Special section: Stationary-phase mutations in microorganisms

Ecological strategies and fitness tradeoffs in *Escherichia coli* mutants adapted to prolonged starvation

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Abstract

Many bacteria in nature are nutritionally deprived, and there has been heightened interest during the past decade in the properties of these bacteria. We subjected five populations of *Escherichia coli* to prolonged starvation in a minimal salts medium, during which time the density of viable cells declined by several orders of magnitude. From each one, we isolated a surviving clone that showed some heritable difference in colony morphology. We then characterized these mutants in two ecologically relevant respects. First, we determined the nature of their selective advantage, if any, during prolonged starvation. (i) Three of the five mutants had significantly lower net death rate when progenitor and mutant clones were starved separately. (ii) Three mutants showed a significant reduction in death rate in mixed culture that was frequency dependent and manifest when the mutant clone was initially rare. This pattern suggests that these mutants fed on some byproduct of progenitor cells (living or dead). (iii) Two mutants caused the death rate of their progenitors to increase significantly relative to the rate measured in the absence of the mutant. This pattern suggests that these mutants had become allelopathic to their progenitors. Thus, three distinct ecological adaptations to prolonged starvation are evident. No advantage was detected for one mutant, whereas two mutants exhibited multiple advantages. Second, we asked whether the starvation-selected mutants were as fit in growth-supporting conditions as their progenitors. All five mutants were inferior to their progenitor during competition in fresh medium. Evidently, there is an evolutionary tradeoff between performance under growth and starvation conditions.

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Introduction

In nature, most bacterial populations are thought to experience periods of famine. Yet, until recently, studies of bacterial physiology and genetics have focussed on cells that were rapidly growing on abundant nutrients. In the past decade, however, there has been heightened interest in the properties of bacteria under starvation. Some of this recent research emerged from the 'directed mutation' debate (Cairns *et al.* 1988; Lenski and Mittler 1993; Sniegowski and Lenski 1995; Foster 1998) and has examined the rate and nature of mutations during starvation (Stahl 1988; Boe 1990; Hall 1990; Mittler and Lenski 1990, 1992; Foster and Cairns 1992, 1994; MacPhee 1993; Maenhaut-Michel and Shapiro 1994; Rosenberg *et al.* 1994; Jayaraman 1995; Sniegowski 1995; Foster 1997; Harris *et al.* 1997; Bhattacharjee and Mahajan 1998). Another interesting line of research seeks to understand the physiological and genetic bases of adaptation to prolonged starvation (Koch 1971; Lange and Hengge-Aronis 1991; Siegele and Kolter 1992; Hengge-Aronis 1993, 1996; Zambrano *et al.* 1993; Huisman and Kolter 1994; Huisman *et al.* 1996; Zambrano and Kolter 1996; Cotter and Miller 1997; Joux *et al.* 1997; Thorne and Williams 1997; Uhde *et al.* 1997; Lazar *et al.* 1998; Pratt and Silhavy 1998; You *et al.* 1998). Our paper builds upon this second line of research.

In this paper, 'stationary phase' refers to the period after a bacterial population has ceased growth owing to the depletion of limiting nutrients. Zambrano *et al.* (1993) isolated some mutants of *Escherichia coli* that could spread through populations maintained for approximately two weeks in a formerly rich medium (LB) without further

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addition of nutrients. By mixing together previously starved and freshly grown cells (carrying different genetic markers), Zambrano *et al.* demonstrated the existence of mutants that could grow even while the progenitor was dying. These mutants were later termed GASP mutants—for growth advantage in stationary phase—and they are presumed to have an advantage in scavenging the nutrients released by dying cells (Zambrano and Kolter 1996). Some GASP mutants have alterations in the *rpoS* gene that reduce, but do not eliminate, the activity of σ^S (Zambrano *et al.* 1993), a transcription factor that is induced by starvation and regulates the expression of many other genes (Lange and Hengge-Aronis 1991; Hengge-Aronis 1993, 1996). Mutations in other genes may also produce a GASP-like phenotype (Huisman *et al.* 1996).

In this study, we obtained five GASP-like mutants of *E. coli* by prolonged starvation in a minimal medium. Rather than characterizing these mutants in terms of genetic and physiological properties, as previous studies have done, we sought to elucidate the ecological 'strategies' employed by these mutants. Specifically, we conducted several experiments to measure the *net* death rate (i.e., death rate minus growth rate) of the starvation-selected mutants and their progenitors during prolonged starvation under several treatments. In one experiment, we compared the death rates of the mutants and progenitors in pure culture. This experiment tests whether the mutants have evolved a greater intrinsic ability to tolerate prolonged starvation. In other experiments, we compared death rates measured in various mixtures to determine whether the mutants have a lower net death rate in the presence of their progenitors, and whether the mutants cause the progenitors to die faster than their intrinsic rate. The former effect would suggest a cross-feeding relationship, whereas the latter effect would indicate an allelopathic interaction. We also sought to determine whether starvation-selected mutants are competitively inferior to their progenitors under conditions of abundant nutrients. To that end, we placed the mutants and their progenitors in competition with one another for fresh medium.

Materials and methods

Bacterial strains: The bacterial strains used as progenitors in this study have been described previously (Lenski *et al.* 1991). Briefly, they are all asexual clones of *Escherichia coli* B, and are stored in glycerol at −80°C. The five progenitors are referred to in this paper simply as 'original' strains O1, O2, O3, O4 and O5, and have strain numbers REL1176, REL1180, REL1192, REL1193 and REL607, respectively. Progenitor O5 is an Ara^+ strain that has no prior history of adaptation to the DM25 medium used in this study. The other four progenitors are Ara^- strains that evolved independently for 300 days (= 2000 generations) in the same DM25 medium used in this study (see below) except by serial transfer into fresh medium every day.

The five mutants are designated in this paper as S1, S2, S3, S4 and S5, where S denotes 'starvation'-selected and the numeral corresponds to the original progenitor. These mutants have strain numbers REL6426, REL6432, REL6430, REL6434 and REL6428, respectively. All five mutants retain the Ara marker state of their progenitors, but each one has noticeable and heritable differences in colony morphology (when spread on TA agar plates: see below) that allow it to be distinguished from its progenitor. Like their progenitors, the mutants are stored at −80°C.

Media and culture conditions: The same liquid culture medium was used in all experiments, including for prolonged starvation as well as growth. That medium is Davis minimal broth supplemented with 25 μg of glucose per ml (Carlton and Brown 1981; Lenski 1988), which we refer to as DM25. Experimental populations were maintained in a shaking incubator at 120 rpm and 37°C. In all experiments, bacteria were enumerated by standard dilution methods on tetrazolium arabinose (TA) agar plates (Lenski 1988). In experiments in which mutants and their progenitors were mixed, the two types were distinguished by differences in their colony morphology on these plates.

Prior to starting every experiment, the following procedures were performed to ensure that all strains were similarly acclimated, so that any reproducible difference must be heritable. Each mutant or progenitor was removed from the freezer; an aliquot of the freezer stock was transferred into a rich broth (LB), where the cells grew for 24 h; that culture was diluted 10,000-fold into DM25, where the cells grew for another 24 h; and then the experiment proper began.

Derivation of starvation-selected mutants: The starvation-selected mutants were obtained in either one (S1, S2 and S4) or two (S3 and S5) stages. First, a number of strains, including O3 and O5, were subjected to starvation for 30 days in DM25 without added nutrients. Preliminary characterization of isolates derived from this first-stage experiment found no compelling evidence for reductions in their death rates measured in pure culture. Therefore, we began a second stage of starvation selection. A number of strains—including O1, O2, O4, and first-stage isolates of O3 and O5—were inoculated into fresh DM25 and then incubated for 49 days, again without adding any nutrients. (The use of serew-capped culture tubes during both stages minimized evaporation, but some occurred nonetheless; sterile distilled water was added to cultures periodically to maintain the original volume. It is unlikely that the bacteria experienced anaerobic conditions, because the culture tubes were opened every second day, there was substantial head space above the culture medium, and the maximum density supported by DM25 is only $\sim 5 \times 10^7$ cells per ml.)

Figure 1 shows the overall population dynamics of the second-stage starvation-selection experiment for the five populations that gave rise to the mutants S1–S5, based on

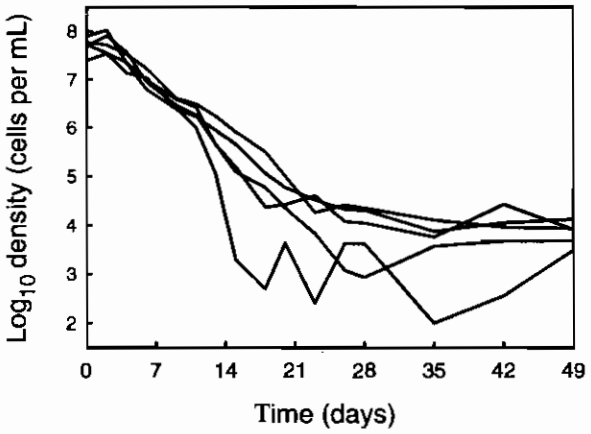

Figure 1. Dynamics of the five populations during the starvation-selection experiment. Each population is indicated by a separate line; the population densities have been $\log_{10}$-transformed. The populations were founded with five different strains of *E. coli* B, which had already diverged from one another as part of another evolution experiment (Lenski *et al.* 1991). At day 49 of the starvation-selection experiment, we isolated from each population a clone that showed a heritable difference in colony morphology from its progenitor.

colony counts obtained on TA agar. All of the populations declined by several orders of magnitude, with the steep decline commencing a few days after the experiment began, but then decelerating about day 30 and levelling off at a density of $\sim 10^4$ cells per ml. Mutants S1–S5 were all obtained on day 49 of the experiment, and they were chosen on the basis of atypical colony morphology (relative to their progenitors) as well as their survival to this point of the experiment. The atypical colony morphology bred true when these mutant clones were restreaked onto fresh TA agar, and these morphological differences gave us confidence that indeed we had genetic mutants (as opposed to any progenitor cells that might have survived without mutations).

It should be noted that the population dynamics observed during prolonged starvation in this minimal medium (figure 1) are qualitatively similar to—but quantitatively very different from—those reported in rich medium (cf. figure 1 in Zambrano *et al.* 1993). In both media, there was a period of stasis around the initial density, followed by a decline and then levelling off at a lower density. However, the decline was much more severe, and the levelling off took much longer to occur, in minimal than in rich medium.

Estimation of death rates: Three different experiments were performed to compare the death rates of mutants and their progenitors under various treatments. All three experiments were performed with cultures in screw-capped tubes grown at 37°C in DM25. Each experiment lasted 15 days; this duration was chosen because it includes a substantial portion of the numerical decline (figure 1), but it does not extend into the period when mutants with altered colony morphologies became abundant in the selection experiment. Samples from experimental populations were spread every second day on TA agar plates, starting the first day after they grew to stationary phase. The natural logarithm of the viable population density (based on colony-forming units) was regressed against time, and the time-averaged death rate was estimated as the slope of the regression. This procedure provides an estimate of the *net* death rate, i.e. the rate of death minus any residual growth. The first experiment compares the death rates of the starvation-selected mutants and their progenitors in pure culture (i.e. each in the absence of the other). The second experiment compares death rates of the mutants measured at two initial frequencies (10% and 90%) in mixed culture with the progenitors. The third experiment compares the death rates of the progenitors in the absence and presence (50%) of their derived mutants. For the last two experiments, the treatment frequencies are based on the ratio of the stationary-phase culture volumes at the time of mixing.

Measurements of cell size: We used a Coulter particle counter (model ZM and channelyzer model 256) to measure average cell volume for each mutant and its progenitor. The raw data were edited to remove debris in very small-size channels (Lenski and Bennett 1993). Measurements were made 24 h after each strain was diluted 1:100 into fresh DM25 medium; this corresponds to about 8–16 h after the bacteria exhausted the glucose in DM25 and entered into stationary phase (Vasi *et al.* 1994).

Estimation of relative fitness in fresh medium: To test whether the starvation-selected mutants had become less fit than their progenitors in fresh medium, we performed short-term competition experiments in DM25 (Lenski *et al.* 1991). In brief, each mutant and its progenitor were separately grown for one day in DM25, then they were mixed 1:1 volumetrically and diluted 1:100 in DM25. An initial sample of the mixture was diluted and spread on TA agar, and a final sample was similarly taken after 24 h. During this period, the mixed population increased ~100-fold in density before entering stationary phase; no discernible death occurs during early stationary phase (Vasi *et al.* 1994). For each competitor, we calculated its net rate of population increase from its initial and final densities, and we then computed relative fitness as the ratio of these values for the derived mutant and its progenitor (Lenski *et al.* 1991).

Results

Ecological strategies of the starvation-selected mutants

Death rate in pure culture: We measured the *net* death rate (i.e. death minus growth) of the starvation-selected mutants and their progenitors in pure culture for 15 days. This experiment was replicated five-fold in complete blocks. Figure 2 shows that two mutants, S1 and S3, had significantly lower death rates in pure culture than did their progenitors; a third mutant, S5, had a marginally significant

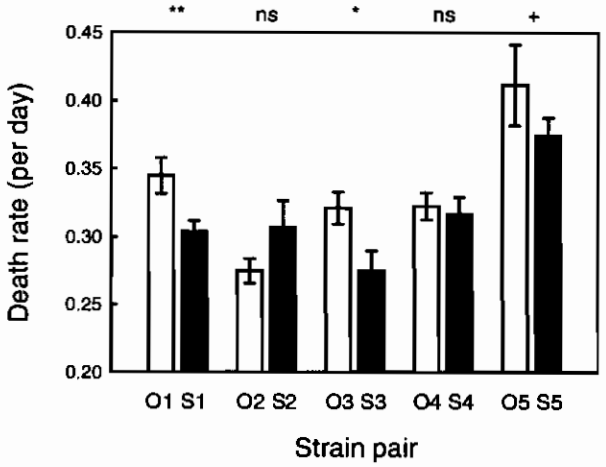

Figure 2. Death rate in pure culture of starvation-selected mutants (S1–S5) and their progenitors (O1–O5). Rates were measured over 15 days. Error bars represent one standard error. For each mutant and progenitor, a one-tailed paired t-test was done to test the hypothesis that the mutant had a lower death rate in pure culture than did its progenitor. Results are summarized above each strain pair by: ns, $0.1 < P$; +, $0.05 < P < 0.1$; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$.

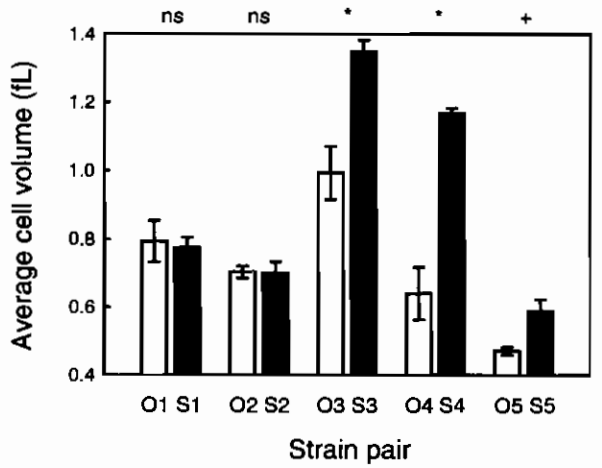

Figure 3. Average cell volume ($1\ \text{fl} = 10^{-15}\ \text{l}$) of starvation-selected mutants (S1–S5) and their progenitors (O1–O5) in early stationary phase. Error bars indicate one standard error. For each mutant and progenitor, a one-tailed paired t-test was done to test the hypothesis that the mutant produced larger cells than did its progenitor. Results are summarized above each strain pair by: ns, $0.1 < P$; +, $0.05 < P < 0.1$; *, 0.01; $P < 0.05$.

reduction. Evidently, at least part of the selective advantage of some of the mutants can be explained without invoking specific pairwise interactions among genotypes, hence implying an evolutionary reduction in the 'intrinsic' death rate.

One plausible explanation for a reduction in the intrinsic death rate is that the starvation-selected mutants might produce larger cells, and contain greater metabolic reserves, than their progenitors. To test this hypothesis, we measured the average cell volume of starvation-selected mutants and their progenitors after they were grown in fresh medium and soon after they entered stationary phase. Replicate estimates of average cell volume were obtained for three independent cultures of each mutant and progenitor in a complete block design. Figure 3 shows that two of the mutants, S3 and S4, made significantly larger eells than their progenitors; the size difference between S5 and its progenitor was marginally significant. However, there was no significant correlation between the evolutionary changes in average cell volume and intrinsic death rate ($r = -0.123$, $n = 5$, one-tailed $P = 0.4223$). Hence, larger cell size does not seem to be the main factor responsible for the lower intrinsic death rate in pure culture of the starvation-selected mutants.

Survival advantage when rare in mixed culture: We compared death rates for the starvation-selected mutants at two different initial frequencies, 10% and 90%, in mixed culture with their progenitors for 15 days. The measurements were replicated five-fold in complete blocks. Figure 4 shows that three mutants—S1, S4 and S5—had significantly lower death rates when they were rare than when they were the predominant genotype in the assays. This outcome suggests a cross-feeding interaction, in which the mutants obtain

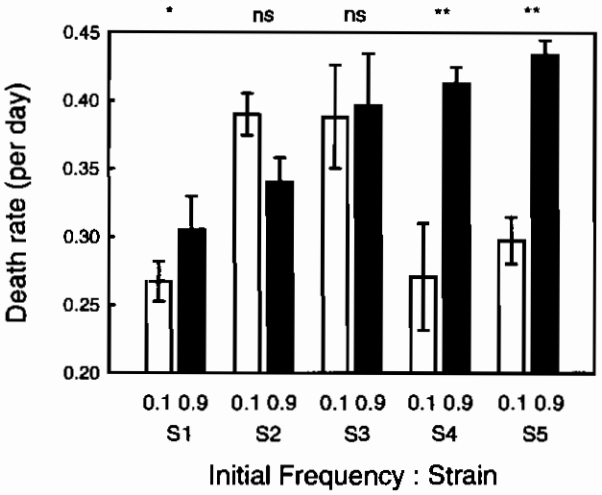

Figure 4. Death rates in mixed culture of starvation-selected mutants (S1–S5) at two different initial frequencies (10% and 90%) with their progenitors. Rates were measured over 15 days. Error bars indicate one standard error. For each mutant, a one-tailed paired t-test was done to test the hypothesis that it had a lower death rate when in the minority (10%) than when in the majority (90%). Results are summarized above each mutant by: ns, $0.1 < P$; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$.

nutrients from their progenitors (including lysed cells) that they cannot obtain so readily from their own type; these nutrients either reduce the death rate of the mutants or permit some offsetting growth.

Killing effect of mutants against their progenitors: We compared death rates over 15 days of the progenitors in pure culture (100%) and in mixed culture (50%) with their derived mutants. Rate estimates were replicated five-fold, but not in a blocked design (the first treatment used data

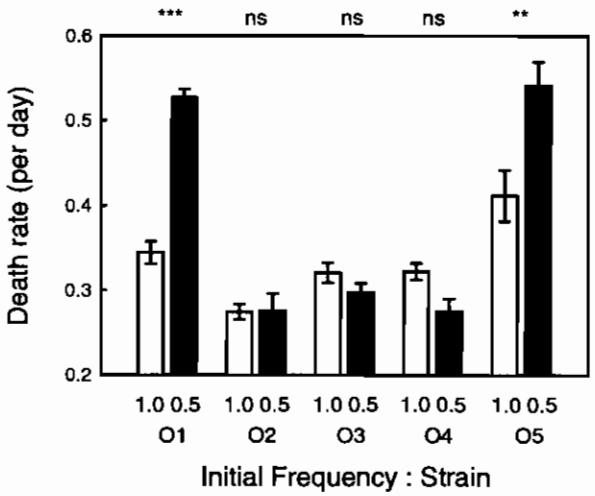

Figure 5. Death rates of progenitors (O1–O5) in pure culture (100%) and mixed (50%) with their starvation-selected mutants. Rates were measured over 15 days. Error bars are standard errors. For each progenitor, a one-tailed t-test was done to test the hypothesis that its death rate was lower when in pure culture (100%) than when mixed (50%) with its derived mutant. Results are summarized above each progenitor by: ns, $0.1 < P$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

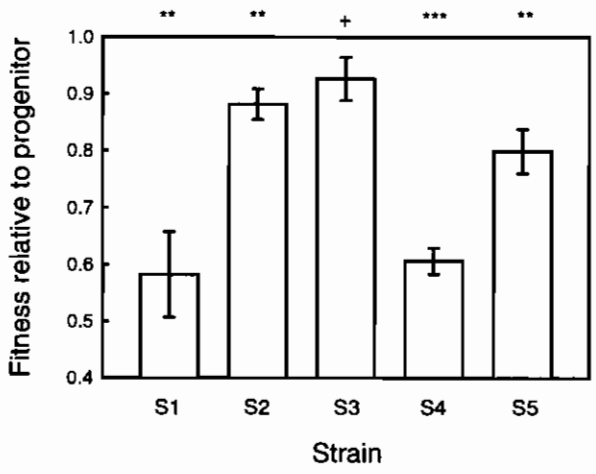

Figure 6. Fitness of each starvation-selected mutant (S1–S5) relative to its progenitor during a one-day competition in fresh DM25 medium. Error bars correspond to one standard error. For each mutant strain, a one-tailed t-test was done to test the hypothesis that its relative fitness in competition for fresh medium was less than 1.0. Results are summarized above each mutant by: +, $0.05 < P < 0.1$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

obtained in the first experiment above). Figure 5 shows that two progenitors, O1 and O5, were subject to significant killing effects; this result indicates that the corresponding derived mutants, S1 and S5, were allelopathic.

Figures 2, 4 and 5 summarize a total of 15 statistical tests: three possible strategies for each of five starvation-selected mutants. It would not be surprising, therefore, if one or two of the tests were significant at the 0.05 level by chance alone. However, seven of 15 tests were significant at the 0.05 level, which itself has an associated probability of < 0.00001 based on the binomial distribution. Thus, the data overall indicate that the mutants have adapted genetically to prolonged starvation.

Starvation-selected mutants are inferior competitors in fresh medium

To address the question of an evolutionary tradeoff in performance between conditions of feast and famine, we estimated the fitness of each starvation-selected mutant relative to its progenitor in fresh DM25 medium. We ran one-day competition experiments in which the combined population grew 100-fold before the glucose was depleted. The competitions were replicated five-fold for each pair of strains. Figure 6 shows that four mutants—S1, S2, S4 and S5—were significantly less fit in competition for fresh medium than were their progenitors; mutant S3 also seemed less fit, but the effect was only marginally significant. Averaging over the five starvation-selected mutants, the mean relative fitness in fresh medium is 0.767 and is significantly below one overall (t statistic = 3.43, four degrees of freedom, one-tailed $P = 0.0133$), despite variation among the five mutants. Evidently, there is a common tradeoff between evolutionary adaptations to prolonged starvation and competitive ability under favourable growth conditions.

Discussion

Previous research on bacterial adaptation to prolonged starvation has focussed on the genetic and physiological properties of starvation-adapted bacteria. In this study, we sought to characterize the ecological strategies employed, and tradeoffs engendered, by *E. coli* mutants selected during prolonged starvation in a minimal medium. Four of the five starvation-selected mutants showed one or more significant advantages (table 1). Mutant S1 exhibited all three ecological strategies that we tested: (i) it had a lower intrinsic death rate in pure culture than did its progenitor; (ii) it had an even lower death rate when mixed with its progenitor; and (iii) it increased the death rate of its progenitor in mixed culture. S5 may also have used all three strategies, although its intrinsic death rate was only marginally significantly lower than that of its progenitor. S3 had a lower intrinsic death rate than its progenitor, while S4 had a significantly lower death rate when it was rare than when it was common. Only S2 showed no significant survival advantage in any of the ecological respects that we tested. Perhaps S2 carries only deleterious mutations. Alternatively, S2 may have certain advantages that are manifest only after very long periods of starvation, because the death-rate assays ran for two weeks, whereas the selection experiment itself lasted seven weeks. In any case, our results confirm prior findings (Siegele and Kolter 1992; Zambrano *et al.* 1993; Zambrano and Kolter 1996) that *E. coli* can evolve enhanced survival capacity under prolonged starvation, and they extend these

Table 1. Summary of the ecological strategies of five starvation-selected mutants of *E. coli*.

	Starvation-selected mutant				
Strategy	S1	S2	S3	S4	S5
Lower intrinsic death rate than progenitor	**	ns	*	ns	+
Survival advantage when rare	*	ns	ns	**	**
Killing effect against progenitor	***	ns	ns	ns	**

ns, $0.1 < P$; +, $0.05 < P < 0.1$; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$. All tests were one-tailed, in the direction predicted by the hypothesis.

findings from a depleted rich medium to a depleted minimal medium. Moreover, our study demonstrates that these starvation-adapted mutants exhibit diverse ecological strategies, either singly or in combination. These diverse strategies presumably indicate distinct mutations in various loci affecting different biochemical pathways, but confirmation of this conjecture awaits formal genetic analysis of the mutants.

Our study also demonstrated an evolutionary tradeoff associated with the mutations that conferred resistance to starvation. Most, if not all, of the starvation-selected *E. coli* mutants were inferior to their progenitors during competition in fresh medium (figure 6). These tradeoffs were typically severe, with the starvation-selected mutants averaging > 20% reductions in their rate of population increase in fresh medium. Tradeoffs in fitness across environments have often been hypothesized as a means for maintaining genetic diversity in spatially and temporally varying environments (e.g. Levins 1968; Slatkin and Lande 1976). Testing for the existence of such tradeoffs is usually difficult, but selection experiments offer a powerful way to do so in suitable organisms such as *Drosophila melanogaster* (Mueller and Ayala 1981; Rose 1984; Rose *et al.* 1987; Chippindale *et al.* 1996) and *E. coli*.

Whether evolutionary tradeoffs exist in *E. coli* appears to depend very much on the nature of the environments that are contrasted. Mutants selected for resistance to certain phages (Lenski and Levin 1985; Lenski 1988) and antibiotics (Schrag and Perrot 1996) are inferior competitors in the absence of those agents. Mutants adapted to low temperature also typically lose fitness at high temperature (Mongold *et al.* 1996), although the reciprocal is not true (Bennett and Lenski 1993). Thus, genetic correlations that govern responses to selection in different environments can be asymmetrical. Indeed, *E. coli* lines that adapted for 2000 generations to an environment with abundant glucose usually became better at surviving prolonged starvation (Vasi *et al.* 1994), whereas we have shown here that most starvation-selected mutants became worse in competition for abundant glucose. Such asymmetrical patterns are unexpected from the standpoint of simple models in which tradeoffs arise from the allocation of internal resources to competing demands. However, asymmetries are less surprising when one considers the several thousand genes and myriad regulatory interactions that are present even in a 'simple' organism like *E. coli*.

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