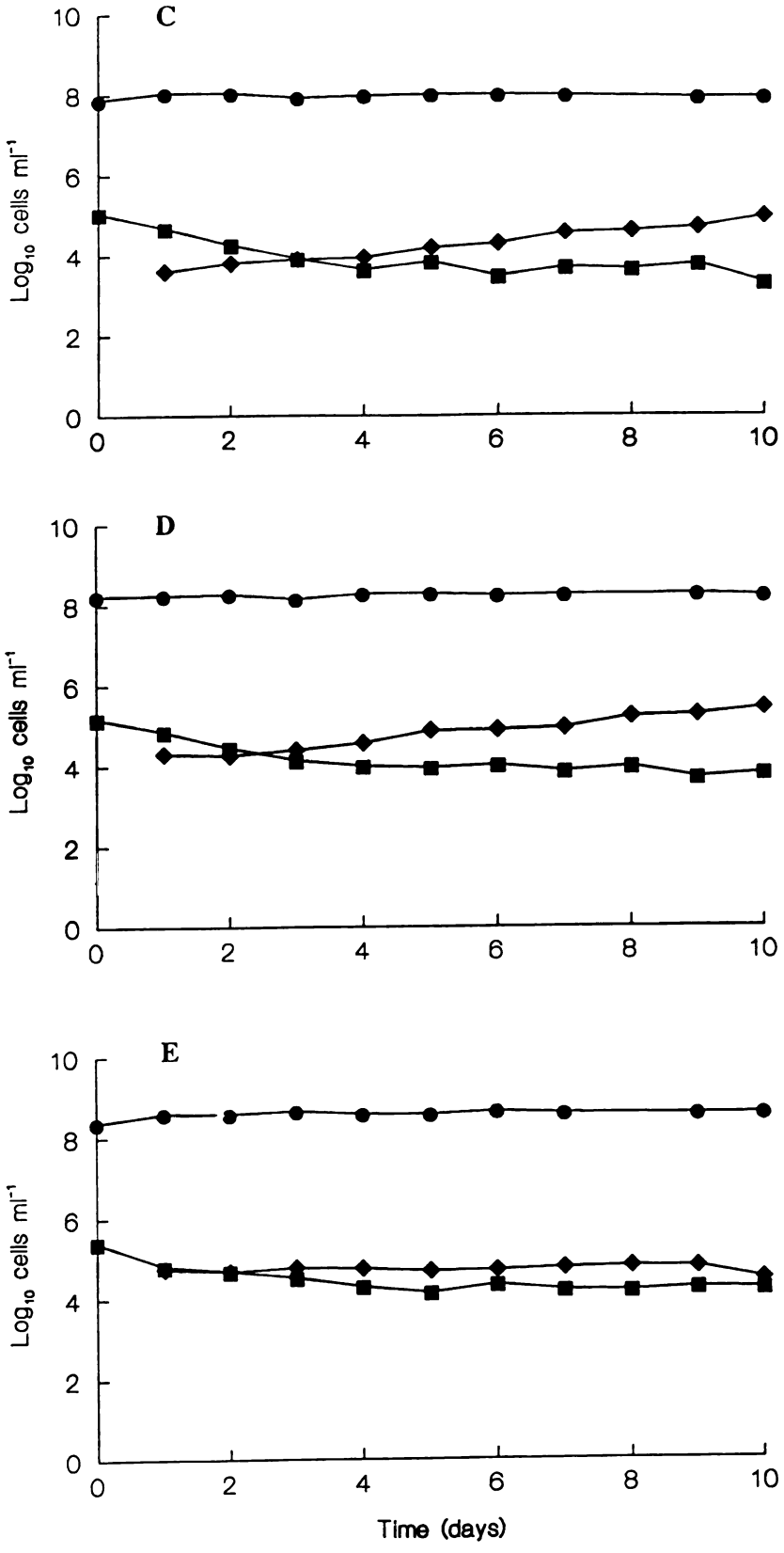


Figure 23. Densities of donors (squares), recipients (circles), and transconjugants (diamonds) of plasmid pB15 during serial transfer at seven different concentrations of glucose: 12.5 (A), 25 (B), 50 (C), 100 (D), 200 (E), 400 (F), and 800 (G) ug ml^{-1}. The plasmid-bearing cell population could increase when rare only at intermediate cell densities (panels C and D). Dashed lines indicate that a cell population fell below the limit of detection.



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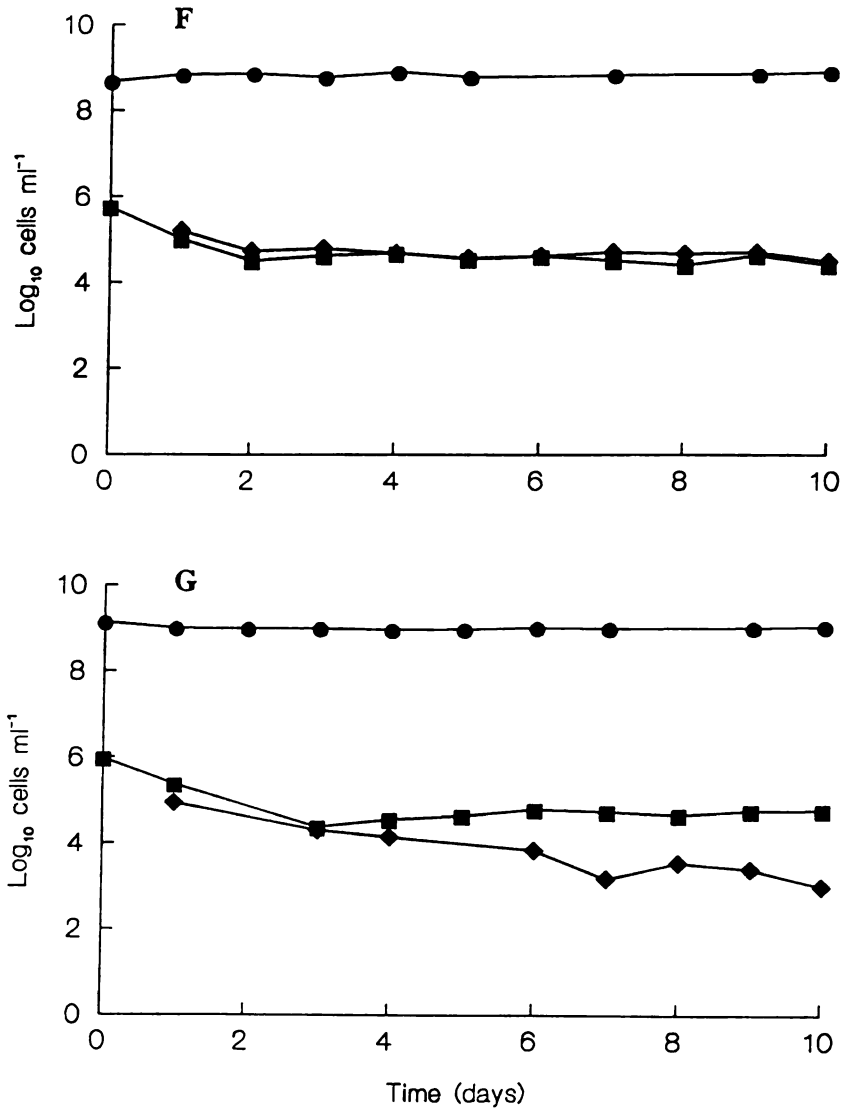

Figure 23 (cont'd).

(Figure 23, panel A). Also as expected, the population of plasmid-bearing cells declined more slowly, and even began to increase, as the density of plasmid-free cells was raised to 5.66×10^7, 9.31×10^7, and 1.74×10^8 by raising the glucose concentration (Figure 23, panels B-D). But unexpectedly, at still higher densities of plasmid-free cells -- 3.71×10^8,

Table 17. Estimates of $\log_{10}$ conjugation rate (γ) for pB15 at three different glucose concentrations, and corresponding cell densities.

Glucose concentration	Final cell density*	Mean estimate of $\log_{10} \gamma$	95% Confidence Interval
5 ug ml^{-1}	1.65×10^7	-9.972	$\pm$ 0.083
50 ug ml^{-1}	8.81×10^7	-10.342	$\pm$ 0.206
500 ug ml^{-1}	8.76×10^8	-11.227	$\pm$ 0.112

*Total density of donors, recipients, and transconjugants at the end of the 24-hour growth cycle, averaged over the six replicates.

6.94×10^8, and 1.06×10^9 -- the plasmid no longer could invade and become established (Figure 23, panels E-G). These data suggest that the rate of horizontal transfer actually declined at higher densities of susceptible hosts, contrary to the mass-action expectation.

To further explore this unexpected effect of host density on horizontal transfer, I estimated the rate constant of conjugative transfer (γ) for pB15 at three concentrations of glucose: DM5, DM50, and DM500. Plasmid-bearing and plasmid-free cells were mixed at

a ~1:100 ratio, in each concentration of resource. The densities of donors, recipients, and transconjugants were determined as above, and γ was then estimated using the formula of Simonsen et al. (1990). Six blocks of assays were performed. As shown in Table 17, γ does in fact decline with increasing susceptible host density (achieved by manipulating glucose concentration). An ANOVA confirmed that the effect of glucose concentration

Table 18. ANOVA of the effect of glucose concentration on $\log_{10}$ conjugation rate (γ) of plasmid pB15.

Source	SS	df	MS	*F*	*P*
Glucose concentration	4.991	2	2.495	103.674	<0.001
Block	0.243	5	0.049	2.021	0.161
Error	0.241	10	0.024		

on $\log_{10} \gamma$ is highly significant (Table 18). These data further indicate that horizontal transfer of pB15 does not behave according to simple mass-action kinetics.

Figure 24 shows the dynamics for the donor, recipient, and transconjugant populations in one representative block of the conjugation rate experiment (Table 17). Interestingly, the number of transconjugants increased substantially between 8 and 10 h of the growth cycle, under all three density treatments. By this time in the cycle, the donors and recipients had evidently made the transition from exponential growth to stationary phase as the medium was depleted of glucose. These observations suggest that significant

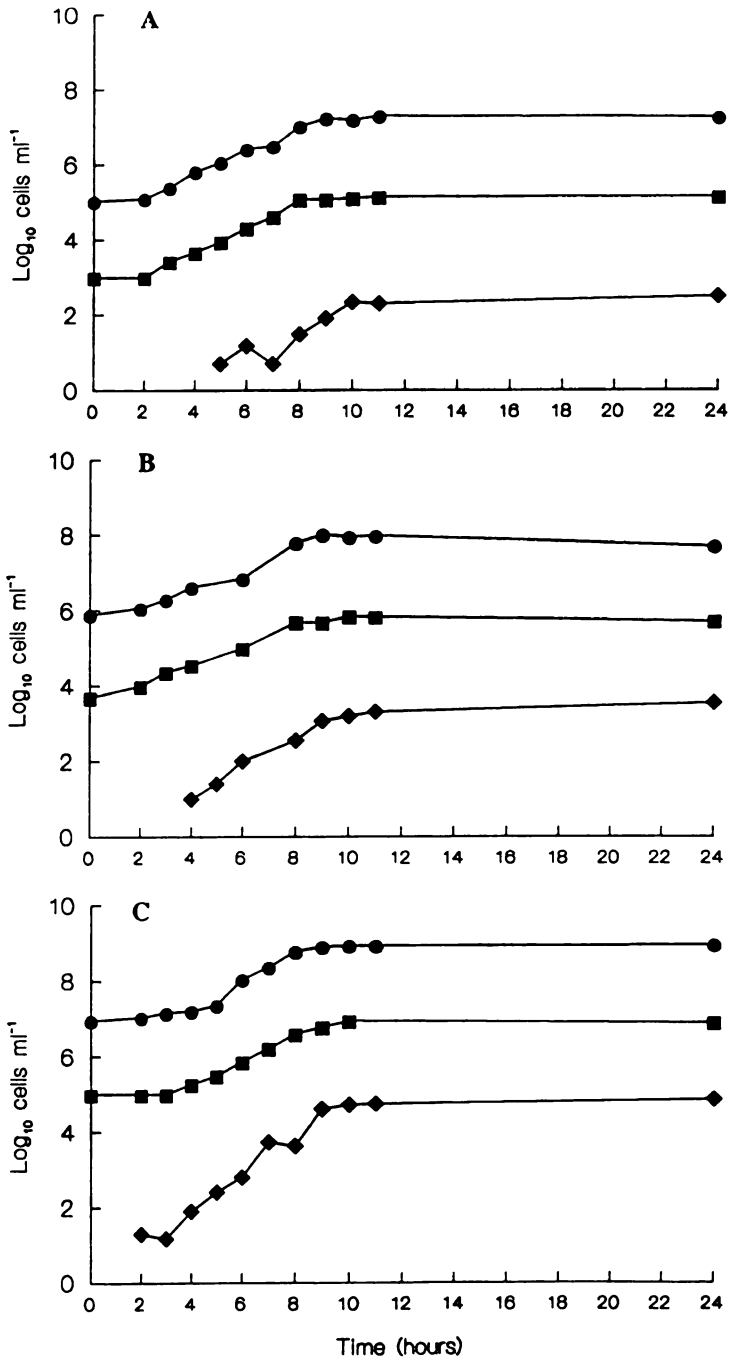

Figure 24. Dynamics of one-day mating experiments with pB15 and *E. coli* B at three glucose concentrations: 5 (A), 50 (B), and 500 (C) ug ml^{-1}. Circles: plasmid-free recipients. Squares: donors harboring plasmid pB15. Diamonds: transconjugants.

plasmid transfer may occur during the transition to stationary phase. By contrast, most models of plasmid population dynamics (Stewart and Levin 1977; Simonsen et al. 1990) assume that the conjugation rate is proportional to growth rate.

DISCUSSION

In Chapter III, I used a simple evolutionary model to predict that plasmids should evolve higher conjugation rates, and become more deleterious to their hosts, when susceptible hosts are common. However, an experiment in which the density of susceptible hosts was manipulated for plasmid pB15 did not support this prediction. A possible explanation for the model's failure to predict the evolutionary response of pB15 to host density is that its conjugal transfer (horizontal transmission) does not obey simple mass-action kinetics.

In this chapter, I performed two experiments to test whether pB15 transmission conforms to mass-action kinetics. Both experiments indicated significant deviations from mass-action. In the first experiment, I observed that the rate of increase in the number of pB15-infected hosts (when pB15 was introduced at a low frequency) was greater at intermediate hosts densities than at either low or high densities (Figure 23). In the second experiment, I estimated the conjugation rate, γ, using the endpoint method of Simonsen et al. (1990). Using plasmid R1, Simonsen et al. found that γ was independent of host density, as expected for simple mass-action kinetics. But for pB15, γ declined by about an order of magnitude as host density was increased by an equivalent amount (Table 17, rows 2 and 3). Thus, for pB15, the product of γ and susceptible host density (which gives the per capita rate of conjugative transmission) was approximately constant over this range, rather than increasing in direct proportion to host density as was expected.

At this juncture, it seems clear that pB15's conjugation rate declines substantially at higher host densities. However, I cannot explain mechanistically why this should be so. Perhaps the simplest explanation is that the conjugation process somehow becomes "saturated" at higher host cell densities, much as bacterial growth reaches a maximum rate that cannot be raised by increasing the concentration of a limiting resource (Monod 1949). However, saturation kinetics cannot explain the apparent maximum rate of plasmid increase at intermediate host densities, as seen in Figure 23. Another possibility is that conjugation rate is not a function of host density *per se* but instead responds to the concentration of glucose, which was varied in order to manipulate host density. That is, higher concentrations of glucose may somehow inhibit the conjugal transfer of pB15. The observation that the number of transconjugants increases unexpectedly during the transition from exponential growth to stationary phase (Figure 24), irrespective of cell density, may be consistent with this explanation, because it suggests that pB15 conjugation is somehow stimulated by the depletion of glucose.

Whatever the precise explanation for these effects, they may explain the failure of the simple mass-action model to predict the evolutionary response of pB15 to experimental manipulations of the density of susceptible, plasmid-free hosts. Although the *genetic* assumption of a tradeoff between rates of horizontal and vertical transmission was fulfilled (Chapter III), the *ecological* assumption that the rate of horizontal transmission is simply proportional to susceptible host density was evidently not satisfied (this Chapter). More generally, models of phenotypic evolution depend on both genetic and ecological assumptions, and the predictions of these models may fail as a consequence of violating either type of assumption.

APPENDIX

APPENDIX

Table 19. Genotypes seen among ten isolates from each recombination treatment population at generation 1,000.

Genotype		Phenotypic[1]						Electrophoretic[1]					Genotype frequency
		Ara	Lac	Tet	Str	T1X	T6	IDH	6-PGD	ADH	MPI	PEP	
Recombinant population[2]													
Ara^{-}1	Rec 1	-	+	s	s	r	s	12	2	2	2	1	0.4
	Rec 2	-	+	s	s	r	s	12	2	2	2	2	0.1
	Rec 3	-	-	s	s	r	s	2	2	2	2	1	0.1
	Rec 4	-	-	s	s	r	s	2	2	1	2	1	0.1
	Rec 5	-	-	s	s	r	s	1	2	2	2	1	0.3
Ara^{-}2	Baseline	-	+	s	r	s	r	1	2	2	2	1	0.2
	Rec 1	-	+	s	r	s	r	12	2	2	2	1	0.2
	Rec 2	-	+	s	r	s	r	1	2	2	2	12	0.3
	Rec 3	-	+	s	r	s	r	12	2	1	2	1	0.1
	Rec 4	+	+	s	r	s	r	1	2	1	2	2	0.1
	Rec 5	-	+	s	r	s	r	2	2	2	2	2	0.1
Ara^{-}3	Rec 1	+	-	r	s	r	s	1	2	2	2	1	0.1
	Rec 2	+	-	r	s	r	s	2	2	12	2	2	0.3

Table 19 (cont'd).

	Rec 3	+	-	r	s	r	s	2	2	2	2	1	0.3
	Rec 4	-	+	r	r	s	r	2	2	1	2	2	0.1
	Rec 5	+	+	r	s	s	r	2	2	2	2	1	0.1
	Rec 6	+	+	r	s	s	r	1	2	1	2	2	0.1
Ara$^-$4	Baseline	-	+	s	r	s	r	1	2	2	2	1	0.8
	Rec 1	-	+	s	r	s	r	12	2	2	2	1	0.2
Ara$^-$5	Rec 1	-	+	s	s	s	r	12	2	2	2	2	0.3
	Rec 2	+	+	s	s	s	s	12	2	1	2	1	0.1
	Rec 3	+	+	s	s	s	s	1	2	2	2	1	0.1
	Rec 4	+	+	s	s	s	s	2	2	2	2	12	0.1
	Rec 5	+	+	s	s	s	s	12	2	2	2	1	0.4
Ara$^-$6	Rec 1	-	-	s	r	r	s	12	2	2	2	1	0.3
	Rec 2	-	+	s	s	r	r	12	2	2	2	1	0.2
	Rec 3	-	+	s	r	s	r	12	2	2	2	1	0.1
	Rec 4	-	+	s	r	s	r	12	2	12	2	1	0.2
	Rec 5	-	+	s	r	s	r	2	2	2	2	1	0.1
	Rec 6	-	+	s	r	s	r	2	2	1	2	2	0.1
Ara$^+$1	Rec 1	-	-	s	r	s	r	1	2	2	2	2	0.1
	Rec 2	+	+	s	r	s	r	2	2	2	2	2	0.5
	Rec 3	+	+	s	r	s	r	12	2	1	2	12	0.1
	Rec 4	+	+	s	r	s	r	12	2	2	2	12	0.2
	Rec 5	+	+	s	r	s	r	2	2	1	2	1	0.1

Table 19 (cont'd).

Ara^+2	Baseline	+	+	s	r	s	r	1	2	2	2	1	0.1
	Rec 1	+	-	s	r	s	r	12	2	2	2	2	0.1
	Rec 2	+	+	s	r	s	r	1	2	1	2	1	0.1
	Rec 3	+	+	s	r	s	r	2	2	1	2	12	0.1
	Rec 4	+	+	s	r	s	r	2	2	2	2	1	0.2
	Rec 5	+	+	s	r	s	r	2	2	12	2	12	0.2
	Rec 6	+	+	s	r	s	r	1	2	12	2	2	0.1
	Rec 7	+	+	s	r	s	r	2	2	2	2	2	0.1
Ara^+3	Baseline	+	+	s	r	s	r	1	2	2	2	1	0.1
	Rec 1	+	+	s	r	s	r	12	2	1	2	12	0.1
	Rec 2	+	+	s	r	s	r	2	2	2	2	1	0.1
	Rec 3	+	+	s	r	s	r	12	2	12	2	12	0.1
	Rec 4	+	-	s	r	s	r	1	2	12	2	12	0.1
	Rec 5	+	-	s	r	s	r	1	2	2	2	1	0.1
	Rec 6	+	-	s	r	r	r	1	2	2	2	1	0.1
	Rec 7	+	+	s	r	s	r	12	2	1	2	1	0.1
	Rec 8	+	+	s	r	s	r	12	2	2	2	1	0.2
Ara^+4	Baseline	+	+	s	r	s	r	1	2	2	2	1	0.4
	Rec 1	+	-	s	r	r	r	2	2	2	2	2	0.1
	Rec 2	+	-	s	r	r	r	1	2	2	2	2	0.1
	Rec 3	+	+	s	r	s	r	12	2	2	2	2	0.1
	Rec 4	+	+	s	r	s	r	1	2	1	2	12	0.1
	Rec 5	+	+	s	r	s	s	1	2	2	2	2	0.1
	Rec 6	+	+	s	r	s	s	1	2	12	2	12	0.1

Table 19 (cont'd).

Ara^+5	Rec 1	+	-	s	r	r	r	12	2	2	2	2	0.1
	Rec 2	+	-	s	r	r	r	12	2	12	2	2	0.1
	Rec 3	+	+	s	r	s	r	2	2	2	2	12	0.4
	Rec 4	+	+	s	r	s	r	1	2	1	2	1	0.1
	Rec 5	+	+	s	s	s	r	1	2	2	2	2	0.1
	Rec 6	+	+	s	s	s	r	2	2	2	2	12	0.1
	Rec 7	+	+	s	r	s	r	12	2	2	2	1	0.1
Ara^+6	Baseline	+	+	s	r	s	r	1	2	2	2	1	0.2
	Rec 1	+	-	s	r	s	r	2	2	12	2	12	0.1
	Rec 2	+	-	s	r	s	r	12	2	2	2	1	0.1
	Rec 3	+	+	s	r	s	r	12	2	12	2	1	0.2
	Rec 4	+	+	s	r	s	r	12	2	1	2	12	0.2
	Rec 5	+	+	s	r	s	r	2	2	2	2	1	0.1
	Rec 6	+	+	s	r	s	r	2	2	12	2	2	0.1

[1] See Table 1 for a description of phenotypic and electrophoretic markers. All genotypes were also Arg^+, Leu^+, Ilv^+.
[2] Ara^-1 through Ara^-6, and Ara^+1 through Ara^+6 denote treatment populations originally composed of Ara^- and Ara^+ recipients, respectively.

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