Tests of Ecological Mechanisms Promoting the Stable Coexistence of Two Bacterial Genotypes

Paul E. Turner, Valeria Souza, Richard E. Lenski

Ecology, Volume 77, Issue 7 (Oct., 1996), 2119-2129.

Stable URL:
http://links.jstor.org/sici?sici=0012-9658%28199610%2977%3A7%3C2119%3ATOEMPT%3E2.0.CO%3B2-1

Ecology, 77(7), 1996, pp. 2119–2129

TESTS OF ECOLOGICAL MECHANISMS PROMOTING THE STABLE COEXISTENCE OF TWO BACTERIAL GENOTYPES[1]

PAUL E. TURNER[2]
Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824-1325 USA

VALERIA SOUZA
Centro de Ecología, Universidad Nacional Autonóma de México, Coyoacán, Ciudad de México, 04510, México

RICHARD E. LENSKI
Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824-1325 USA

Abstract. A series of competition experiments with two genotypes of *Escherichia coli* showed that each genotype was favored when it was the minority, allowing their coexistence in a stable polymorphism. In these experiments, glucose was the sole source of carbon provided, and its concentration was limiting to population density. Thus, the stable polymorphism did not conform to a simple model of competitive exclusion. In similar experiments also with glucose as the sole resource, we considered two hypotheses that might explain the observed coexistence: (1) a strictly demographic trade-off, 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 that acts as a second resource for which the other genotype is the better competitor. Although there was a demographic trade-off, the advantage to the superior competitor at high glucose concentrations was too large (given the initial concentration of glucose used in these experiments) to allow the second genotype to invade when rare at the observed rate. Therefore, the second genotype must have had some other advantage that allowed it to readily invade a population of the superior competitor for glucose. Indeed, the second genotype could increase in abundance after glucose was depleted, but only in the presence of the superior competitor for glucose, thus implicating a cross-feeding interaction. These results confirmed earlier studies showing that populations of *E. coli* can maintain ecologically relevant genetic diversity even in a simple environment.

Key words: bacteria; coexistence; competition; cross-feeding; demographic trade-off; Escherichia coli; *frequency-dependent selection; polymorphism.*

INTRODUCTION

Various forms of frequency-dependent selection have long been hypothesized to maintain genetic polymorphisms in natural populations (Cain and Sheppard 1954, Fisher 1958, Haldane and Jayakar 1963, Ayala and Campbell 1974, Levin 1988, Hori 1993). Because of the long generation times of most organisms, however, researchers must often assume (rather than prove) that a stable polymorphism exists. Also, the complexity of natural environments often makes it difficult to identify the ecological mechanisms responsible for frequency-dependent selection. (It should be noted that density-dependent processes underlie most mechanisms of frequency-dependent selection. We emphasize frequency-dependence because the simplest criterion for stable coexistence of two genotypes is the ability of each type to invade, when rare, an established population of the other type.)

Laboratory experiments with *Drosophila* provide conclusive evidence that the fitness of a genotype may depend on its relative abundance, or frequency (e.g., Wright and Dobzhansky 1946, Kojima 1971, Van Delden et al. 1978). Given control over selective factors in the environment as well as short generation times, laboratory studies with model organisms would seem to allow precise determination of the underlying causes of stable genetic polymorphisms. However, this is not necessarily the case: frequency-dependent selection involving the ADH (alcohol dehydrogenase) locus in laboratory populations of *D. melanogaster* has been clearly demonstrated, but never fully explained (Van Delden 1982).

Because of their short generation times, 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 asexual and laboratory environments typically provide only a single limiting re-

[1] Manuscript received 28 June 1995; revised 1 December 1995; accepted 2 January 1996; final version received 29 January 1996.

[2] Present address: Department of Zoology, University of Maryland, College Park, Maryland 20742 USA.

source. For these reasons, it is often assumed that experimental populations of bacteria are subject to takeover by a single genotype that harbors a mutation conferring some selective advantage (Atwood et al. 1951, Dykhuizen 1990). Thus, polymorphisms in experimental populations of bacteria are often assumed to exist only transiently, while an advantageous mutant is replacing its ancestor. This assumption is in accord with the competitive-exclusion principle, which states that two competitors cannot stably coexist on a single limiting resource (Gause 1934, Hardin 1960).

In apparent violation of this simple model leading to competitive exclusion, the evolution of stable polymorphisms in laboratory populations of bacteria has been clearly demonstrated. For example, three genotypes of *Escherichia coli* were observed to coexist in glucose-limited chemostat culture (Helling et al. 1987). It was later shown that these three genotypes had evolved a complex system of cross-feeding interactions that depended on differential patterns of excretion and utilization of two alternative growth substrates, acetate and glycerol, in addition to glucose (Rosenzweig et al. 1994). Other studies have demonstrated stable coexistence of bacterial genotypes mediated by viruses (Chao et al. 1977) and by detoxification of antibiotics (Lenski and Hattingh 1986).

In theory, stable coexistence of two bacterial genotypes is also possible under a serial-transfer regime in an environment that contains only a single limiting resource (Stewart and Levin 1973). Serial culture is analogous to a seasonal environment, in that resources are abundant at the beginning of a transfer cycle but become scarce as the population approaches its saturation density. Coexistence requires a demographic trade-off, such that one genotype has a growth-rate advantage when the limiting resource is abundant whereas the other genotype has an advantage when the resource is sparse. Levin (1972) postulated that such a trade-off was responsible for the stable coexistence that he observed between strains of *E. coli* B and K12 in serial culture. Although Levin did not rule out the possibility that cross-feeding was also involved, a strictly demographic trade-off is a theoretically tenable mechanism for stable coexistence in a fluctuating environment (Stewart and Levin 1973, Tilman 1982).

In a study to examine the effect of recombination on the dynamics of bacterial evolution (Turner 1995), we sometimes observed two or more distinct genotypes co-occurring in populations of *E. coli.* Choosing one such pair of genotypes for further study, we observed that they could coexist indefinitely in an environment in which glucose was provided as the limiting nutrient (nitrogen, phosphorus, etc., being provided in excess). Because this environment was free of viruses and antibiotics, we propose that either a cross-feeding interaction or a demographic trade-off in growth rates may have allowed stable coexistence. Of course, these two hypotheses are not mutually exclusive, so that coexistence might be explained by the two mechanisms jointly.

Cross-feeding.—According to this hypothesis, one genotype is competitively superior for the limiting resource that is exogenously supplied, but it excretes some metabolite into the medium that disproportionately enhances the growth of a second genotype. Assuming that the amount of metabolite produced is proportional to the density of the primary competitor, then a cross-feeder benefits from being in the minority. Similarly, the primary competitor benefits when the population contains a high proportion of cross-feeders due to its intrinsic competitive superiority for the resource that is exogenously supplied. Therefore, the relative fitnesses of both genotypes will be decreasing functions of their own frequencies, so that each genotype has a higher relative fitness when it is in the minority. Hence, competition is fiercest among individuals of the same genotype and a stable polymorphism may arise from the inability of either genotype to displace the other.

Demographic trade-off.—According to this hypothesis, one genotype is competitively superior when the sole limiting resource is at high concentration, whereas the other genotype is superior in competition when that resource is scarce. In the simple case of the Monod model (1949), each genotype'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 resource conversion efficiency. A necessary (but not sufficient) condition for coexistence is that one genotype has a higher maximum growth rate, V_{max}, while the other genotype has a higher ratio V_{max}/K_s Each genotype will then have an advantage at a different stage of the serial transfer cycle and the two genotypes may stably coexist, each having a net advantage when it is rare (Fig. 1).

We now present a series of experiments with two recombinant genotypes of *E. coli* that seek to establish the existence of a stable equilibrium and then to determine the contributions of a demographic trade-off and a cross-feeding interaction to their dynamics.

Materials and Methods

Bacterial strains.—Turner (1995) describes the experiment from which the two genotypes used in this study were obtained. Briefly, 12 treatment populations were founded from independently derived clones of *Escherichia coli* B that had been serially propagated in the selective environment for 7000 generations (Lenski and Travisano 1994). These populations were serially propagated for another 1000 generations (150 d), during which time they were allowed to undergo recombination with donor strains every fifth day. The donors were four Hfr (high frequency of recombination) strains of *E. coli* K12, which are genetically dis-

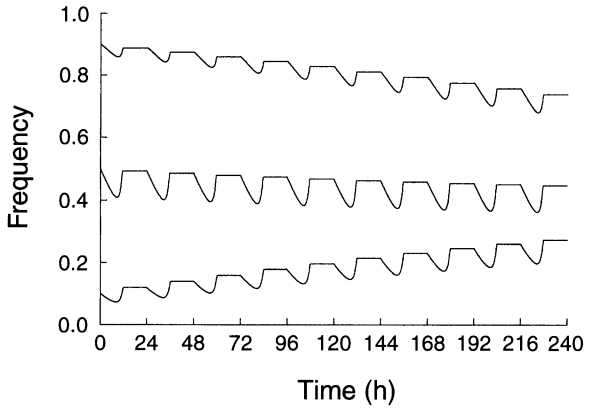

FIG. 1. Numerical simulations showing stable coexistence of two bacterial genotypes on a single resource in a seasonal environment, mediated by a demographic trade-off. One genotype (*a*) has $V_{max} = 0.4\ h^{-1}$ and $K_s = 0.1\ \mu g/mL$, whereas the other genotype (*b*) has $V_{max} = 0.59$ and $K_s = 7.5\ \mu g/mL$; resource conversion efficiency $c = 5 \times 10^{-7}\ \mu g$ for both. The vertical axis shows the frequency of genotype *a*. At the beginning of the simulation, and after every 24 h thereafter, the competing populations were diluted 1:100 into fresh medium that contained 25 μg/mL of the limiting resource. In each 24-h period, there were three phases during which (1) the resource concentration was sufficiently high that the genotype with the higher V_{max} (*b*) had an advantage, (2) the resource concentration had been reduced so much that the genotype with the lower K_s (*a*) had an advantage, and (3) the resource had been exhausted to the point that neither genotype could grow. The three curves differ only in the initial frequency of the competing genotypes; each genotype 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).

tinct from *E. coli* B (Table 1; see also Selander and Levin 1980). These donors were all auxotrophs, meaning that they were unable to synthesize at least one amino acid essential for growth in the environment. Thus, the Hfr strains were able to donate genes via recombination but were unable to survive and out-compete the prototrophic *E. coli* B recipients (which could synthesize all their own amino acids).

For the purpose of this study, we isolated two recombinant genotypes (REL4397 and REL4398) at generation 1000 from one of the treatment populations (Ara-3). Using nine physiological and five electrophoretic markers, it was evident that both recombinant genotypes contained a mixture of the markers present in *E. coli* B and K12 (Table 1). The two genotypes also differed from one another in several respects, including their ability to utilize the sugar lactose. Thus, REL4397 (Lac^+) and REL4398 (Lac^-) formed white and red colonies, respectively, when they were spread on tetrazolium-lactose (TL) indicator plates (Levin et al. 1977). Both recombinant genotypes are prototrophic and so can grow without supplemental amino acids. From here on, we 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 thiamine hydrochloride (at 2×10^{-3} μg/mL) and glucose at a specified concentration. For example, DM25 indicates DM supplemented with glucose at 25 μg/mL, which yields $\approx 5 \times 10^7$ cells/mL 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

TABLE 1. Genetic markers for the two recombinant genotypes of *Escherichia coli* used in this study, as well as their parental donor and recipient strains.

Strain	Phenotypic†									Electrophoretic‡				
	Ara	Lac	Tet	Str	Arg	Leu	Ilv	T1X	T6	IDH	6-PGD	ADH	MPI	PEP
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
REL291	−	−	r	s	−	+	+	r	r	2	1	1	1	2
REL296	+	−	r	s	−	−	+	r	s	2	1	1	1	2
REL298	+	−	r	s	−	−	+	r	r	2	1	1	1	2

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

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

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. Prior to every assay, each genotype was grown separately for (at least) 1 d in the experimental medium as a preconditioning step to ensure that 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 1-d growth cycle. Initial and final densities of each competitor were estimated by spreading cells on TL plates, which distinguish the two genotypes by colony color.

The fitness of one genotype relative to the other was obtained, first, by calculating the realized Malthusian parameter for each competitor, and then by computing the ratio of their Malthusian parameters (Lenski et al. 1991). Let $N_i(0)$ and $N_i(1)$ be the densities of one genotype initially and after 1 d, respectively. Then the average rate of increase, or realized Malthusian parameter, for that genotype is $m_i = \ln[N_i(1)/N_i(0)]/(1\text{ d})$. The fitness of one genotype relative to another (W_{ij}) is estimated simply as the ratio of their Malthusian parameters during direct competition, or $W_{ij} = m_i/m_j$. A fitness differential between two genotypes may be caused by differences in the duration of lag phase, maximum growth rate, affinity for resource as it becomes limiting, survival at stationary phase, or any combination thereof (e.g., Vasi et al. 1994).

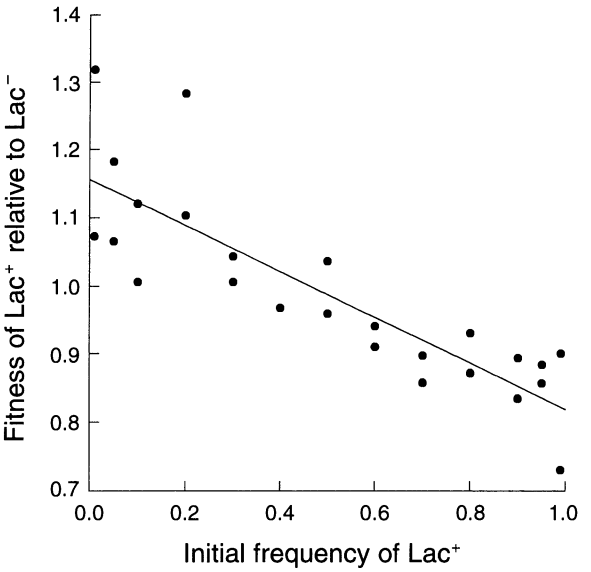

FIG. 2. The fitness of recombinant *Escherichia coli* genotype Lac$^+$, relative to genotype Lac$^-$, is a decreasing function of its own frequency. Relative fitness was calculated as the ratio of Malthusian parameters estimated during competition over 1-d growth cycles in DM25 (Davis Minimal medium containing glucose at 25 μg/mL), 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 1.

RESULTS

Demonstration of the stable equilibrium

Evidence for frequency dependence.—We first sought to establish that each recombinant genotype, Lac$^+$ and Lac$^-$, could increase in frequency when it was initially rare. To that end, we performed fitness assays in DM25 medium in which the two genotypes were mixed at thirteen different initial frequencies of Lac$^+$: 0.99, 0.95, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, and 0.01. Assays were replicated twice for each initial frequency.

Two aspects of the results (Fig. 2) are of particular interest. First, there was compelling evidence that the relative fitness of each genotype was a decreasing function of its own initial frequency (slope = -0.338, $t = -7.855$, df = 24, $P < 0.001$). Second, these data allowed us to predict the equilibrium frequency of Lac$^+$ and Lac$^-$ in DM25. By definition, equilibrium will occur when the two genotypes 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 0.45.

Evidence for stable equilibrium.—To further establish the existence of a stable polymorphic equilibrium, Lac$^+$ and Lac$^-$ were allowed to compete during daily serial transfers in DM25 for 200 generations (30 d). Each genotype was separately preconditioned and then they were mixed at five initial frequencies (0.9, 0.75, 0.5, 0.25, 0.1), with three-fold replication. After 16 d, we temporarily suspended this experiment by adding 3 mL glycerol to each culture flask and then storing the cultures at −80°C (to place cells in a state of suspended animation). We restarted the experiment 2 wk later by inoculating from each thawed freezer sample into Luria broth (Sambrook et al. 1989) for 1 d. Cultures were then diluted into DM25 for 1 d of re-acclimation before continuing the experiment proper for another 14 d in DM25. (Two replicates were terminated at days 19 and 27 due to contamination.) During the entire 30-d competition experiment, we spread samples taken from stationary-phase cultures (i.e., at the end of the 24-h growth cycle) onto TL plates to determine the densities of the Lac$^+$ and Lac$^-$ genotypes.

As shown in Fig. 3, 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. The perturbation resulted in a systematic advantage to the Lac$^+$ competitor, but this effect was transient and disappeared as the experiment continued. By day 30 of the competition experiment, the frequency of Lac$^+$ averaged 0.509 ± 0.012 (mean ± 1 SE), which

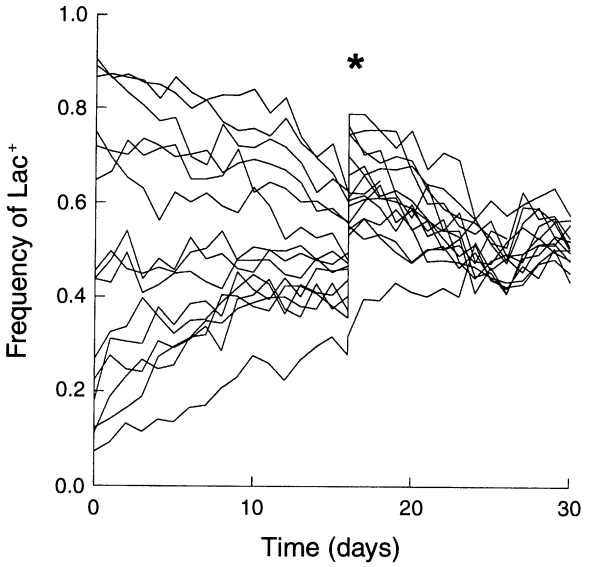

FIG. 3. Starting from different initial frequencies, *E. coli* genotypes Lac$^+$ and Lac$^-$ establish a stable polymorphism in DM25. After 30 d, observed frequencies agree rather well with the equilibrium predicted from 1-d competition experiments (Fig. 2). The asterisk (*) indicates a perturbation when the experiment was put in a freezer for temporary storage.

agreed well with the equilibrium predicted from the regression of relative fitness on initial frequency (Fig. 2). We concluded that frequency-dependent selection was strong enough to drive the genotypes toward a stable equilibrium ratio, at which point the two genotypes were of equal fitness.

Evaluation of the hypothesis of demographic trade-off

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

Lac$^+$ and Lac$^-$ gave estimates for V_{max} that averaged 0.9225 ± 0.0137 and 0.977 ± 0.0081 h^{-1}, respectively (means ± 1 SD). This difference in V_{max} was statistically significant (t = 7.651, 8 df, $P < 0.001$).

Estimation of affinity for limiting resource.—K_s is the concentration of resource at which a genotype 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 genotype's affinity for a limiting resource. To determine whether Lac$^+$ and Lac$^-$ differed in their affinity for glucose, we sought to estimate K_s for each genotype.

We 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 genotype were removed from the freezer and propagated in DM25 to acclimate them to growth on glucose. We then transferred inocula into DM media containing glucose concentrations of 0.01, 0.025, 0.1, 0.25, or 1 μg/mL; the number of cells added was adjusted to allow for 100-fold growth in cell numbers at the food concentration available.

As a control for growth on possible contaminating resources, we also inoculated cells into DM without any glucose. Cultures were propagated by 1:100 serial dilution for 7 d. Immediately prior to each dilution, samples were spread onto TL plates to estimate cell densities. We then regressed ln N against time to determine the lowest glucose concentration at which each genotype could sustain a constant population size.

We found that Lac$^+$ was able to replace itself in the face of serial dilution at glucose concentrations of ≥0.1 μg/mL. Let $V = (1/N)(dN/dt)$ be some submaximal rate of increase. The 100-fold daily increase (to offset dilution) implies that $V > \ln(100)/24 \text{ h} = 0.19 \text{ h}^{-1}$. From the Monod 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 $K_s < 0.39$ μg/mL. Similarly, the inability of Lac$^+$ to persist in DM0.025 implies that $K_s > 0.10$ μg/mL. In contrast, we found that Lac$^-$ was able to sustain itself in the face of 1:100 daily serial dilution only at glucose concentrations of ≥0.25 μg/mL. Using V_{max} for Lac$^-$ as estimated above, it follows similarly that K_s for that genotype lies between 1.04 and 0.41 μg/mL.

Our results were consistent with a demographic trade-off between V_{max} and K_s. Lac$^-$ had a higher maximum growth rate than Lac$^+$, whereas Lac$^+$ had the greater affinity for glucose and could evidently grow faster at very low glucose concentrations. However, the existence of such a trade-off is not sufficient to explain stable coexistence, which also depends on the input concentration of limiting resource and on the dilution factor (Stewart and Levin 1973).

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

The following points summarize the results of many numerical simulations. We first used the mean estimates for each genotype's V_{max} and considered the effects of different K_s values within the bounds of our uncertainty. We found that Lac$^-$ competitively excluded Lac$^+$ unless the difference in their K_s values was rather near the maximum allowed by the experimental uncertainty.

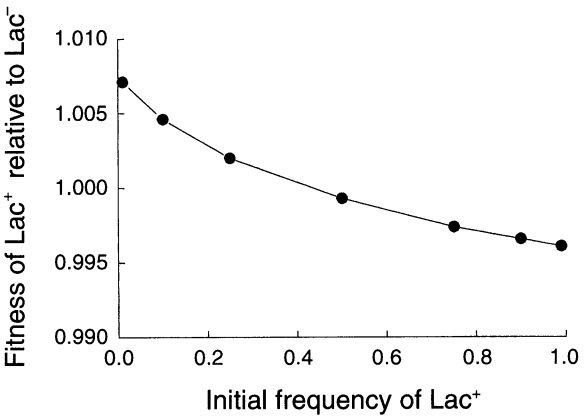

FIG. 4. Numerical simulations of the fitness of *E. coli* genotype Lac$^+$ relative to Lac$^-$, as a function of the initial frequency of Lac$^+$, assuming only a demographic trade-off between growth rates at high and low glucose concentrations. For Lac$^+$, V_{max} = 0.9225 h^{-1} and K_s = 0.12 μg/L. For Lac$^-$, V_{max} = 0.977 h^{-1} and K_s = 0.9 μg/L. For both genotypes, the glucose conversion efficiency $c = 5 \times 10^{-7}$ μg. Simulations assume the combined population is diluted 1:100 into medium containing glucose at 25 μg/mL and 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 trade-off that is consistent with the estimates for each genotype's V_{max} and K_s allows stable coexistence. However, the predicted frequency dependence was much weaker than was observed in experiments (see Fig. 2).

However, there was a small region of stable coexistence. For example, coexistence resulted if Lac$^+$ had V_{max} = 0.9225 h^{-1} and K_s = 0.12 μg/mL while Lac$^-$ had V_{max} = 0.977 h^{-1} and K_s = 0.9 μg/mL (Fig. 4). Next we considered the effect of uncertainty in our estimate of the difference in V_{max} between the two genotypes. Still, we could find at most a small region of stable coexistence in terms of K_s values. Even more importantly, in those cases where coexistence was observed, the strength of the resulting frequency-dependence was very weak, with fitness advantages for the rarer genotype on the order of only 1% (Fig. 4). This finding contrasts with the much stronger frequency-dependence observed in the actual experiments, where each genotype had a fitness advantage of >10% when it was rare (Fig. 2). Of course, it was possible to give a much larger advantage to Lac$^-$ when it was rare by using values well within the confidence limits for both genotypes' growth parameters. However, in those cases, Lac$^-$ competitively excluded Lac$^+$.

From these results, we conclude that a demographic trade-off between growth rates at high and low glucose concentrations contributes only slightly, at best, to the observed coexistence between the Lac$^-$ and Lac$^+$ genotypes. Therefore, we also sought to evaluate the possible importance of cross-feeding interactions involving the differential secretion and utilization of metabolic by-products.

TABLE 2. ANOVA of effects of initial frequency and glucose concentration on fitness of Lac$^+$ relative to Lac$^-$ genotypes of *Escherichia coli*.

Source of variation	df	MS	*F*	*P*
Initial frequency	4	0.058	11.223	<0.001
Glucose concentration	4	0.053	10.277	<0.001
Interaction	16	0.019	3.623	<0.001
Error	98†	0.005		

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

Based on our analysis thus far, Lac$^-$ is evidently the superior competitor for glucose except at very low concentrations, and such low (but non-zero) concentrations occur for too short a period during the growth cycle to provide Lac$^+$ with a sufficient offsetting advantage to explain its ability to readily invade an established population of Lac$^-$. Therefore, we would anticipate that any cross-feeding interaction must favor Lac$^+$ if it is to explain the stable coexistence.

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. That is, at low glucose concentrations, the population density of the metabolite-producing genotype would be reduced, with a concomitant reduction in the concentration of metabolite and hence a diminished opportunity for cross-feeding (see also Rosenzweig et al. 1994). To investigate this possibility, we examined the influence of glucose concentration on the frequency dependence of relative fitness. We performed fitness assays in which the two genotypes 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 genotypes were removed from the freezer into DM1000 and acclimated for 2 d in the competition medium; then they competed for 1 d. Each treatment combination was replicated five-fold, but two replicates were excluded due to contamination.

An ANOVA indicated that the interaction between glucose concentration and initial frequency had a highly significant effect on relative fitness (Table 2). As shown in Fig. 5A, the fitness of each genotype is a decreasing function of its own frequency when genotypes are competed at glucose concentrations of 25, 250, and 1000 μg/mL. At all three of these concentrations, the one-tailed regression of fitness on initial frequency is highly significant (DM25: slope = −0.300, t = −10.349, df = 23, $P < 0.001$; DM250: slope = −0.254, t = −5.097, df = 23, $P < 0.001$; DM1000: slope = −0.171, t = −3.992, df = 23, $P < 0.001$). But at the lower glucose concentrations of 1 and 2.5 μg/mL, the dependence of relative fitness on initial

frequency broke down, with Lac$^+$ having a small advantage over Lac$^-$ regardless of frequency (Fig. 5B). Contrary to the conditions required for stable coexistence, there was no significant negative regression of fitness on frequency (DM1: slope = -0.048, $t = -0.776$, df = 21, $P = 0.448$; DM2.5: slope = 0.124, $t = 2.219$, df = 23, $P = 0.982$). We also performed all pairwise comparisons among the slopes of relative fitness on initial frequency obtained at the different glucose concentrations ([5 × 4]/2 = 10 comparisons in all). We employed the sequential Bonferroni criterion (Rice 1989) to compute significance levels. The three higher concentrations (DM25, DM250, and DM1000) yielded a set of slopes that did not differ significantly from one another, nor were the slopes from the two lower concentrations (DM1 and 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 genotype was manifest at glucose concentrations of ≥25 μg/mL, but it broke down at much lower concentrations.

We also note that the observation that Lac$^+$ prevailed in competition at low glucose concentrations is consistent with the demographic trade-off documented above. Averaging over all initial frequencies, Lac$^+$ had a fitness of 1.060 (with 95% CL of 1.022, 1.098) relative to Lac$^-$ when the two genotypes competed in DM1. This relative fitness implies a difference in K_s values between the two genotypes that is considerably smaller than was used in numerical simulations to obtain stable coexistence based on a demographic trade-off (Fig. 4). For example, if one uses the point estimates of V_{max} for each genotype and K_s values of 0.5 and 0.37 μg/mL for Lac$^-$ and Lac$^+$, respectively, then numerical simulation yields the observed 6% advantage for Lac$^+$ in DM1, with nearly identical fitnesses over the entire range of initial frequencies. If one also simulates competition in DM25 using these same parameter values, then there is no stable coexistence but instead Lac$^-$ wins in competition with Lac$^+$ regardless of its initial frequency. Thus, the relatively small advantage of Lac$^+$ in DM1 adds further support to our earlier inference that a demographic trade-off, by itself, cannot explain the stable coexistence. Moreover, the finding that the conditions for stable coexistence broke down at low glucose concentrations is consistent with the cross-feeding hypothesis. However, we have not yet shown explicitly that Lac$^+$ does indeed benefit from a cross-feeding interaction in DM25.

Evidence for cross-feeding after glucose has been depleted.—At a glucose concentration of 25 μg/mL, and in the absence of frequency-dependent forces, Lac$^-$ had a pronounced advantage owing to its higher V_{max}. We therefore sought to determine at what phase of the population growth cycle Lac$^+$ made up for this deficiency. To that end, we performed fitness assays in

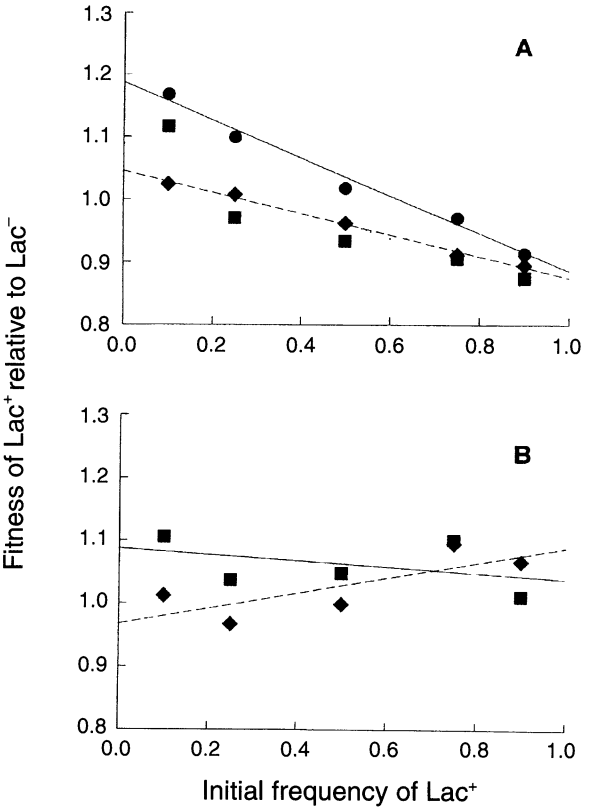

FIG. 5. Fitness of Lac$^+$, relative to Lac$^-$, as a function of its initial frequency, in DM media containing five different glucose concentrations. (A) In medium containing glucose at concentrations of 25 (●, ——), 250 (■, ·····), or 1000 (◆, – – –) μg/mL, each genotype had an advantage when rare such that there existed a stable polymorphism. (B) In medium containing glucose at concentrations of 1 (■, ——) or 2.5 (◆, – – –) μg/mL, there was no evidence that the rarer genotype had an advantage. Each point is the mean of five replicates. Lines indicate least-squares regressions.

which the two genotypes were mixed at three initial frequencies of Lac$^+$ (0.1, 0.5, and 0.9), with elevenfold replication, in DM25. We also had treatments comprising entirely Lac$^+$ and entirely Lac$^-$ cells, each with three-fold replication. Samples were spread onto TL plates at 0, 12, and 24 h to determine the densities of Lac$^+$ and Lac$^-$.

Fig. 6 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. As shown repeatedly, the relative fitnesses of these two genotypes are frequency dependent. However, Fig. 6 also shows that Lac$^+$ has a systematic advantage relative to Lac$^-$ between 12 and 24 h. An ANOVA shows that the effects of initial frequency and sampling interval are highly significant (Table 3).

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 (5×10^{-7} μg), glucose should have been thoroughly depleted from the culture

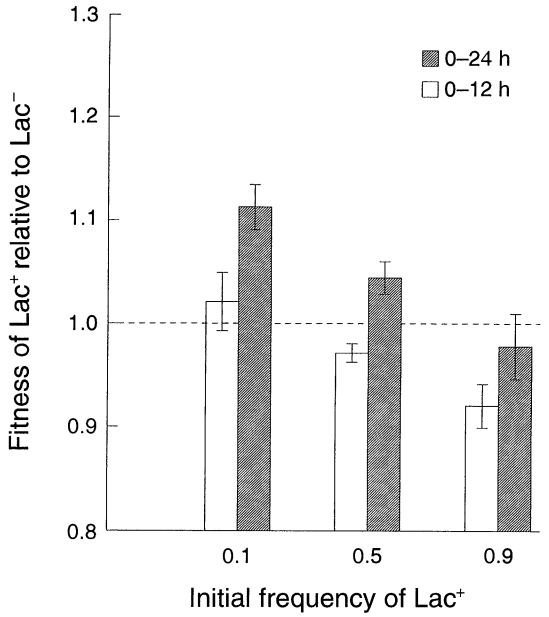

FIG. 6. Fitness of Lac$^+$, relative to Lac$^-$, as a function of its initial frequency, in DM25, calculated between 0 and 12 h and between 0 and 24 h. The fitness of Lac$^+$ relative to Lac$^-$ increased between 12 and 24 h, long after glucose had been exhausted (see *Results: Evaluation of the cross-feeding hypothesis: Evidence for cross-feeding . . .*), implying either differential mortality or growth on metabolic by-products. Each bar represents the mean (± 1 SE) of 11 replicates; the ANOVA is given in Table 3.

medium within the first 8–10 h, even allowing for a lag phase of 1 or 2 h prior to growth (see Vasi et al. 1994). Therefore, the finding that Lac$^+$ gained a significant advantage between 12 and 24 h suggests either that Lac$^+$ was growing on some metabolite (and at a faster rate than Lac$^-$) or that Lac$^-$ was dying (and at a faster rate than Lac$^+$), or perhaps both.

To evaluate these two alternative explanations, we computed the absolute rate of change in population density between 12 and 24 h for each genotype. A positive or negative value would indicate net growth or death, respectively. Fig. 7 shows that, here in the absence of the other genotype (i.e., the initial frequency of Lac$^+$ equals either 0 or 1), each genotype is subject to some cell death, although Lac$^+$ and Lac$^-$ do not differ significantly in their death rates (t = 1.161, df = 4, P = 0.310). However, in the presence of Lac$^-$, Lac$^+$ experienced substantial net growth between 12 and 24 h (Fig. 7). 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. Thus, between 12 and 24 h, each genotype benefits in absolute terms from the presence of the other genotype (Fig. 7), although Lac$^+$ has a large advantage in relative terms (Fig. 6).

We conclude that cross-feeding interactions occurred after glucose had been depleted from the culture medium. These interactions clearly favored Lac$^+$, but the fact that each genotype benefitted from the other's presence between 12 and 24 h suggests that two (or more) metabolic by-products may be involved. In any case, the large advantage to Lac$^+$ after the glucose had been depleted offset the earlier advantage of Lac$^-$ in competition for glucose and allowed their stable coexistence.

TABLE 3. ANOVA of effects of initial frequency and final sample time (12 or 24 h) on fitness of Lac$^+$ relative to Lac$^-$ genotypes of *E. coli*. The experiment was performed in medium containing glucose at 25 μg/mL.

Source of variation	df	MS	F	P
Initial frequency	2	0.077	13.644	<0.001
Final sample time	1	0.091	16.113	<0.001
Interaction	2	0.002	0.286	0.752
Error	60	0.006		

DISCUSSION

From an experimental study of the effects of recombination on the dynamics of bacterial evolution (Turner 1995), we isolated two distinct recombinant genotypes of *E. coli*. In this paper, we first demonstrated that each of these genotypes had a competitive advantage when it was rare, such that the two genotypes could stably coexist (Figs. 2 and 3). Such coexistence was unex-

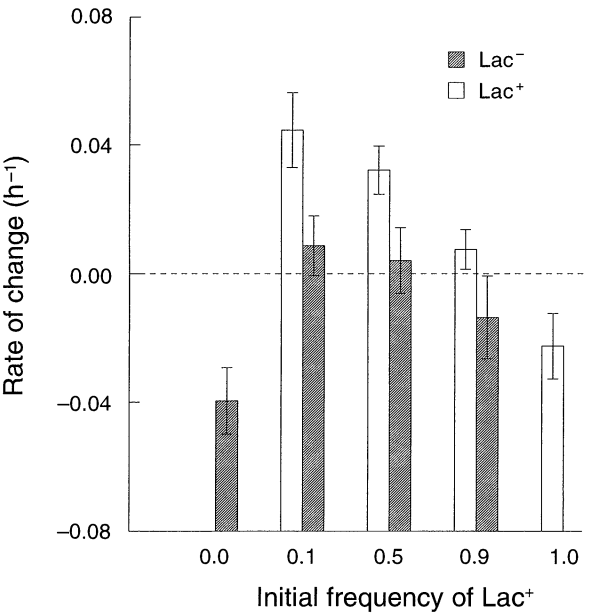

FIG. 7. Net rate of change in viable cell density for Lac$^+$ and Lac$^-$ in DM25 between 12 and 24 h, after glucose had been exhausted from the medium. In the absence of the other genotype, each genotype declined due to death. However, Lac$^+$ experienced net growth between 12 and 24 h when Lac$^-$ was present. Lac$^-$ also benefitted from the presence of Lac$^+$ between 12 and 24 h, although Lac$^+$ had a large advantage in relative terms (see also Fig. 6). Each bar represents the rate of change in viable cell density between 12 and 24 h (mean ± 1 SE) with eleven-fold replication for initial frequencies of 0.1, 0.5, and 0.9 and with three-fold replication for initial frequencies of 0 and 1.

pected since the populations were provided with only a single resource (glucose), which was limiting to population density. Therefore, we then sought to determine the ecological mechanisms responsible for the coexistence of these two genotypes.

We considered two mechanistic hypotheses to explain the stable coexistence. One hypothesis depends on the fact that the experimental environment was seasonal, such that the glucose concentration fluctuated temporally as a consequence of periodic transfers of the bacteria into fresh medium. In a seasonal environment, two genotypes may stably coexist on a single limiting resource (Fig. 1) if one of them has an advantage when the resource is common and the other has a sufficiently large opposing advantage when the resource has become sparse (Stewart and Levin 1973, Tilman 1982). According to the other hypothesis, an additional resource is generated by the metabolic activities of the organisms themselves. In that case, two 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.

We demonstrated a demographic trade-off between growth rates at high and low concentrations of the resource glucose. That is, genotype Lac$^-$ was superior in competition for abundant glucose whereas genotype Lac$^+$ was the better competitor for sparse glucose. However, the expected magnitude of each genotype's advantage when rare, based on this trade-off, was much too small to explain the observed strength of frequency dependence (Fig. 4 vs. Fig. 2) and, in fact, may even have been too weak to allow stable coexistence. The model using a demographic trade-off fails because low (but non-zero) concentrations of glucose occurred for too short a period during the daily growth cycle to provide Lac$^+$ with a sufficient advantage to explain its success in the face of the higher maximal growth rate of Lac$^-$. Thus, although there was a trade-off between growth rates at high and low glucose concentrations, it could not have been primarily responsible for the stable polymorphism that we observed. There must have been some other advantage for Lac$^+$ to explain its ability to invade an established population of Lac$^-$.

We also demonstrated a cross-feeding interaction between the two genotypes. After glucose was depleted by cell growth, each genotype, when grown by itself, experienced a net decrease in population density due to cell death. However, when the two genotypes were grown together, each genotype did better—after the glucose was depleted—than when it was alone (Fig. 7). Lac$^+$ had a significant advantage relative to Lac$^-$ in this cross-feeding interaction (Fig. 6). Thus, Lac$^+$ could offset its disadvantage relative to Lac$^-$ in competition for abundant glucose by its superior ability to acquire and assimilate one or more metabolic by-products.

The major opposing factors that allowed the stable coexistence thus were as follows: (1) Lac$^-$ grew more quickly than its competitor when glucose was abundant, but (2) Lac$^+$ was the superior competitor for some metabolite that allowed additional cell growth after glucose had been depleted. Beyond these two major factors, we also identified two other factors that may have contributed to the stable polymorphism, although to a lesser degree: (3) Lac$^+$ was the superior competitor for very low (but non-zero) concentrations of glucose, but such concentrations were experienced only briefly during the daily growth cycle. (4) Lac$^-$ also benefitted (in absolute terms) from the presence of its counterpart after glucose had been depleted, although it was at a relative disadvantage in this phase of the dynamics.

In addition to the inherent interest in determining the ecological mechanisms responsible for the stable coexistence of competitors in this simple model system, we believe that our results have several other more general implications, which we will briefly address in turn. First, this system illustrates how organisms could, through their own biological activities, change a simple environment into one that is more complex. And this environmental complexity, in turn, allowed for diversity to be stably maintained where it could not otherwise persist. As eloquently stated by Rosenzweig et al. (1994: 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 Turner (1995) studied recombinant genotypes bearing many easily distinguishable genetic markers (see Table 1), we had the opportunity to observe polymorphisms that might otherwise have been overlooked. In a related study with populations that were evolving in a strictly asexual fashion, and which lacked readily screened genetic markers, Lenski et al. (1991) saw no conspicuous evidence for complex ecological interactions of the sort observed in this study. It will be interesting to examine those asexual populations more thoroughly to discern whether some stable polymorphisms might have been overlooked. If such polymorphisms are not observed in those strictly asexual populations, it raises the interesting question of whether the opportunities for stable polymorphisms to evolve are greater in a system that allows for recombination between strains that are already more divergent genetically. It is perhaps relevant that Levin (1972) observed stable coexistence between *E. coli* B and K12 strains in environmental conditions similar to those that we employed, since the recombinant genotypes used in this study were hybrids of *E. coli* B recipients and K12 donors (Table 1). Although Levin did not conclusively establish the ecological mechanisms that mediated coexistence between *E. coli* B and K12, it seems plausible that they might be the same mechanisms seen in this study.

Third, many evolutionary ecologists seem to regard it as adequate to show that there exists a trade-off be-

tween two ecological attributes (e.g., r vs. K) in order to explain stable coexistence of the corresponding genotypes or species. But while such trade-offs are generally *necessary* for stable coexistence, they may not be *sufficient.* In our study, we observed a demographic trade-off between growth rates at high and low glucose concentrations. Such a trade-off 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 magnitude of the trade-off as well as the extent of fluctuations in resource concentration and population density. In fact, the concentration-mediated trade-off for the two genotypes in this study could not fully explain the observed strength of the frequency-dependent advantage that each genotype had when rare (Figs. 2 and 4). When possible, therefore, experimental analyses of the ecological mechanisms that promote stable coexistence should move beyond merely demonstrating that a requisite trade-off exists and seek to establish the quantitative agreement between observed and predicted dynamics. While we were able to fulfill this objective for our model of a demographic trade-off, we could not perform a quantitative analysis of the cross-feeding hypothesis owing to our uncertainty with respect to the identity of the relevant metabolites and the parameters governing their rates of production and consumption.

Finally, there is a long-standing interest in ecology concerning the factors responsible for maintaining greater or lesser diversity of species within communities (Connell and Orias 1964, MacArthur and Wilson 1967, Connell 1978, Huston 1979). According to one hypothesis, communities with greater primary production are more diverse than less productive communities (see, e.g., Brown and Davidson 1977), which might contribute to the latitudinal cline in ecological diversity (Fischer 1960, Pianka 1966). One can imagine several possible mechanistic bases for such a relationship. For example, 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 reduced, then the density of the producer species may be reduced to a level where the secondary resource is not abundant enough to maintain the second species. In fact, the stable coexistence of bacterial genotypes that we 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.

ACKNOWLEDGMENTS

We thank Lynette Ekunwe and Sue Simpson for help with some of the experiments. We also thank Julian Adams, Al Bennett, Guy Bush, Ted Case, Dan Dykhuizen, Dennis Fulbright, Andy Jarosz, Doug Taylor, Mike Travisano, and two anonymous reviewers for helpful comments and discussion. This research was supported by an NSF grant (DEB-9421237) to R. E. Lenski and by the NSF Center for Microbial Ecology (BIR-9120006).

LITERATURE CITED

Atwood, K. C., L. K. Schneider, and F. J. Ryan. 1951. Periodic selection in *Escherichia coli.* Proceedings of the National Academy of Sciences, USA **37**:146–155.

Ayala, F. J., and C. A. Campbell. 1974. Frequency-dependent selection. Annual Review of Ecology and Systematics **5**: 115–138.

Blythe, S. P., W. S. C. Gurney, P. Maas, and R. M. Nisbet. 1990. Program and model building guide for SOLVER (Rev. 4). Applied Physics Industrial Consultants, Glasgow, Scotland.

Brown, J. H., and D. W. Davidson. 1977. Competition between seed-eating rodents and ants in desert ecosystems. Science **196**:880–882.

Cain, A. J., and P. M. Sheppard. 1954. Natural selection in *Cepaea.* Genetics **39**:89–116.

Carlton, B. C., and B. J. Brown. 1981. Gene mutation. Pages 222–242 *in* P. Gerhardt, editor. Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C., USA.

Chao, L., B. R. Levin, and F. M. Stewart. 1977. A complex community in a simple habitat: an experimental study with bacteria and phage. Ecology **58**:369–378.

Connell, J. H. 1978. Diversity in tropical rain forests and coral reefs. Science **199**:1302–1310.

Connell, J. H., and E. Orias. 1964. The ecological regulation of species diversity. American Naturalist **98**:399–414.

Dykhuizen, D. E. 1990. Experimental studies of natural selection in bacteria. Annual Review of Ecology and Systematics **21**:373–398.

Fischer, A. G. 1960. Latitudinal variations in organic diversity. Evolution **14**:64–81.

Fisher, R. A. 1958. The genetical theory of natural selection. Second edition. Dover, New York, New York, USA.

Gause, G. F. 1934. The struggle for existence. Dover, New York, New York, USA.

Haldane, J. B. S., and S. D. Jayakar. 1963. Polymorphism due to selection depending on the composition of a population. Journal of Genetics **58**:318–323.

Hansen, S. R., and S. P. Hubbell. 1980. Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. Science **207**: 1491–1493.

Hardin, G. 1960. The competitive exclusion principle. Science **131**:1292–1297.

Helling, R. B., C. N. Vargas, and J. Adams. 1987. Evolution of *Escherichia coli* during growth in a constant environment. Genetics **116**:349–358.

Hori, M. 1993. Frequency-dependent natural selection in the handedness of scale-eating cichlid fish. Science **260**:216–219.

Huston, M. 1979. A general hypothesis of species diversity. American Naturalist **113**:81–101.

Kojima, K. 1971. Is there a constant fitness value for a given genotype? No! Evolution **25**:281–285.

Lenski, R. E., and S. E. Hattingh. 1986. Coexistence of two competitors on one resource and one inhibitor: a chemostat model based on bacteria and antibiotics. Journal of Theoretical Biology **122**:83–93.

Lenski, R. E., and M. Travisano. 1994. Dynamics of adaptation and divergence: a 10,000-generation experiment with bacterial populations. Proceedings of the National Academy of Sciences, USA **91**:6808–6814.

Lenski, R. E., M. R. Rose, S. C. Simpson, and S. C. Tadler.

1991. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. American Naturalist **138**:1315–1341.

Levin, B. R. 1972. Coexistence of two asexual strains on a single resource. Science **175**:1272–1274.

———. 1988. Frequency-dependent selection in bacterial populations. Philosophical Transactions of the Royal Society, London B **319**:459–472.

Levin, B. R., F. M. Stewart, and L. Chao. 1977. Resource-limited growth, competition, and predation: a model and experimental studies with bacteria and bacteriophage. American Naturalist **111**:3–24.

MacArthur, R. H., and E. O. Wilson. 1967. The theory of island biogeography. Princeton University Press, Princeton, New Jersey, USA.

Monod, J. 1949. The growth of bacterial cultures. Annual Review of Microbiology **3**:371–394.

Neidhardt, F. C., J. L. Ingraham, and M. Schaechter. 1990. Physiology of the bacterial cell. Sinauer, Sunderland, Massachusetts, USA.

Pianka, E. R. 1966. Latitudinal gradients in species diversity: a review of concepts. American Naturalist **100**:33–46.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution **43**:223–225.

Rosenzweig, R. F., R. R. Sharp, D. S. Treves, and J. Adams. 1994. Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli*. Genetics **137**:903–917.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Second edition. Cold Spring Harbor Laboratory, Plainview, New York, USA.

Selander, R. K., and B. R. Levin. 1980. Genetic diversity and structure in *Escherichia coli* populations. Science **210**: 545–547.

Stewart, F. M., and B. R. Levin. 1973. Partitioning of resources and the outcome of interspecific competition: a model and some general considerations. American Naturalist **107**:171–198.

Tilman, D. 1982. Resource competition and community structure. Princeton University Press, Princeton, New Jersey, USA.

Turner, P. E. 1995. Associations between bacteria and conjugative plasmids: model systems for testing evolutionary theory. Dissertation. Michigan State University, East Lansing, Michigan, USA.

Van Delden, W. 1982. The alcohol dehydrogenase polymorphism in *Drosophila melanogaster*: selection at an enzyme locus. Evolutionary Biology **15**:187–222.

Van Delden, W., A. C. Boerema, and A. Kamping. 1978. The alcohol dehydrogenase polymorphism in populations of *Drosophila melanogaster*. I. Selection in different environments. Genetics **90**:161–191.

Vasi, F., M. Travisano, and R. E. Lenski. 1994. Long-term experimental evolution in *Escherichia coli*. II. Changes in life-history traits during adaptation to a seasonal environment. American Naturalist **144**:432–456.

Wright, S., and T. Dobzhansky. 1946. Genetics of natural populations. XII. Experimental reproduction of some of the changes caused by natural selection in certain populations of *Drosophila pseudoobscura*. Genetics **31**:125–156.