Long-Term Experimental Evolution in *Escherichia coli*. VIII. Dynamics of a Balanced Polymorphism

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ABSTRACT: We describe the short- and long-term dynamics of a phenotypic polymorphism that arose in a population of *Escherichia coli* while it was serially propagated for almost 20,000 generations in a glucose-limited minimal medium. The two types, designated *L* and *S*, differ conspicuously in the size of the colonies they form on agar plates as well as the size of their individual cells, and these differences are heritable. The *S* type reached a detectable frequency (>1%) at generation 6,000, and it remained above that frequency throughout the subsequent generations. In addition to morphological differences, *L* and *S* diverged in important ecological properties. With clones isolated at 18,000 generations, *L* has a maximal growth rate in fresh medium that is ~20% higher than that of *S*. However, experiments with conditioned media demonstrate that *L* and *S* secrete one or more metabolites that promote the growth of *S* but not of *L*. The death rate of *L* during stationary phase also increases when *S* is abundant, which suggests that *S* may either secrete a metabolite that is toxic to *L* or remove some factor that enables the survival of *L*. One-day competition experiments with the clones isolated at generation 18,000 show that their relative fitness is frequency dependent, with each type having an advantage when rare. When these two types are grown together for a period of several weeks, they converge on an equilibrium frequency that is consistent with the 1-d competition experiments. Over the entire 14,000-generation period of coexistence, however, the frequency of the *S* type fluctuated between approximately 10% and 85%. We offer several hypotheses that may explain the fluctuations in this balanced polymorphism, including the possibility of coevolution between the two types.

Keywords: Escherichia coli, frequency-dependent selection, experimental evolution, stable polymorphism.

The use of bacteria and other microorganisms to address questions of fundamental ecological and evolutionary importance has substantially increased in recent years (Dykhuizen 1990; Lenski 1995). Among the reasons for this are that certain microbes are easy to grow, have large populations with short generation times, and possess simple genetic systems that can be manipulated. These attributes allow one to create defined genotypes to measure their performance and fitness (Dykhuizen and Hartl 1980; Chao and Levin 1981; Dykhuizen and Dean 1990; Elena and Lenski 1997), as well as to study natural selection acting on spontaneous mutants (Helling et al. 1987; Lenski et al. 1991; Bennett et al. 1992; Lenski and Travisano 1994; Velicer et al. 1998). One can also construct communities to examine the dynamics and stability of ecological interactions (Chao et al. 1977; Rosenzweig et al. 1994; Bohannan and Lenski 1997). A potential concern is that these systems are so artificial that they may prevent the emergence of complexity and thereby limit the insights that can be drawn from them. In this article, and following earlier studies (Helling et al. 1987; Rosenzweig et al. 1994; Turner et al. 1996; Treves et al. 1998), we demonstrate the emergence of a stable polymorphism even in a simple environment. Moreover, we show that the dynamics of this polymorphism become increasingly complex over the long term.

Bacteria reproduce asexually, and it is often assumed that their evolution on a single limiting resource will consist of a temporal series of replacements by ever more fit genotypes via the process of periodic selection (Atwood et al. 1951; Koch 1974; Levin 1981). Each selective replacement creates a bottleneck of one contributing genotype in a haploid asexual population, eliminating all genetic variation. Accordingly, polymorphisms are presumed to be transient and indicative of selective sweeps in progress. The competitive exclusion principle (Hardin 1960) also implies that populations propagated on a single limiting resource will be monomorphic. Thus, two processes—one genetic (periodic selection) and the other ecological (competitive exclusion)—should maintain monomorphism in asexual microbial populations as they evolve under simple laboratory regimes.

Certain circumstances, however, can promote the evo-

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lution of stable polymorphisms in asexual populations. For example, Chao et al. (1977) observed the evolution of *Escherichia coli* mutants that were resistant to viral infections, which then stably coexisted with their sensitive progenitors in a predator-mediated manner. Helling et al. (1987) demonstrated the emergence of stable polymorphisms in *E. coli* populations that were propagated in a chemostat on a single resource; they later showed that the polymorphisms were maintained by cross-feeding interactions, in which secondary resources are secreted as metabolic by-products of a primary resource (Rosenzweig et al. 1994). Turner et al. (1996) observed the coexistence of two *E. coli* strains in a serial transfer regime; a cross-feeding interaction and a trade-off in relative growth rate at high and low resource concentrations were both implicated (see also Levin 1972). This study examines the emergence, ecological mechanisms, and evolutionary dynamics of a stable polymorphism that arose during a long-term evolution experiment with *E. coli* (Lenski et al. 1991; Lenski and Travisano 1994; Vasi et al. 1994; Travisano et al. 1995; Elena et al. 1996; Travisano and Lenski 1996; Elena and Lenski 1997). In that experiment, 12 replicate populations were serially propagated in a glucose-limited minimal medium. Previous articles in this series reported on the dynamics of genetic adaptation and on the extent of variation within and among the evolving populations. The replicate populations exhibit substantial differences from one another in certain phenotypic traits, such as average cell size and performance in novel environments. By contrast, they are very similar, but not identical, to one another in the extent of their fitness improvement measured in the selective environment itself.

Throughout the 20,000 generations of this experiment, there emerged an interesting temporal pattern of fitness variation within the evolving populations. During the initial 2,000 generations—the period of most rapid adaptation—the extent of within-population variation in fitness corresponded closely to the level predicted by Fisher's fundamental theorem from the observed rate of adaptation (Lenski et al. 1991). In other words, it was unnecessary to invoke any variation in performance during this early phase beyond the transient variation that must occur whenever beneficial mutations sweep through a population. After 10,000 generations, however, the situation had become more complex; the rate of genetic adaptation declined substantially relative to the earlier phase, whereas the variation in performance among clones within a population remained high (Elena and Lenski 1997). Only about 1% of the within-population variation in performance could then be explained by ongoing selective sweeps, whereas previously all the variation could be thus explained. Another modest fraction, about 10%, of the within-population variation for fitness at generation 10,000 could be attributed to deleterious mutations, which had become more common after some of the replicate populations had evolved much higher mutation rates during this period (Sniegowski et al. 1997). Most of the variation in performance was attributed instead to frequency-dependent selection of the form that promotes balanced polymorphism—that is, when the fitness of a genotype is higher when that genotype is rare than when it is common. This form of frequency dependence has often been invoked to explain the maintenance of stable polymorphisms in nature (Ayala and Campbell 1974; Levin 1988). In experiments in which marked clones were reintroduced at variable frequencies into the populations from which they had been sampled, it was shown (Elena and Lenski 1997) that the marked clones had, on average, higher fitness when they were rare than when they were common in all six of the study populations. In five of the populations, the average advantage when rare was small (~1%), but in one population the fitness advantage when rare was much greater (~5%).

This article focuses on the population that showed the most extreme frequency-dependent selection. We demonstrate that there are two predominant morphs in this population, and we confirm that each morph does indeed have a strong selective advantage when it is rare, such that there exists a stable polymorphism. We show that the two morphs can be distinguished on the basis of several phenotypic differences, and we examine which differences can explain the stable polymorphism. Finally, we document when the balanced polymorphism arose during the population's history, and we show that this seemingly stable polymorphism has in fact exhibited unexpectedly complex dynamics over the duration of its existence.

Material and Methods

Bacterial Strains

The genotypes used in this study were derived from a single clone of *Escherichia coli* B that has been serially propagated for almost 20,000 generations in glucose-limited medium (see Lenski et al. 1991 for a description of the ancestral strain). Throughout this long-term experiment, samples of the evolving populations have been periodically spread as individual cells onto petri plates to examine the populations for possible contamination. Two distinct morphotypes were identified in one of the evolving populations (Ara-2) at generation 18,000. This population had previously been shown to harbor significant genetic variation in performance that was apparently maintained by frequency-dependent selection (Elena and Lenski 1997). The two morphotypes differed in their colony size and time of appearance on tetrazolium-arabinose (TA) indicator agar

plates incubated at 37°C. Per liter, the TA plates contain 10 g tryptone, 1 g yeast extract, 5 g NaCl, 16 g agar, 10 g arabinose, and 1 mL of a 5% stock of tetrazolium (2,3,4-triphenyltetrazolium chloride). The large type (*L*) produced visible colonies ~24 h after plating, whereas colonies of the small type (*S*) were visible only after ~48 h. Representative clones of each morphotype from generation 18,000 were subcultured on two TA plates before storage in 15% glycerol at −80°C. This procedure ensured that we had obtained a single clone of each type, and it also indicated that the distinctive colony morphologies of *L* and *S* were heritable and stable. We determined that neither *S* nor *L* were contaminants by examining their phenotypes with respect to markers specific to the experimental populations (Lenski et al. 1991). We present additional data in "Results" to show that phenotypic differences between *L* and *S* are heritable.

The *L* and *S* clones were isolated from a population that was founded by an ancestral strain unable to metabolize arabinose; consequently, both *S* and *L* were phenotypically Ara^-. To facilitate counting the *L* and *S* clones during competition experiments, we isolated Ara^+ mutants of both types by plating ~10^8 cells on minimal-arabinose agar (Lenski 1988). The Ara^+ mutants retained their characteristic colony morphologies. They are designated S^+ and L^+, and they too were stored in 15% glycerol at −80°C. When samples from competition experiments are spread on TA agar, Ara^- strains produce red colonies, whereas Ara^+ colonies are white (Miller 1992).

Growth Conditions

Unless otherwise noted, bacteria were cultured in Davis minimal medium supplemented with thiamine hydrochloride (at 2×10^{-3} μg mL^{-1}) and glucose at 25 μg mL^{-1} (DM25). This medium supports a stationary-phase population density of ~5×10^7 cells mL^{-1}. In all experiments, 10-mL cultures were maintained in 50-mL Erlenmeyer flasks placed in a rotary shaker at 37°C and 120 rpm. Each day, 0.1 mL was transferred from the stationary-phase culture into 9.9 mL of fresh medium. Regrowth following this 100-fold dilution allowed ~6.64 generations of binary fission per day ($\log_2 100 \approx 6.64$). In competition experiments, specified ratios (based on culture volume) of each genotype were mixed and then diluted, so that each culture received the same initial density of cells as in the long-term evolution experiment.

Competition Experiments and Fitness Estimation

Competition experiments were performed to determine the relative fitness of the *L* and *S* clones. Before doing so, however, it was necessary to test the neutrality of the Ara^+ mutants of both *L* and *S* relative to their isogenic Ara^- progenitors. Before every fitness assay, each competitor was separately grown for one full day in DM25; this acclimation step ensured that the competitors were in similar physiological states and at similar densities. Following the acclimation step, either S^+ and S^- or L^+ and L^- were mixed at a 1 : 1 ratio and diluted 1 : 100 into fresh DM25. Hence, the acclimation and dilution steps mimic the serial-transfer cycle that prevailed during the long-term evolution experiment. Initial and final (after 1 d) densities of each competitor were determined from counts on TA agar after appropriate dilution. The fitness of one genotype relative to the other was calculated as the ratio of their Malthusian parameters, which for each genotype was estimated by $m_i = \ln(N_i[1]/N_i[0])/(1\text{d})$, where $N_i(0)$ and $N_i(1)$ are initial and final densities, respectively (Lenski et al. 1991). We performed 13 replicate competition experiments for each morphotype. The fitness of S^- relative to S^+ was 1.004 ± 0.012 ($\bar{X} \pm$ SE), and the fitness of L^- relative to L^+ was 1.022 ± 0.022. Neither value is significantly different from 1.0 (*S*, $t = 0.32$, df = 12, $P = .755$; *L*, $t = 1.04$, df = 12, $P = .320$), indicating that the Ara marker is effectively neutral on each background. In all subsequent competitions, we used only S^+ and L^-, which henceforth are denoted simply as *S* and *L*.

To examine frequency-dependent interactions between *S* and *L*, we used the same protocol as described earlier, except that the competition experiments were inoculated at three different initial ratios of *S* and *L*, which were 9 : 1, 1 : 1, and 1 : 9. Each treatment was replicated 10-fold.

Measurements of Maximum Growth Rate and Average Cell Size

The maximum growth rate, V_m, of each genotype was measured under standard culture conditions. The glucose concentration (25 μg mL^{-1}) in DM25 has been shown to be well above that which limits growth rate (Vasi et al. 1994). Cells were enumerated using a Coulter electronic particle counter (model ZM and channelyzer model 256). Four replicate cultures of each clone were grown to stationary phase in DM25, and each culture was then diluted 100-fold into fresh DM25. Beginning 2 h after transfer, cell counts were obtained every half-hour until the rate of population growth began to slow appreciably because of depletion of the limiting glucose. The maximum growth rate of each culture was estimated by regressing the natural logarithm of population density against time, using only those time points over which population density increased log-linearly.

The Coulter counter was also used to measure average cell size during stationary phase in DM25. For each ge-

notype, 10 replicate cultures were allowed to complete the standard 24-h propagation cycle; the glucose is depleted within the initial 6–10 h, so the cells have entered stationary phase when their sizes are measured. The individual cell volumes of 10^4-10^5 bacteria were obtained from each culture. For the purpose of statistical analysis, the mean cell size from each independent culture was the unit of replication.

Results

L *and* S *Differ in Average Cell Size*

The two genotypes, *L* and *S*, were originally distinguished by the size of their colonies and the time of their appearance on agar plates. They also differ in the average volume (1 fL = 10^{-15} L) of individual cells measured at stationary phase. The average cell size for *L* is 1.251 ± 0.051 fL ($\bar{X}$ ± SE, based on 10 replicate cultures), whereas for *S* it is 0.700 ± 0.008. This difference is highly significant using Welch's approximate *t*-test, which takes into account their unequal variances ($t = 10.619$, df = 9, $P < .0001$). Thus, the *S* genotype has smaller individual cells, as well as smaller colonies, than does the *L* type. This reproducible morphological differentiation at the cellular level further indicates the heritable nature of the polymorphism.

Maximum Growth Rate of L *Is Greater than That of* S

We measured the maximum exponential growth rates of both genotypes in DM25, with fourfold replication of paired cultures. The maximum growth rate for *L* is 1.079 ± 0.003 h^{-1} ($\bar{X}$ ± SE), while that for *S* is only 0.896 ± 0.009 h^{-1}. This difference is highly significant (paired $t = 17.80$, df = 3, $P = .0004$).

Stability of the Polymorphism

The population sample from which the clones *L* and *S* were isolated contained high frequencies of both morphotypes. The finding that *L* has a significantly higher maximum growth rate than does *S* tends to argue against the stable maintenance of the polymorphism because maximum growth rate is a very important component of fitness in the serial transfer regime used for the evolution experiment (Vasi et al. 1994). One reasonable interpretation, therefore, is that the polymorphism is transient, with the superior genotype *L* having been caught in the middle of its selective replacement of the inferior genotype *S*. Alternatively, *S* may have some countervailing advantage at some other stage of the growth cycle, which may allow it to persist despite its lower maximum growth rate. If that is the case, then the relative fitness of *S* and *L* in competition over the entire growth cycle may depend on their initial frequencies, and the polymorphism may be maintained at some stable equilibrium.

Relative Fitness Is Frequency Dependent. To test whether the fitness of *S* relative to *L* depends on their relative abundance, we performed competition experiments at three different starting ratios of *S* and *L* (1 : 9, 1 : 1, and 9 : 1), each with 10-fold replication. Figure 1 shows that the fitness of *S* relative to *L* is >1.0 when *S* is rare, whereas the same relative fitness is <1.0 when *S* is common. An ANOVA indicates that the effect of initial frequency on relative fitness is significant ($F = 19.35$, df = 2, 27, $P < .0001$). Thus, each genotype has a selective advantage when it is rare, which implies a stable equilibrium.

Existence of a Stable Equilibrium. We then propagated mixtures of *S* and *L* by daily serial dilution in DM25 for 20 d (~130 generations). Samples from each mixed population were spread onto TA agar every day to determine the frequency of both types. Figure 2 shows that each genotype can invade the other when it is initially rare and that the two genotypes converge on a stable equilibrium. The final frequency of the *S* genotype was 0.612 ± 0.043 ($\bar{X}$ ± SE, based on all nine mixtures at day 20). Thus, it is clear that frequency-dependent selection maintains a stable polymorphism despite the large advantage that accrues to the *L* genotype during exponential growth in pure culture. Evidently, the *S* genotype must have an offsetting advantage elsewhere in the serial transfer regime.

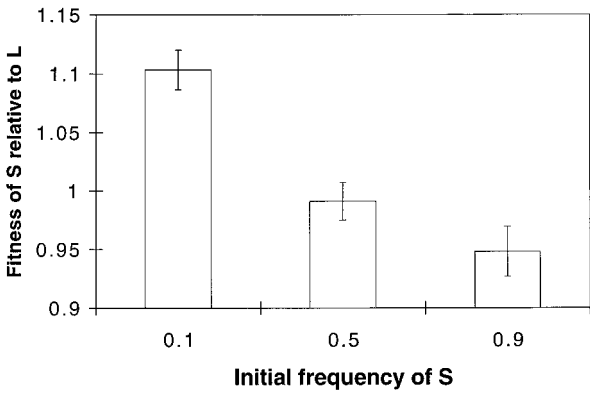

Figure 1: Frequency-dependent relative fitness in short-term competition experiments. The fitness of genotype *S* relative to genotype *L* is shown as a function of the initial frequency of *S*. Each value is the mean of 10 observations; error bars are SEs. See text for ANOVA.

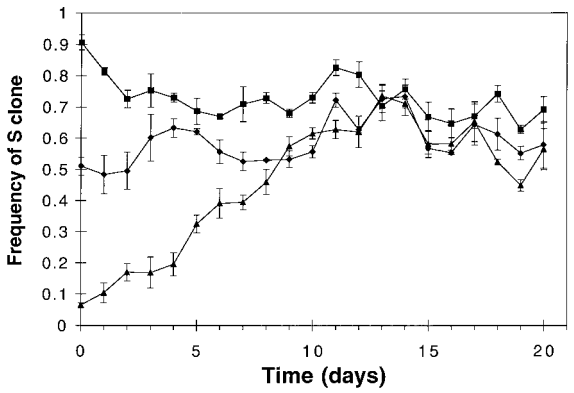

Figure 2: Convergence on a stable equilibrium over 20 serial transfer cycles (≈130 generations). Genotype *S* is able to invade when rare, but it declines in frequency when it is initially common, leading to a balanced polymorphism. Three replicate trajectories were run starting from each of three initial conditions; error bars are SEs.

S *Has Advantages in Both Growth and Stationary Phases.* We sought to determine when, during the serial transfer cycle, *S* compensates for its lower maximum growth rate. To that end, we sampled from the same competition experiments (used to infer frequency dependence) at an intermediate time point, 8 h, as well as at the start and finish of the daily growth cycle. We chose 8 h because that is the approximate duration of the growth phase, given an initial lag period of 1–2 h and six to seven cell divisions with a doubling time of ~1 h. Thus, at ~8 h, the cultures would exhaust the glucose in the medium and enter stationary phase, where they would remain for the rest of the daily cycle (Vasi et al. 1994). We then computed the fitness of *S* relative to *L* over just the first 8 h of growth as well over the entire 24-h cycle.

Figure 3 shows the fitness of *S* relative to *L* over these two time intervals for the three initial ratios. There are three conclusions from this experiment. First, the fitness of *S* relative to *L* is frequency dependent, with *S* having an advantage only when it is rare. This pattern is true over the first 8 h alone ($F = 10.67$, df = 2, 27, $P = .0003$; nonparametric Kruskal-Wallis test, $P = .008$), as well as over the full 24-h cycle ($P < .0001$, as reported earlier). Second, *S* appears to have an advantage when rare even in those first 8 h ($t = 2.084$, df = 9, $P = .0668$), despite its much lower growth rate when grown by itself in DM25. Third, *S* gains a further advantage between 8 and 24 h, when growth has diminished owing to glucose depletion. Paired comparisons of the fitness values obtained over the two different intervals are significant with all three initial frequencies combined (mean difference = 0.0316, $t = 2.5313$, df = 29, $P = .0170$). This late-arising advantage is especially strong when *S* was initially common (for S = 0.1, mean difference = 0.0167, $t = 0.5424$, df = 9, $P = .6007$; for S = 0.5, mean difference = 0.0244; $t = 1.9899$, df = 9, $P = .0778$; for S = 0.9, mean difference = 0.0537, $t = 2.9856$, df = 9, $P = .0153$). Evidently, *S* has advantages in both growth and stationary phases that offset its lower rate of exponential growth in pure culture.

S *Affects the Death of* L *in Stationary Phase.* The preceding analysis does not show whether the stationary-phase advantage of *S* relative to *L* is a consequence of differential growth or death. To address that issue, we analyzed the same data in terms of absolute (rather than relative) rates of change in population density between 8 and 24 h. Figure 4 shows that the changes over this interval are mostly due to the death of *L*, rather than continued growth by *S*, at least when *S* is initially abundant. An ANOVA indicates a significant effect of initial frequency on the rate of numerical change for *L* ($F = 4.742$, df = 2, 27, $P = .017$), but not for *S* ($F = 2.034$, df = 2, 27, $P = .150$). The fact that *L* declined in density only when *S* was abundant suggests either that *S* produces some metabolite that is toxic to *L* or that *S* removes a substance that promotes the survival of *L*. Our results cannot distinguish between these two hypotheses.

Cross-Feeding of S *on Metabolites during Growth Phase.* In addition to its survival advantage in stationary phase, *S* also has an advantage when rare during the growth phase, which offsets its slower growth in pure culture. A plausible explanation is cross-feeding, whereby *S* may be able to use

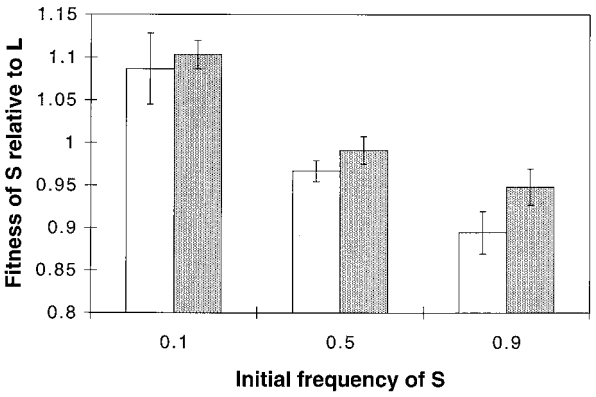

Figure 3: Frequency-dependent advantages for genotype *S* in both growth and stationary phases. The fitness of *S* relative to *L* is shown over two different portions of the population growth cycle and as a function of its initial frequency. *Open bars,* growth phase (0–8 h); *filled bars,* growth and stationary phases combined (0–24 h, shown previously in fig. 1). Each value is the mean of 10 observations; error bars are SEs. See text for statistical analyses.

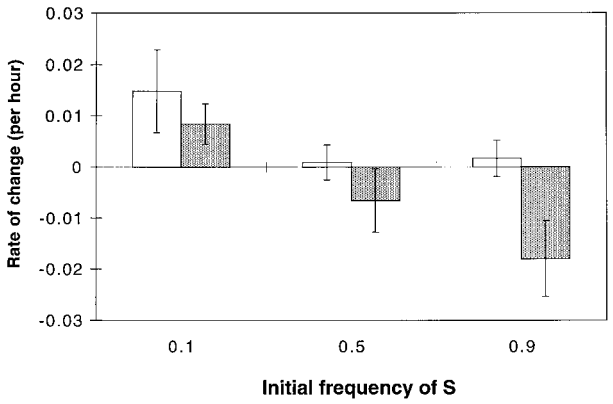

Figure 4: Genotype *S* increasing the death of *L* in stationary phase. The absolute rates of change in both population densities during stationary phase (8–24 h) are shown as a function of the initial frequency of *S*. *Open bars,* genotype S; *filled bars,* genotype *L*. Each value is the mean of 10 observations; error bars are SEs. See text for statistical analyses.

(more effectively than *L*) one or more by-products of glucose metabolism that *L* secretes into the medium. To examine this possibility, we prepared conditioned media that contained the secretions of each genotype, and we then measured the maximum growth rate of each type in these media. Cultures of each genotype were grown separately to stationary phase (either 8 or 24 h) and then filtered through 0.45-μm filters to remove all cells. Because the time course of the accumulation and degradation of metabolites during stationary phase is unknown, we prepared conditioned media using filtrates made near the start (8 h) and at the end (24 h) of stationary phase. In all cases, the conditioned media were composed of a filtrate reconstituted with glucose to 25 μg mL^{-1} (the same concentration as in fresh DM25). We prepared five different media in all: fresh DM25 (which serves as a control, the results for which were given earlier), *L*8, *L*24, *S*8, and *S*24 (the letter indicates the genotype that produced the filtrate, and the numeral the number of hours the genotype spent to produce the filtrate). Each medium was prepared in four independent batches to preclude any spurious effects of variation among batches.

Following the usual acclimation step, all five media were separately inoculated with each genotype, with fourfold replication (corresponding to the independently prepared batches and treated as blocks in the statistical analyses). The maximum growth rate of each genotype in every medium was obtained as before. Figure 5 summarizes data that support three conclusions. First, *L* has a significantly higher maximum growth rate than does *S* in the unconditioned DM25 medium, as reported earlier. Second, the growth rate of *L* is unaffected by any conditioning of the media by either genotype ($F = 0.965$, df = 4, 12, $P = .4615$). Third, by contrast, the growth rate of *S* is significantly influenced by conditioning of the media ($F = 61.19$, df = 4, 12, $P < .0001$). A Tukey-Kramer test indicates that eight of 10 pairwise contrasts are significant ($P < .05$). The growth rates of *S* in the different media can therefore be ranked as follows: *L*24 = *L*8 > *S*24 = *S*8 > DM25. Evidently, both *S* and especially *L* secrete metabolites that promote the growth of *S*, but *L* does not effectively use these metabolites, and so the cross-feeding occurs specifically from *L* to *S*.

Long-Term Dynamics of the Polymorphism

The preceding experiments demonstrate that two clones, *S* and *L*, isolated at generation 18,000 of an evolution experiment, can stably coexist with one another. These experiments also reveal two ecological mechanisms, involving cross-feeding and differential death in stationary phase, that allow *S* to persist despite the much faster exponential growth by *L* in pure culture. Given the evident rapidity with which the two clones approach their joint equilibrium (fig. 2), one might imagine that these two types have been at this equilibrium for a long time. However, one cannot exclude alternative scenarios—for example, their relative abundance may fluctuate over time because of further evolution of one or both types. To examine this issue, and to ascertain when the polymorphism arose, we examined the "fossil record" of this population, from which large samples were obtained every 500 gen-

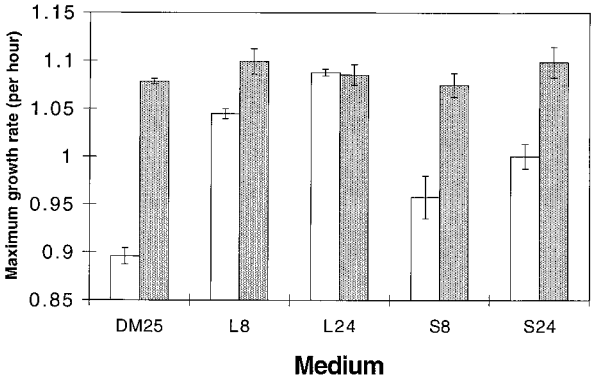

Figure 5: Cross-feeding of genotype *S* (but not genotype L) on metabolites secreted into the culture medium. The maximum growth rates of *S* (*open bars*) and *L* (*filled bars*) are shown in five different culture media. DM25 is the control medium, whereas the other four have been supplemented with filtrates obtained by growing either *L* or *S* for either 8 or 24 h. Each value is the mean of four observations; error bars are SEs. See text for statistical analyses.

erations, then stored frozen at −80°C (Lenski et al. 1991; Lenski and Travisano 1994).

Aliquots from the frozen stocks were revived, acclimated to growth conditions, and then spread on the same TA agar plates on which the polymorphism was noted at 18,000 generations. For each 500-generation interval, five separate plates (several hundred colonies) were scored as either *S* or *L*, on the basis of the timing of their appearance: as noted previously, *L* colonies generally appear after 24 h, whereas *S* colonies become visible only after 48 h. (For some 200 colonies, we confirmed that assignments based on colony appearance were corroborated by differences in average cell size measured with a Coulter counter. For the same 200 clones, we also confirmed these assignments by running restriction digests and using insertion sequences as genetic probes; we observed characteristic differences in the genetic "fingerprints" of the *S* and *L* morphotypes using this approach; D. E. Rozen, D. Schneider, M. Blot, and R. E. Lenski, unpublished data.)

Figure 6 shows the frequency of the *S* type between 0 and 19,500 generations at 500-generation intervals. These data indicate that the *S* morphotype initially invaded *L*, rather than the other way around. They also show that the polymorphism is quite ancient, with the *S* type being common by generation 6,500 and remaining so throughout the duration. Of course, the *S* type must have arisen earlier to have become common by that time; when a mutant lineage first appears, its frequency is $1/N_e$, where the effective population size (adjusted for the bottlenecks during serial dilution) in the long-term evolution experiment is $\sim 3 \times 10^7$ (Lenski et al. 1991). If we assume that *S* had a relative fitness of 1.1 during its initial invasion, as it does when rare at generation 18,000 (see fig. 1), then it would have taken ~200 generations for *S* to have increased from a single individual to ~3% at generation 6,000. Further extrapolating from the convergence on the stable equilibrium (see fig. 2), it should then have taken another 100 generations (15 d) or so for *S* to have increased to its equilibrium frequency of ~60%. But that approach to the equilibrium did not occur; the frequency of *S* did not even reach 50% in the next 1,000 generations, yet it then continued to increase to >80%. We can also estimate the time-averaged fitness of *S* relative to *L* during the period between 6,000 and 7,500 generations (Dykhuizen 1990), when its frequency increased from ~3% to ~86%. That estimate is 1.005, and extrapolating back assuming this much lower fitness yields an estimated origin several thousand generations earlier, around generation 2,000 or so. Thus, while we know that both types were present by generation 6,000, we remain ignorant of the time of origin of the *S* morphotype, owing to the uncertainty about its selective advantage when it first invaded.

It is also clear from these data that the polymorphism

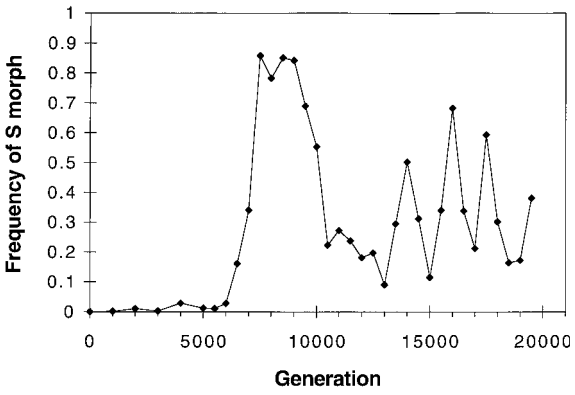

Figure 6: Long-term dynamics of the polymorphism. Each point shows the frequency of the *S* morph, obtained by scoring several hundred bacteria as *S* or *L* on the basis of their resulting colony morphology. From the binomial distribution, 95% confidence limits extend only a few percentage points in either direction. Despite the short-term stability of the polymorphism (fig. 2), it is unstable over much longer intervals. See "Discussion" for several alternative explanations for these fluctuations.

is very dynamic through time and has not simply remained at the equilibrium frequency that obtains from ecological interactions over the relatively short term (fig. 2). These fluctuations are not merely statistical noise. Each datum in figure 6 is based on a sample size of several hundred colonies; from the binomial distribution, the 95% confidence intervals should in every case encompass only a few percentage points in each direction, yet the observed frequencies of the *S* morphotype vary between ~10% and ~85% after generation 6,500. Despite the dramatic oscillations in relative frequency, calculations indicate that differences in relative fitness of <1% would be sufficient to explain even the most rapid of these fluctuations, given that they are manifest over thousands of generations. In "Discussion," we present several scenarios that might explain these changes in the relative abundance of the *S* and *L* types.

Discussion

We observed the emergence of two distinct morphotypes, *L* and *S*, in an evolving population of the bacterium *Escherichia coli*. This population was founded from a single haploid cell, and it lacks any mechanism for genetic exchange; hence, it is strictly asexual (Lenski et al. 1991). The two types show a number of heritable differences, including the appearance of their colonies on agar plates, the average size of their individual cells, and several important demographic properties. We showed that the *S* type invaded the ancestral *L* type, with the *S* type achieving polymorphic frequency (>1%) at generation 6,000 (fig. 6).

We calculate that the *S* type may have arisen, by mutation, anywhere from hundreds to thousands of generations earlier, depending on different assumptions about its initial rate of invasion. Between generations 6,000 and 19,500, the two types coexisted. Such coexistence is not without precedent (Helling et al. 1987; Rosenzweig et al. 1994; Treves et al. 1998) but is nonetheless unexpected on simple ecological and population genetic grounds. In ecological terms, the culture medium used for the experimental evolution contained glucose as the sole carbon and energy input, and it was density limiting (Hansen and Hubbell 1980; Tilman 1982). On population genetic grounds, the asexual condition of the bacteria implies that each successive sweep of a beneficial mutation should purge all genetic variation from the evolving population (Muller 1932; Atwood et al. 1951).

Using *L* and *S* clones isolated after 18,000 generations, we examined, first, the dynamic stability of their coexistence and, second, the ecological mechanisms responsible for the interaction. We showed that the interaction between these clones was dynamically stable by two different approaches. First, 1-d competition experiments showed that each type, when rare, had a fitness >1 relative to the other (fig. 1). Second, over the course of a few weeks (~100 generations), the two types converged on the same final relative abundance (about three *S* to two *L*) regardless of their initial frequencies (fig. 2).

The *L* genotype has a much higher maximum growth rate in the culture medium, DM25, than does *S* (fig. 5, left-most pair). Maximum growth rate is a very important fitness component in the serial transfer regime employed during our long-term evolution experiment (Vasi et al. 1994). If all else had been equal, this difference would have led to the competitive exclusion of *S* by *L*. However, the *S* clone had two opposing advantages that allowed it to invade and coexist with the *L* clone (indeed, *S* was numerically dominant at the resulting equilibrium). One of these advantages is that *L* dies during stationary phase after the glucose has been exhausted, whereas *S* does not (fig. 4). In fact, the death rate of *L* increases when *S* is more abundant, which suggests that *S* may produce some metabolite that is toxic to *L* or that *S* removes some factor from the medium that sustains the viability of *L*. We cannot distinguish between these two possibilities based on the evidence at hand. However, allelopathic production of a toxin, by itself, would not provide a selective advantage to an invading genotype in a mass-action environment (Chao and Levin 1981). This consideration may indirectly favor the hypothesis that *S* depletes some nutrient necessary for the survival of *L*. Second, both *L* and (to a lesser degree) *S* secrete one or more metabolites into the medium that increase the growth rate of *S* but that do not promote the growth of *L* (fig. 5).

This latter mechanism echoes the earlier findings of Helling et al. (1987), Rosenzweig et al. (1994), and Treves et al. (1998), who demonstrated the evolutionary emergence of cross-feeding interactions among *E. coli* genotypes growing in chemostat culture. The physiological mechanism of cross-feeding in their populations involved an increased rate of glucose uptake coupled with the secretion of acetate; a mutation causing semiconstitutive overexpression of acetyl CoA synthetase then allows a second genotype to persist as a specialist on the secreted acetate (Rosenzweig et al. 1994). Treves et al. (1998) have found that the mutation resulting in overexpression of acetyl CoA synthetase arose repeatedly in replicate chemostat cultures.

This reproducibility contrasts with our own work, wherein strong frequency dependence apparently evolved in only one of six replicate populations examined (Elena and Lenski 1997). There may be a simple ecological explanation for this difference between the two studies in the propensity of evolving bacterial populations to give rise to balanced polymorphisms based on cross-feeding interactions. Such interactions are sensitive to the concentration of metabolites in the medium, which in turn depend on bacterial density and ultimately the amount of resource put into the system. In the chemostat experiments, bacteria were propagated on a medium that contained fivefold more glucose than the one used in our experiments; moreover, cells were diluted 100-fold each day in our serial transfer regime, whereas the bacteria were continuously maintained at their maximum density in the chemostat populations (Helling et al. 1987; Lenski et al. 1991). This hypothesis could be tested by varying the glucose concentration and examining its effect on the emergence of stable polymorphisms mediated by cross-feeding interactions.

Consideration of physiological mechanisms may also help explain the difference between chemostat and serial transfer regimes in their propensity to promote cross-feeding interactions. Catabolite repression is a physiological process in bacteria that causes sequential rather than simultaneous use of multiple substrates for growth (Harder and Dijkhuizen 1982). In *E. coli,* catabolite repression ensures that the available glucose is exploited before other less profitable resources are used. The strength of repression increases with the concentration of preferred resource as well as the growth rate of the population. In chemostats, bacteria hold the glucose concentration to a much lower level than the concentration experienced during the growth phase in the serial transfer regime; and the chemostat populations grow much more slowly than their counterparts during the exponential growth phase of the serial transfer regime. This difference in the strength of catabolite repression between chemostat and serial transfer populations may influence the phenotypic expression of

mutants that can exploit metabolic by-products, perhaps amplifying the selective effect of metabolite concentration noted earlier.

In our experiment, as in the chemostat studies, the stably coexisting types evolved from a common ancestor and diverged while they were sympatric (indeed, in a thoroughly mixed environment). The ecological opportunity for this evolutionary divergence evidently depended on generation, by the organisms themselves, of a diverse resource base from one that was otherwise homogeneous (Rosenzweig et al. 1994; Rainey and Travisano 1998). The *S* type emerged from the *L* type, and the cross-feeding interaction clearly benefits *S* at the expense of *L*. However, as noted, such a strong frequency-dependent interaction and polymorphism do not appear to have evolved in five other replicate populations that were founded with the same ancestral strain and evolved under identical conditions (Elena and Lenski 1997). This difference in outcome was evident even though the effective population size and number of generations were so large that all simple mutations should have occurred multiple times—but in different chronological order—in each evolving population (Lenski and Travisano 1994). Taken together, these observations suggest that two or more genetic events may have been necessary for the emergence of the balanced polymorphism, some of them perhaps similar to those events reported by Rosenzweig et al. (1994) in the chemostat populations. First, the lineage that gave rise to *L* may have had a mutation that increased its rate of glucose utilization, but at the expense of efficient metabolism, which led to the coincident loss of metabolites to the medium (owing to enhanced secretion or diminished reacquisition). Then the lineage that produced *S* may have benefited from a mutation that enabled it to scavenge and use these metabolites. Perhaps the property of *S* that increases the death of *L* evolved still later. This scenario is similar to a model of ecological succession in which an early successional species alters the environment so as to facilitate the invasion by a later species. An important difference is that, in these bacterial experiments, the invader evolves in situ. In both cases, the frequency of the early species or genotype is depressed by the invader, with the fate of the former—either extinction or coexistence—depending on the specifics of their interaction.

Long-Term Dynamics of a "Stable" Polymorphism

The sequence of events presented in the previous paragraph is merely a scenario, at present, but it serves to illustrate two points. First, it shows the interest in determining the number and timing of the genetic events that led to the emergence of the balanced polymorphism of the *L* and *S* types. Second, it emphasizes the fact that a polymorphism that is stable over the short term (fig. 2) may exhibit more complex dynamics on a longer time scale (fig. 6); indeed, that is what we observed. The ratio of the *S* and *L* types fluctuated ~60-fold over several thousand generations, whereas clones of these types that were isolated at one point in time rapidly converged on a stable equilibrium (fig. 2). Our study is the first one with sufficient temporal duration to show such pronounced fluctuations in a "stable" polymorphism. A major focus of our future research on this polymorphism will be to determine the cause of these fluctuations. We can formulate four distinct hypotheses to account for the fluctuations, which we hope to distinguish eventually by appropriate experiments.

H_1*: Environmental Fluctuations.* The fluctuations in relative abundance could reflect fluctuations in environmental variables—in the absence of any further genetic change in either *L* or S—despite our best effort to maintain a constant environment. For example, the equilibrium frequency of *S* might vary from 10% to 90% even over a very slight temperature range (say, 1°C). The samples that were characterized in figure 6 were analyzed at the same point in time, so variation in conditions at the time of analysis is not a factor. But the samples were taken at different points in time, and therefore, the fluctuations in relative abundance could reflect subtle fluctuations in the environment. If this strictly ecological hypothesis were true, then *L* and *S* clones isolated from various time points should give the same equilibrium when they are run at the same time, but blocks of experiments run at different times may give different equilibriums. In some sense, this is the null hypothesis from an evolutionary perspective. The three alternative hypotheses described later all invoke evolutionary changes, in which *L* and *S* clones isolated at one time point have heritable differences in demographic traits from their counterparts isolated at other time points.

H_2*: Multiple Origins of* S. The derived morphotype, *S*, may not be monophyletic but instead may have been repeatedly derived from the *L* lineage. Thus, one can imagine that *L*1 gave rise to *S*1 and that the two types achieved a balanced polymorphism based on cross-feeding for some period. After that, a beneficial mutation arose in *L*1 that created *L*2, and the advantage of *L*2 in terms of competing for glucose was so strong that it not only replaced *L*1 but also caused the extinction of *S*1. Nonetheless, *L*2 may have continued to secrete useful metabolites, so that a cross-feeding mutant *S*2—derived from *L*2—could readily invade. This hypothesis can be tested by finding enough molecular genetic markers to construct a phylogeny that resolves whether *S* clones isolated later in the experiment are more closely related to *S* clones from early in the

experiment, supporting monophyly, or whether *S* clones from different time points are more closely related to various *L* clones than to one another, which implies multiple origins of *S*.

H_3: *Adaptation to General Conditions.* The derived type, *S*, may be monophyletic, but both *L* and *S* continually adapt to general aspects of their environment, such as temperature or pH of the medium. These adaptations allow *L*2 to replace *L*1, and they shift the equilibrium away from *S* toward *L*, but they must not cause the extinction of *S*1. Later, *S* adapts genetically to the environment, giving rise to *S*2 and shifting the equilibrium back toward *S*, but without driving *L* to extinction. Repeated rounds of adaptation thus produce fluctuations in relative abundance. This hypothesis can be tested by competing genetically marked variants of strains isolated at earlier and later time points. For example, *L*2 should outcompete *L*1, and *S*2 should outcompete *S*1, under this hypothesis. However, the fitness advantage of later isolates relative to their earlier counterparts should presumably be small relative to the advantage that each type (*S* or *L*) has when rare, so that neither type drives the other extinct.

H_4: *Coevolutionary Red Queen.* This hypothesis is essentially the same as H_3, except that instead of independent genetic adaptation of each lineage to the general culture environment, the adaptations are coevolutionary in nature. For example, *L*2 might replace *L*1, not because *L*2 is any better in competition with *L*1 in isolation, but because *L*2 is better at resisting an allelopathic effect of *S*. Distinguishing between the evolutionary and coevolutionary hypotheses (H_3 vs. H_4) will require comparing, for example, the fitness of *L*2 relative to *L*1 in the absence of any *S*, in the presence of *S*1, and in the presence of *S*2.

A related line of inquiry concerns the fact that the polymorphism emerged after 2,000 generations, by which time most of the overall adaptation relative to the ancestral strain had already taken place. Several beneficial mutations of large effect swept through each evolving population during the first 2,000 generations of the long-term experiment, whereas later sweeps were more infrequent and had less dramatic effects on fitness (Lenski and Travisano 1994). This deceleration presumably occurred because the populations, as they became better adapted, had fewer avenues available for further improvements of a similar magnitude. It is possible that *S*-type mutants started to invade the population in a frequency-dependent manner, well before the successful invasion around generation 6,000, but these early invaders might have been purged by mutations of strong beneficial effect that continued to sweep through the *L* background. Only after the strongest beneficial mutations were incorporated into *L*—such that further generally beneficial mutations would be insufficient to disrupt an emerging polymorphism—could the *S* type become common enough to be detected and, moreover, persist by its own further evolution (or coevolution). In effect, the actual history might be some composite of H_2 and H_3 (or H_4). More generally, we intend to perform experiments across all of the replicate evolving populations to determine whether frequency-dependent interactions became more important over time, as this composite scenario would suggest.

Coda

Many ecological and genetic simplifications are made in experimental studies such as this one. These include environmental constancy, the absence of any other species, a single founding genotype, no sexual recombination, and a focal organism that is much simpler than many others. Yet, despite all these simplifications, polymorphisms emerge over relatively short periods, and further complexities are evident over somewhat longer timescales. As in previous experiments with bacteria (Helling et al. 1988; Rosenzweig et al. 1994; Turner et al. 1996; Rainey and Travisano 1998; Treves et al. 1998), we observed the evolution of stable interactions among genotypes that had evolved from a common ancestor. But unlike these earlier studies, we also showed that these interactions could be destabilized over longer periods by subtle environmental or genetic changes. That such long-term complexities are seen even in simple model systems suggests that they might help to illuminate the evolution of polymorphism, and even speciation and extinction, in macro- and microorganisms alike (Schluter 1996; Reznick et al. 1997; Rainey and Travisano 1998; Wilson 1998).

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