This is to certify that the

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POPULATION GROWTH, DYNAMICS AND EVOLUTION IN *ESCHERICHIA COLI:* THEORETICAL AND EXPERIMENTAL STUDIES

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Farida Vasi Attar

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Richard E. Lenski
Major professor

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POPULATION GROWTH, DYNAMICS AND EVOLUTION IN *ESCHERICHIA COLI:* THEORETICAL AND EXPERIMENTAL STUDIES

By

Farida Vasi Attar

A DISSERTATION

Submitted to
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DOCTOR OF PHILOSOPHY

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Department of Ecology, Evolution and Behavioral Biology

2000

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ABSTRACT

POPULATION GROWTH, DYNAMICS AND EVOLUTION IN *ESCHERICHIA COLI:* THEORETICAL AND EXPERIMENTAL STUDIES

By

Farida Vasi Attar

Studies of bacterial population growth and dynamics have focused primarily on exponentially growing cells in chemostat cultures. In this dissertation, I focus on aspects of population dynamics that are less well understood, i.e., lag phase and stationary phase, and in a more complicated environment, i.e., batch culture. Simple mathematical models are used to examine the effects of the lag phase, exponential growth rate, resource uptake capacity and death rate on the population dynamics and evolution of *Escherichia coli* in a periodic environment. I report on the following projects. I. Changes in life-history traits during adaptation to a periodic environment. II. Dynamical consequences of the lag phase for population growth in a seasonal environment. III. Two factors that may influence the duration of the lag phase: starvation status and population density. IV. Selection and analysis of life-history traits of mutants selected during prolonged starvation.

I dedicate this d
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I dedicate this dissertation to Aqa Maula Syedna Mohammed Burhanuddin Saheb (T.U.S) and my parents and family, who support me fully in all my endeavors.

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ACKNOWLEDGMENTS

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Chapter 1

LONG-TERM EXPERIMENTAL EVOLUTION IN *ESCHERICHIA COLI*: CHANGES IN LIFE-HISTORY TRAITS DURING ADAPTATION TO A PERIOIDIC ENVIRONMENT*

INTRODUCTION

For decades, a major focus in evolutionary ecology has been to elucidate the adaptive significance of variation in life histories (Fisher, 1930; Cole, 1954; Lack, 1966; MacArthur & Wilson, 1967; Levins, 1968; Pianka, 1970; Luckinbill, 1978; Charlesworth, 1980; Mueller & Ayala, 1981; Reznick, 1983; Rose, 1984; Caswell, 1989; Partridge & Sibly, 1991; Rose, 1991; Stearns, 1992). Here, we intend life-histories to include not only age-specific patterns of survivorship and reproduction but also differences in demographic responses to such environmental factors as population density and resource availability. According to all of these various formulations of life-history theory, a key issue is the relative importance of different forms of reproductive contributions made at different ages or under different ecological circumstances to the overall growth of a population. In an expanding age-structured population, for example, reproduction late in life is discounted relative to reproduction at an earlier age, since the earlier progeny will themselves begin to reproduce sooner than the later progeny (Fisher, 1930; Lenski & Service, 1982).

Beyond distinguishing genetic and environmental influences, a fundamental empirical challenge is determining the extent to which observed variation in life-history

* This chapter is presented verbatim from Vasi *et al.*, 1994.

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traits actually reflects adaptation to different environments. Alternatives to strictly "adaptationist" explanations for variation in life-history (or any other) traits include the effects of independent genetic ancestries, which may constrain subsequent evolution, as well as the effects of stochastic processes such as random mutation and genetic drift, which can cause divergence even when initially identical populations evolve in identical environments (Wright, 1932, 1982; Gould & Lewontin, 1979; Wade, 1979, Cohan, 1984; Barton & Rouhani, 1987; Clarke *et al.*, 1988; Lenski, 1988; Cohan & Hoffman, 1989; Mani & Clarke, 1990; Harvey & Pagel, 1991; Lenski *et al.*, 1991).

We are conducting a long-term experimental study of evolutionary adaptation and divergence, using the bacterium *Escherichia coli*. Twelve populations, each founded from the same clone, were serially propagated for 2,000 generations (300 d) in replicate environments that subjected the bacteria to alternating periods of feast and famine (Lenski *et al.*, 1991). We have previously assessed the rate and extent of genetic adaptation to this seasonal regime (see Stewart & Levin, 1973) by competing the ancestral and derived genotypes in the experimental environment. (The ancestral genotype was stored in a frozen state. Prior to each competition experiment, both genotypes were allowed to acclimate physiologically to the experimental environment.) Relative fitness was calculated as the ratio of the realized Malthusian parameters during the competition experiments. After 2,000 generations, the mean fitness of the derived populations relative to their common ancestor had increased by ~35%. However, any divergence in competitive fitness among the replicate populations was quite small (and might be explained simply by stochastic variation in the timing of equivalent favorable mutations in the replicate populations). In other words, the replicate populations were remarkably similar in the extent of their evolutionary improvement, even though the genetic variation available for selection was derived entirely from new mutations that occurred independently in each population.

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In this article, we seek to identify the changes in life-history traits that were responsible for the demonstrable adaptation of these bacterial populations to the seasonal environment. Our measure of relative fitness takes into account the differential survival and reproductive success of the ancestral and derived genotypes over an entire growth cycle consisting of several more or less distinct phases: (i) a lag phase upon transfer to fresh medium, prior to the commencement of cell replication; (ii) a period of sustained exponential growth, during which the available resource concentration is little affected because of the low population density; (iii) a transition period, in which the limiting resource becomes progressively depleted and the rate of population growth is correspondingly diminished; and (iv) a stationary phase, in which the lack of resources prevents replication but death may occur. The demographic parameters that govern these phases can therefore be regarded as *components* of fitness, whereas fitness itself is integrated over the entire growth cycle (just as the fitness of an organism with a complex life cycle is an integrated function of its age- or stage-specific fitness components). In addition to these fitness components, we measure certain other life-history traits, including cell size and numerical yield in pure culture. We address whether the changes in life-history traits were similar between the replicate populations, or whether the populations achieved similar fitness gains but by quite different underlying changes in their demography. We also examine whether the observed changes in life-history traits correspond to the opportunity for selection on those traits that can be calculated from a simple model of resource-based competition in a seasonal environment.

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MATERIALS AND METHODS

Bacterial Strains

The genotypes used in this study have been described previously (Lenski *et al.*, 1991). Briefly, the common ancestor is a strictly asexual strain of *Escherichia coli* B. The ancestor has two forms, Ara^- and Ara^+, which differ from one another by a single mutation and which can be distinguished by their colony color on tetrazolium-arabinose (TA) indicator agar. Six populations of each arabinose marker type were founded from single cells and then propagated in a serial transfer culture regime (see below). Contamination from external sources was excluded on the basis of genetic markers that distinguished the common ancestor from wild bacteria. Cross-contamination was excluded by propagating Ara^- and Ara^+ lines in a strictly alternating sequence and observing no encroachment of genotypes with the inappropriate marker state. At 2,000 generations (300 d), genotypes were chosen at random from each of the 12 populations. The ancestral and derived genotypes are stored indefinitely at –80°C, so that their properties can be directly compared at any time.

In this study, we used a single clone (genotype) from each of the 12 populations. Previous work has shown that there is very little within-population variation for fitness (Lenski *et al.*, 1991), consistent with the expectation of a purging effect caused by selection in an asexual population. Our interest here is to evaluate the parallelism versus divergence of demographic parameters and other life-history traits for independently derived lineages rather than formally to partition genetic variation into its within- and between-population components.

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Culture Conditions

Unless otherwise noted, the culture conditions used to measure life-history traits in this study are the same as the conditions used during the long-term evolution of the twelve populations (Lenski *et al.*, 1991). Briefly, this standard environment consists of a glucose-limited minimal salts medium (25 μg glucose ml^{-1}) maintained in a shaking incubator at 37°C. Each day, the populations were diluted 100-fold into fresh medium; they then grew until they had exhausted the available resources (within less than 10 h, as compared to the 24-h transfer interval). The 100-fold daily growth corresponds to ~6.6 ($\log_2 100$) generations of binary fission. To ensure that genotypes were comparably acclimated to the experimental regime prior to assays of competitive fitness or any of the life-history traits, we cultured bacteria for 1 day in a rich broth after removing them from the freezer, and then they were conditioned for 1 day (one complete cycle of lag, exponential growth, transition, and stationary phases) in the standard glucose-limited minimal medium.

Assays of Relative Fitness

The fitnesses of the derived genotypes relative to their common ancestor were assayed in competition experiments under the standard culture conditions and using the methods described previously (Lenski *et al.*, 1991). Briefly, in each competition experiment, a derived genotype was competed against the ancestral genotype of the opposite marker state. The Ara marker itself is selectively neutral within ±1% under the standard culture conditions. Following separate conditioning in the standard culture medium, the two competitors were each diluted 1:200 into fresh medium and allowed to compete for 1 day; the competition experiment therefore encompassed the complete cycle of lag, exponential growth, transition, and stationary phases. Initial and final densities of

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each competitor were estimated by plating onto TA agar. The Malthusian parameter for each competitor is given by:

$$m = \log_e \frac{N_f}{N_0}$$

where N_o and N_f are initial and final densities, respectively, and m is therefore a time-average (geometric mean) over the entire growth cycle. The fitness, W, of a derived genotype relative to the common ancestor is expressed as the ratio of their Malthusian parameters during a competition experiment. (Lenski *et al.*, (1991) describe the relationship between relative fitness, which is dimensionless, and the selection rate constant, which has units of inverse time.) Five estimates of W were obtained for each derived genotype in sets of complete blocks. These same formulae were also used to calculate relative fitnesses in numerical simulations (see below).

Estimation of Demographic Parameters and Life-History Traits

Measurements of population density. Estimation of the various demographic parameters required measurements of population density over time. Depending on the particular experiment, we obtained population estimates from viable cell counts based on colony forming units, from spectrophotometric measurements of optical density, or from particle counts using a Coulter electronic particle counter (model ZM and channelyzer model 256). At fairly high cell densities, spectrophotometric measurements are easier to obtain, while particle counts are probably the most accurate method. However, neither spectrophotometry nor particle counts are effective at either very low density of in distinguishing between viable and nonviable cells, when plate counts become necessary.

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Maximal growth rate. The maximal growth rate, V_m, for each genotype was estimated under the standard culture conditions, except that the medium contained 1,000 μg glucose ml^{-1}. (This high glucose concentration is used for estimating V_m in order to be very close to the asymptotic maximal growth rate that is assumed by the Monod model. After the bacteria were conditioned, they were diluted 1:100 into fresh medium, and the population densities were sampled approximately every half-hour by measuring the absorbance (proportional to density) of the culture with a spectrophotometer. The maximal growth rate was estimated by regressing $\log_e$-transformed absorbance against time during exponential growth phase. (Absorbance depends on the size of cells as well as their number; however, this dependence does not affect the estimate of V_m provided that cell size remains constant during exponential growth. We will show later that cell size differs between exponential growth and stationary phases; however, cell size appeared to be constant within the period of exponential growth used to estimate V_m.) Seven estimates of V_m were obtained for each genotype (including both marker states for the ancestor) in sets of complete blocks.

Resource concentration supporting growth at half the maximum rate. The glucose concentration, K_S, that supports growth at half the maximum rate provides a measure of a genotype's affinity for glucose as this limiting resource becomes depleted during the transition phase. Preliminary experiments established 0.1 μg glucose ml^{-1} as a concentration at which all of the genotypes used in this study grew at roughly

$$\frac{V_m}{2},$$

which provided a suitable concentration for accurately estimating K_S. After the bacteria were conditioned at this concentration, they were diluted 1:100 into fresh medium, and the population densities were sampled approximately every half-hour by spreading

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diluted cultures on TA agar. Densities were $\log_e$ transformed, and a sub-maximal rate of increase, V, was estimated for each genotype as the slope of the repression line during exponential growth phase. Using the V_m for each genotype (as estimated above), K_S was then calculated from the model of Monod (1942, 1949):

$$V = V_m\left(\frac{S}{S + K_S}\right)$$

so that

$$K_S = S\left(\frac{V_m}{V - 1}\right)$$

where S is the resource concentration (here, 0.1 μg ml^{-1}). For each genotype (including both marker states for the ancestor), we obtained two estimates of K_S in sets of complete blocks.

Duration of the lag phase. Each genotype was grown under standard culture conditions and densities were estimated approximately every hour, using an electronic particle counter. Cultures were diluted 100-fold into an isotonic solution, and the particles in 0.05 ml of the resulting dilution were counted and sized. The frequency distributions for particle sizes exhibited a distinct trough between background particles and bacterial cells. The distributions were edited to eliminate the background counts, which yielded cell densities that agreed well with densities based on viable plate counts. The duration of lag phase, L, was estimated by subtraction, as follows. First, for each growth curve, we measured the initial population density, N_0. Second, we assumed that a population was near the midpoint of exponential-phase growth by the time that its density was ~$10N_0$. The two population sizes sampled on either side of $10N_0$ were designated N_1 and N_2, respectively, and the corresponding sample times at which they were observed, t_1 and t_2.

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Third, using the V_m and K_S for each genotype (as estimated above), we inferred the hypothetical times, T_1 and T_2, required to reach densities N_1 and N_2, respectively, *if* there was no lag phase prior to exponential growth. That is,

$$T_i = \frac{\log_e(\frac{N_i}{N_0})}{V},$$

where

$$V = V_m(\frac{S}{S + K_S}).$$

Finally, the duration of lag phase was then estimated as the average difference between hypothetical and actual times to reach the densities on either side of $10N_0$:

$$L = \frac{(t_1 - T_1 + t_2 - T_2)}{2}$$

We obtained three estimates of L for each genotype (including both marker states for the common ancestor), in sets of complete blocks.

Death rate during stationary phase. Death rates during stationary phase, D, were estimated for each genotype under standard culture conditions. Death rates were obtained over the period of 11-24 hours after transfer into fresh medium. By 11 hours, all genotypes have entered stationary phase, as indicated by both direct observation of population densities and computation of expected dynamics using the previously estimated growth parameters. Densities of viable cells were measured by spreading diluted samples onto TA plates approximately every 2 hours during this period. The natural logarithm of cell density was regressed against time during stationary phase, and

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the regression coefficient provides an estimate of the corresponding death rate. Three independent estimates of D were obtained for each genotype (including both marker states for the common ancestor), in sets of complete blocks.

Death rate during prolonged starvation. During the 2,000 generations of evolution of the experimental populations, cells were transferred into fresh medium every 24 hours, and so death rates over longer periods of starvation, D', were not directly subject to natural selection. Nonetheless, these death rates might have changed as a correlated response to selection on some other traits, and so we also assayed the death rates of all genotypes over the period of 1-14 days (24-336 hours) after transfer into fresh medium. Densities of viable cells were measured by spreading appropriately diluted cultures onto TA plates at least every other day during this period. The natural logarithm of cell density was regressed against time during prolonged starvation, and the regression coefficient provides an estimate of the corresponding death rate. Four independent estimates of D' were obtained for each genotype (including both marker states for the common ancestor), in sets of complete blocks.

Numerical yield in pure culture. We define the numerical yield, Y, of a genotype as the number of viable cells per unit of limiting resource, when that genotype is grown in isolation (i.e., in the absence of any competitor). The reciprocal of this yield therefore provides a measure of the efficiency of conversion of the limiting resource into cell numbers during population growth. Yields in the standard culture medium were estimated at 24 hours after transfer into fresh medium by viable cell counts on TA agar plates. In principle, one could adjust the yield obtained at the end of the growth cycle for cell death that occurred during stationary phase. In fact, however, there was no discernible cell death during this period (see Results), and so no adjustment was made. Five independent estimates of Y were obtained for each genotype (including both marker states for the common ancestor), in sets of complete blocks.

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Average cell size. Cell sizes were measured electronically (see above), which indicated the volume displaced by a particle rather than its mass. Size distributions were edited to remove background particles, which were generally distinctly smaller than the cells (see above). The particle-sizer was calibrated using the modal size of highly uniform Coulter latex microspheres with 0.82 μm diameter (0.29 fl, where 1 fl = 10^{-12} ml). Three independent estimates of the average cell size at the end of stationary phase, Z_S, where obtained for each genotype (included both marker states for the ancestor), in sets of complete blocks. We also obtained three independent estimates of the average cell size during the middle of exponential-phase growth, Z_e, for each genotype (including both marker states for the ancestor), in sets of complete blocks.

Statistical Considerations

In evaluating whether the derived genotypes had, as a group, changed relative to their common ancestor, the twelve independently derived lineages were the appropriate unit of replication. In this context, the repeated measures obtained for each genotype were averaged, and they served only to improve the realized precision of our measurements. (We also averaged measurements across the two marker-state variants of the common ancestor.) Except for relative fitness, we employed two-tailed *t*-tests for comparing one value (the average of two marker states for the common ancestor) with the mean of many values (for the 12 independently derived genotypes), which provides $n_1 + n_2 - 2 = 11$ *df* (Sokal & Rohlf, 1981, pp. 229-231). For relative fitness, we simply compared the mean for the derived genotypes with the value of 1.0 (by definition, the relative fitness of the ancestor), which also provides $n - 1 = 11$ *df*.

In evaluating whether the derived lineages had diverged significantly from one another, the replicate measures were essential for estimating the relevant error variance.

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Analyses of variance were performed to test for the significance of between-genotype variation, Var_G, which was estimated as the difference in the group and error mean-squares, divided by the number of replicates assays (=blocks) performed per group (Sokal & Rohlf, 1981, pp. 217). Of course, the common ancestor was not included in these ANOVAs, because the question concerns the divergence of the derived lineages.

Model of Bacterial Population Dynamics

The model used in this study is one of resource-based population growth in a seasonal environment (Stewart & Levin, 1973), which has been modified to take into account a lag phase prior to exponential growth and to allow cell death during stationary phase. Let the density of bacteria be denoted by N (cells ml^{-1}) and the concentration of the limiting resource by S (μg glucose ml^{-1}). For numerical analysis, we integrate the dynamics in three parts. The first part consists of the lag phase, during which time we assume that a population neither grows nor consumes resources:

$$\frac{dN}{dt} = \frac{dS}{dt} = 0$$

The second part consists of both the exponential and transitional growth phases, during which time the population increases and resources are consumed at rates depending on the parameters of the Monod model:

$$\frac{dN}{dt} = \left[V_m \frac{S}{(S + K_S)} \right] N$$

and

$$\frac{dS}{dt} = -\left(\frac{1}{Y}\right)\left(\frac{dN}{dt}\right)$$

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where V_m (h^{-1}), K_S (μg glucose ml^{-1}), and Y (cells μg^{-1} glucose) are the maximal growth rate, the resource concentration at which growth rate is half maximum, and the numerical yield, respectively. The third part consists of the stationary phase, during which time the cell population may decline due to death, while the resource concentration remains constant:

$$\frac{dN}{dt} = -DN$$

and

$$\frac{dS}{dt} = 0,$$

where D (h^{-1}) is the death rate.

The first part runs from $t = 0$ to $t = L$, where L (h) is the duration of the lag phase and t is the time after transfer into fresh medium. The second part then runs until $S = 0$, that is, until the resource has been exhausted. The third part runs until $t = 24$ h, at which time the cycle is completed. If two genotypes are competing, they may enter the second part at distinct times, depending on the duration of their lag phases. Because competing genotypes share the same pool of limiting resource, they necessarily enter the third part simultaneously.

In our numerical analyses we sought to duplicate the conditions of our experiments. We therefore began with a medium containing 25 μg ml^{-1}, then added to this $1/100^{th}$ of a corresponding volume of a stationary phase cell culture. Thus the initial resource concentration and population density were

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In the case of two competing genotypes, each one has an initial density of only

$$N(0) = 0.005\ Y(25).$$

Equations were integrated numerically using SOLVER.SWV, which uses the fourth-order Runge-Kutta method of integration and allows discrete switch-points in dynamic models (Blythe *et al.*, 1990). We used time-steps of 0.0005 - 0.01 h in the numerical integrations, depending on the precision required for solution of a particular problem. The criterion for the end of the second part, $S = 0$, is approached asymptotically in a truly continuous model, so that one must begin the third part when the resource concentration reaches some arbitrary threshold criterion. In a simulation involving two competing genotypes, their relative fitness over the entire cycle can be expressed using the same formulae used to estimate relative fitness empirically (see above).

RESULTS

Ancestral Traits and Opportunity for Selection

Table 1 gives estimates of the growth parameters for the common ancestor. The maximal growth rate V_m corresponds to a doubling time ($\log_e 2 / V_m$) of a little less than one hour. The duration of the lag phase prior to growth, L, is well over one hour. Also

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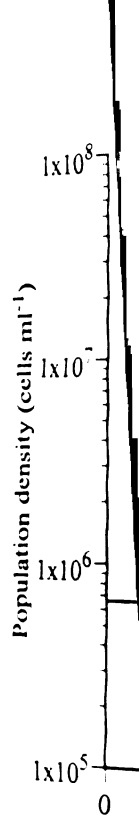
Table 1. Ancestral Fitness Components and Proportional Selection Gradients

Fitness Component	Ancestral Value, X	Selection gradient, $(X/W)(\partial W/\partial X)$	Maximum Fitness
L	1.5264 h	-0.2554	1.3051 ($L = 0$)
V_m	0.7726 h^{-1}	1	∞ ($V_m = \infty$)
K_S	0.0727 $\mu g\ ml^{-1}$	-0.0066	1.0069 ($K_S = 0$)
D *	(0) h^{-1}	0	1 ($D = 0$)

* The actual estimate of D was negative, but this value was not significantly different from zero. The population dynamic model does not allow negative death rates (i.e., growth after the glucose has been exhausted), and so D has been set to zero in these analyses.

K_S is very low relative to the initial resource concentration (25 μg glucose ml^{-1}); in fact, the ancestral genotype is predicted to grow at half its maximum rate when 99.7% of the glucose has been used. The estimated death rate during stationary phase, D, is slightly negative, suggesting the possibility of continued growth at a slow rate (and hence some deficiency in the model of population dynamics). However, this trend is not statistically significant. (It is also possible that some cell death occurred but was offset by continued cell division. However, the methods we employed do not allow this distinction to be made.) Figure 1 shows a numerical simulation of the dynamics of the ancestral genotype and the limiting resource over the course of one 24 hour cycle, assuming neither growth nor death during stationary phase.



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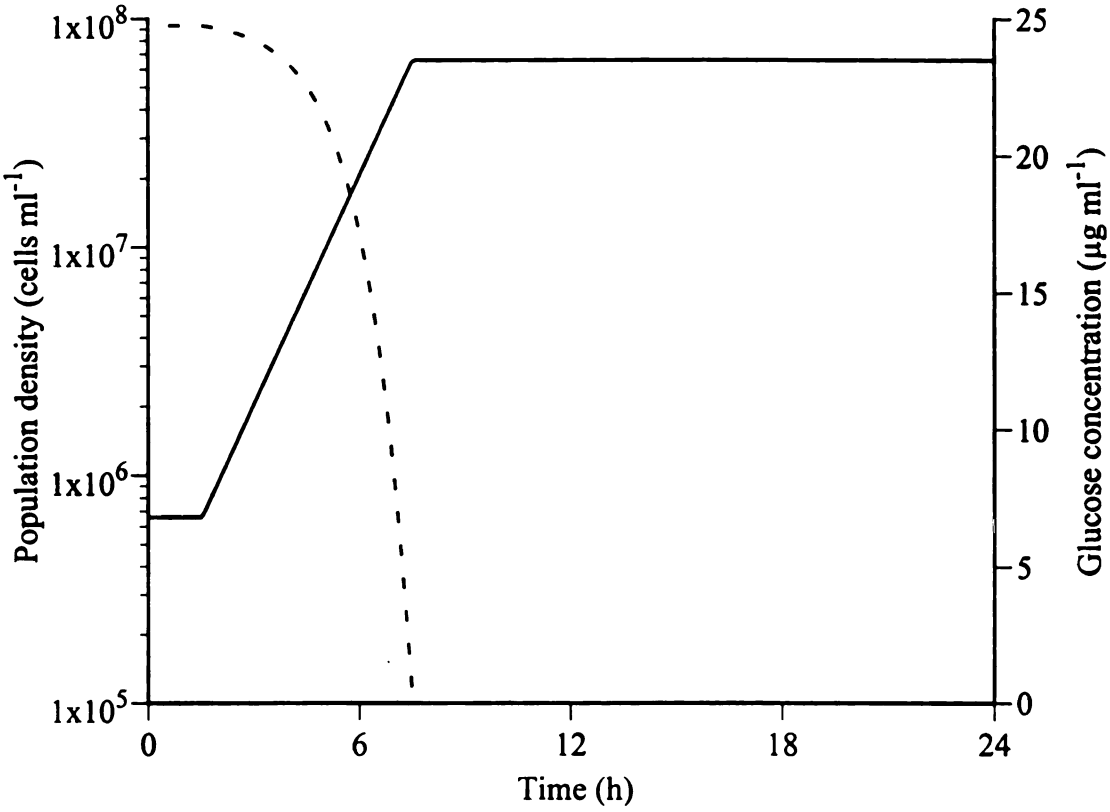

Figure 1. Numerical integration of the dynamics of a bacterial population (left axis, solid line) and the limiting resource (right axis, dashed line), using the parameters estimated for the common ancestor (Table 1) and the standard culture conditions.

Some, but not all published estimates of K_S for *E. coli* strains grown in minimal media with glucose as the sole carbon source are more than an order of magnitude higher than our own estimates (Monod, 1949; Shehata & Marr, 1971; Luckinbill, 1984); but see Shehata & Marr 1971 for an estimate within a few percentage points of our estimate). Therefore, we were concerned with the possibility that our method of estimating K_S might somehow be biased. One possible bias is that we might have measured cell division without concomitant cell growth, which may occur transiently after transfer into fresh medium. This explanation would imply that the ancestral genotype could not indefinitely sustain population growth consistent with our estimate of K_S. A second possible bias is that we might have measured growth on some organic contaminants of the medium or the glassware, or slight growth on citrate that is included in the medium

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(which some bacteria other than *E. coli* can utilize as a carbon source), rather than on glucose. This explanation would imply that our ancestral genotype could grow at a similar rate in a medium with less or even no added glucose. We excluded both of these artifacts by showing that our bacteria can indefinitely sustain a 100-fold daily increase at glucose concentrations 0.04 μg ml^{-1} and higher, but not at concentrations of 0.02 g ml^{-1} and below. The 100-fold daily increase implies $V > \log_e 100 / 24\ h = 0.192\ h^{-1}$. Given that $V = V_m S / (S + K_S)$ and with $V_m = 0.77\ h^{-1}$, this rate of increase can be sustained in medium that contains 0.04 μg glucose ml^{-1} only if $K_S < 0.12$ μg glucose ml^{-1}. By the same logic, the failure of the bacteria to persist in medium containing 0.02 μg glucose ml^{-1} implies that $K_S > 0.06$ μg glucose ml^{-1}. If K_S were lower than this value, then the bacteria should have persisted even at this low concentration. We conclude that our low estimate of K_S is quite accurate.

One can formalize the intensity of selection acting directly on each fitness component as follows. We begin with a genotype having the growth parameters estimated for the common ancestor (except death rate during stationary phase, which is set to zero, since the model does not allow for continued growth after the limiting resource has been exhausted). We then introduce a second genotype that is identical except for a very small change in one parameter. Next, we simulate competition between these two genotypes over an entire growth cycle and compute the fitness of the improved genotype relative to the ancestor, as described in the Materials and Methods. The selection gradient for any trait X is defined as the partial derivative of fitness with respect to that trait (cf. Lande, 1982), $\partial W / \partial X$, which we have obtained by the limit of $\Delta W / \Delta X$ as X goes to zero. This gradient therefore reflects the direct selection acting on each fitness component, with the other components held constant. (In effect this method ignores any interaction between the demographic parameters by assuming that each one can be changed independently of the others.) To facilitate comparisons among the

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selection gradients for the several fitness components (which have different units), we have scaled these to reflect the proportional sensitivity of fitness to each component, rendering them all dimensionless quantities:

$$G_X = \left(\frac{X}{W}\right)\left(\frac{\partial W}{\partial X}\right),$$

where $W = 1$ for the ancestor (by definition). (One can obtain the proportional selection gradients for L and V_m analytically; D. E. Dykhuizen, personal communication.) The proportional selection gradients are given in Table 1.

The proportional selection gradients are equivalent to "elasticities" in population projection matrices (deKroon *et al.*, 1986) and to "control coefficients" in biochemical models of metabolic flux (Kacser & Burns, 1979; Dykhuizen *et al.*, 1987). It is frequently the case in such analyses that the sum of the proportional sensitivities is equal to one (deKroon *et al.*, 1986; Dykhuizen *et al.*, 1987; Mesterton-Gibbons, 1993); however that is not always true, and is demonstrably not the case for the proportional selection gradients considered here (Table 1). The failure of the proportional sensitivities to sum to one implies that W is not a homogeneous function of the fitness components (Mesterton-Gibbons, 1993); this inhomogeneity may be a consequence of the nonequilibrium nature of the population dynamics in a seasonal environment.

The proportional selection gradient for the maximum growth rate, V_m, is about four times steeper than the corresponding gradient for the duration of lag, L, and more than 100 times steeper than the gradient for K_S, which governs the reduction in growth rate as the resource becomes depleted. The death rate, D, cannot be reduced below zero according to the model, and so its proportional selection gradient is zero.

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The selection gradient for the numerical yield, Y, is also zero. This result may seem puzzling at first glance, but in fact it is straight-forward: any increase in the efficiency of conversion of resources into cell numbers that does *not* simultaneously increase growth rate cannot enable one genotype to out-compete another, because such a change would (in a physically unstructured environment) concomitantly leave more resource for use by the competitor. One might reformulate the model by replacing V_m in the equation of population growth with the product of a maximum rate of resource uptake ($U_m = V_m/Y$) and yield (Y), such that selection would act both on U_m and Y. We have not done so because we did not measure U_m.

In addition to these proportional selection gradients, we can calculate the theoretical maximum improvement in fitness that could be achieved by changing any one of the parameters from the ancestral state (Table 1). There is no theoretical limit to V_m, nor to the fitness that can be achieved by increasing V_m. But L, K_S, and D cannot be less than zero. Hence, there is a maximum improvement in fitness that can be achieved by changing any of these parameters, with the greatest room for improvement in L (~30%), only slight room for improvement in K_S (<1%), and no room whatsoever for improvement in D.

Of course, average cell sizes (Z_S and Z_e) and the death rate during prolonged starvation (D') do not enter directly into the dynamic model, and hence selection gradients are not applicable, unless specific couplings between fitness components and these traits are assumed. Also, these selection gradients do not take into account possible tradeoffs or other couplings between the demographic parameters that do enter into the model. However, one can readily see that selection might favor an allele that improved one of the parameters subject to strong selection (e.g., V_m), even if it compromised one of the other parameters subject to weaker selection (e.g., K_S).

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Changes in Life-History Traits

Table 2 compares the average values of the life-history traits for the ancestral and derived genotypes. The derived genotypes improved, on average, by ~35% in fitness relative to the common ancestor. Contributing to this fitness increase were ~15% improvement in the maximal growth rate (V_m) and ~20% shorter lag phase (L). The resource concentration at which growth rate was half maximum (K_S) was ~20% higher in the derived genotypes, which has a negative impact on fitness. The death rate during stationary phase (D) did not change significantly. Thus, the improvements were in the two demographic parameters with the greatest opportunity for selection.

The numerical yield (Y) of the derived genotypes was reduced by ~30% relative to the common ancestor. That is, whereas the derived genotypes increase in abundance relative to the common ancestor during competition (as indicated by their higher relative fitness), the derived genotypes yield *fewer* cells per unit resource when they are grown in isolation. At least two distinct hypotheses could account for this result: the derived genotypes may produce larger cells, so that the total biovolume is not reduced; or in the course of their more rapid growth, the derived genotypes may burn the glucose less efficiently or produce more metabolites that inhibit growth, so that the total biovolume is also reduced. Table 2 shows that the derived genotypes were in fact, ~85% larger in average cell volume in stationary phase (Z_S). Thus, the total biovolume produced over the entire growth cycle (expressed as the product of Y and Z_e, was even greater (~100%) than the difference in stationary phase.

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Table 2. Changes in Fitness Components and Other Life-History Traits during 2,000 Generations of Evolution in a Seasonal Environment

Life-History Trait	Mean for Common Ancestor	Mean for Derived Genotypes	t_s	P
Relative fitness				
W	(1)	1.3486	12.450	***
Fitness components:				
L (h)	1.5264	1.2470	-2.458	*
V_m (h^{-1})	0.7726	0.8887	5.504	***
K_S ($\mu g\ ml^{-1}$)	0.0727	0.0880	2.215	*
D (h^{-1})	-0.0127	0.0029	1.243	ns
Other life-history traits:				
Y ($\times 10^6\ \mu g^{-1}$)	2.6352	1.8386	-5.137	***
D' (h^{-1})	0.0216	0.0156	-2.745.	*
Z_S (fl)	0.3546	0.6549	4.412	**
Z_e (fl)	0.5678	1.1357	4.704	***

NOTE. -- For all traits except relative fitness, the null hypothesis is that the mean values are equal for the common ancestor and the derived genotypes. For relative fitness, the null hypothesis is that mean fitness of the derived genotypes is 1. Significance is based on two-tailed probability of rejecting the null hypothesis using the t-distribution with 11 degrees of freedom. ns $P > 0.05$, * $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, *** $P < 0.001$.

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Although the death rate during stationary phase (D) did not change significantly, the derived genotypes had ~30% lower death rate during prolonged starvation (D').

Parallelism Versus Divergence in Life-History Traits

Table 3 summarizes the ANOVAs to test for variation among the twelve independently derived genotypes in each of the fitness components and other life-history traits. There was no significant heterogeneity among the derived genotypes in their fitnesses relative to the common ancestor (W).

The derived genotypes also showed no significant genetic variation in their maximal growth rates (V_m), which contributed to their fitness improvement, or in the resource concentrations that allow half maximum growth rate (K_S), which were uniformly worse than that of their ancestor. There was, however, significant heterogeneity in the duration of lag phase (L), which improved, on average, relative to the ancestor, and in death rates during stationary phase (D), which did not change on the average from the ancestral state.

Among other life-history traits, numerical yield (Y), death rate during prolonged starvation (D'), and average cell sizes (Z_S and Z_e) all exhibited significant heterogeneity among the independently derived genotypes.

Sufficiency of the Demographic Model

Are the demonstrable improvements in maximal growth rate (V_m) and duration of lag phase (L) sufficient to explain the ~35% improvement in fitness relative to the common ancestor? Using our demographic model, we simulated competition between a genotype with the ancestral parameters and each of 12 genotypes with parameters

Table 3. Genetic Variation in Fitness Components and Other Life-History Traits Among the 12 Independently Derived Genotypes

Table 3. Genetic Variation in Fitness Components and Other Life-History Traits Among the 12 Independently Derived Genotypes

	Group		Block		Error			
Life-history traits	MS	df	MS	df	MS	df	Var_G	*P*
Relative fitness:								
W	7.63×10^{-3}	11	5.77×10^{-3}	4	6.27×10^{-3}	44	2.72×10^{-4}	ns
Fitness components:								
L (h)	3.58×10^{-2}	11	2.05×10^{0}	2	1.54×10^{-2}	22	6.79×10^{-3}	*
V_m (h^{-1})	2.88×10^{-3}	11	1.47×10^{-2}	6	2.55×10^{-3}	66	4.67×10^{-5}	ns
K_S ($\mu g\ ml^{-1}$)	9.58×10^{-5}	11	1.82×10^{-4}	1	1.08×10^{-4}	11	(0)	ns
D (h^{-1})	4.33×10^{-4}	11	8.10×10^{-4}	2	1.81×10^{-4}	22	8.40×10^{-5}	*

Table 3. (cont'd)

Table 3. (cont'd)

Other life-history traits								
Y (μg^{-1})	1.11×10^{11}	11	4.20×10^{11}	4	4.46×10^{10}	44	1.33×10^{10}	*
D' (h^{-1})	1.75×10^{-5}	11	2.34×10^{-6}	3	1.17×10^{-6}	33	4.09×10^{-6}	***
Z_S (fl)	1.28×10^{-2}	11	3.40×10^{-3}	2	6.12×10^{-4}	22	4.07×10^{-3}	***
Z_e (fl)	4.04×10^{-2}	11	1.23×10^{-2}	2	4.64×10^{-3}	22	1.19×10^{-2}	***

NOTE. -- MS and df indicate mean squares and degrees of freedom, respectively. The between-genotype variance component, Var_G, is estimated as $(MS_{group} - MS_{error}) / n$, where n is the number of replicate assays (= blocks) per group. Estimates of Var_G may be negative but the true Var_G must be zero or positive. Level of significance for rejecting the null hypothesis that $Var_G = 0$ is based on the F-test, with degrees of freedom corresponding to the group and error mean squares. ns $P > 0.05$, * $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, *** $P < 0.001$.

corresponding to the independently derived types. Overall, the mean fitness of the derived competitors relative to their common ancestor, using the model and the corresponding parameter estimates, was 1.21, compared with the mean fitness of 1.35 obtained from the actual competition experiments. For all 12 derived genotypes, the simulated fitness was less than the actual fitness, which is highly unlikely by chance ($p = 0.5^{12} < 0.001$).

One potentially artifactual explanation for this effect is that all of the simulations use the same parameters for the common ancestor, and so all share some errors in estimation that might produce concordance in the deviations between simulated and actual competitions. This explanation is not tenable, however, since the uncertainties in the parameter estimates for the ancestral genotype are too small to account for any of the deviations between simulated and actual fitnesses. To see this, recall that the fitness components for the common ancestor were independently estimated for each of the two marker-state variants of the common ancestor (Ara^- and Ara^+). The simulated relative fitness for these two ancestral competitors differs by only ~0.01 from the measured relative fitness (data not shown), whereas the simulations between the derived and ancestral genotypes differ by > 0.09 in all twelve cases. Evidently, the discrepancy between the relative fitnesses predicted by the estimated parameters in the simple model of competition and the observed fitnesses is real, with the model accounting for ~60% [(1.21 - 1) / (1.35 - 1)] of the actual improvement in competitive ability. In the Discussion, we will consider possible explanations for the remaining 40% of the fitness gain.

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Genetic Correlations between Traits

We calculated the product-moment correlations between the estimated values of the eight fitness components and other life-history for the 12 independently derived genotypes. Only two of the 28 pairwise correlations are individually significant, and only one of these (between Z_S and Z_e) is significant at $P < 0.05$ after correcting for multiple tests using the sequential Bonferroni criterion (Rice, 1989). However, these genetic correlations between extant populations do not necessarily tell us very much about the correlated responses that occurred within populations in the past.

DISCUSSION

We have been investigating the changes that occur during long-term evolution in a simple experimental system. Twelve bacterial populations, founded from the same ancestral genotype, were propagated for 2,000 generations in replicate environments, which subjected the bacteria to alternating periods of feast and famine. The derived genotypes increased in their competitive fitness in this seasonal environment by ~35%, on average, relative to their common ancestor (Lenski *et al.*, 1991). There was very little divergence among the derived genotypes in their competitive fitnesses relative to one another, despite their dramatic gains relative to their common ancestor and even though their improvements depended entirely on new mutations that occurred independently in the replicate populations (Lenski *et al.*, 1991). However, the populations may have attained similar fitnesses, but by different underlying changes in their life-histories.

Therefore, in this study, we sought to determine which demographic parameters were responsible for the improved competitive fitness of the derived genotypes relative to

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their common ancestor, and whether similar life-history changes had occurred among the independently evolving populations. We also addressed whether the observed changes corresponded to those expected from the relative opportunities for selection acting on the various parameters in a simple mathematical model of resource-based population growth in a seasonal environment. In addition, we examined whether the observed changes were sufficient, within the context of this model, to account for the improvements in fitness that were measured by the competition experiments.

Changes in Fitness Components and Other Life-History Traits during Adaptation to a Seasonal Environment

We believe it is important to distinguish two types of life-history traits. First, there are fitness components per se, which are those demographic parameters that directly determine rates of change in the relative abundance of competing genotypes. If one has sufficient knowledge of these demographic parameters, then one should be able to predict the outcome of competition between genotypes, provided that one's demographic model is sufficiently realistic to encompass the relevant dynamics. In age-structured models of life-history evolution, the age-specific survivorship and fecundity schedules provide these fitness components (Service & Lenski, 1982). In models based on Lotka-Volterra dynamics, the fitness components are subsumed by exponential growth rates, carrying capacities, and competition (Pianka, 1970; Gill, 1972). In this paper, fitness components are represented by the terms defining population dynamics in a resource-based model of competition in a seasonal environment: the duration of lag (L), the maximum growth rate (V_m), the resource concentration at which the growth rate is half maximum (K_S) and the death rate during stationary phase (D). Fitness itself is therefore some function of these parameters, but it need not be equally sensitive to all of them. Moreover, the effect of

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each parameter on fitness will depend upon features of the environment (including the initial resource concentration, the dilution factor, and the frequency of transfers into fresh medium) as well as on the values of the other parameters.

Second, one may believe that other traits underlie fitness (based on intuition, empirical data, or other knowledge of the organism), but lack any theoretical basis to predict the outcome of competition. Traits such as body size or running speed, for example, may correlate with, and even predict empirically, survival and reproductive success. But these traits do not enter into any population dynamic model, except to the extent that they may be used to predict one or more fitness components that do (Arnold, 1983).

Populations of *Escherichia coli* that were maintained for 2,000 generations in a seasonal environment showed systematic changes in several fitness components as well as other traits that are presumably physiologically relevant to these components. In particular, the derived genotypes adapted by increasing their maximum growth rates (V_m) and by reducing their lags prior to growth upon renewal of a previously exhausted resource (L).

The derived genotypes also had increased the resource concentration necessary to sustain growth at half the maximum rate (K_S), seemingly a maladaptive response. However, the growth rate asymptotically approaches V_mS/K_S, as S goes to zero, so that K_S becomes more important at low resource concentrations, but V_m does not become any less important. In fact, the average ration, V_m/K_S, for the derived genotypes is very close to that ratio for the ancestral genotype. Thus, the derived genotypes are not actually less fit that the ancestors, but they are better adapted to exploiting abundant glucose.

The derived genotypes also showed no improvement in their death rate during stationary phase (11-24 h); if anything, they are worse than their progenitor in this

respect. However, the derived genotypes do have improved survival during prolonged starvation (1-14 d). But this trait cannot be considered a fitness component per se, since it is manifest only in environments different from the environment in which the populations evolved. (Had they evolved in an environment in which transfers were made only every 14 days, then this would properly be a fitness component and would enter into a dynamic model of competition in that environment.)

The derived genotypes also had much lower numerical yields (Y) than their common ancestor. Yield enters into the dynamic model of competition, but it does not directly affect fitness in a mass-action environment. However, in a structured environment, in which the probability of extinction of a local deme or the number of dispersing propagules may be functions of population size, numerical yield could well affect the outcome of competition. Our results therefore suggest a possible conflict between intrademic selection, favoring demographic traits that evidently correlate with a reduction in numerical yield, and interdemic selection, favoring higher (Wade, 1979; Wade, 1980). This reduction in numerical yield is evidently due to a change in the size of the individual bacteria, which became systematically larger in the derived genotypes, rather than by less efficient conversion of resource into biomass. In fact, the total biovolume, expressed as the product of numerical yield and average cell size at stationary phase (YZ_S), was greater for the derived genotypes than for their common ancestor.

Our preconception had been that evolving cells would become smaller (and more numerous), in order to increase the ratio of surface area to volume and hence the effective rate of uptake of the limiting resource. But clearly, this preconception was wrong. We can imagine at least four (post hoc) explanations for the evolution of larger cell size. First, survival during stationary phase may have improved for larger cells. The derived genotypes, which had larger cells, survived better during prolonged starvation. However, there was no measurable cell death (or improvement therein) over the length of stationary

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phase that the cells experienced during their experimental evolution. Hence, this hypothesis cannot account for the evolution of larger cells, even if it does plausibly explain the observed reduction in death rate during prolonged starvation. Second, larger cell size may provide the physiological basis for the shorter duration of lag phase upon transfer into fresh medium. That is, larger cells may be less depleted of metabolic reserves during stationary phase (even if the smaller celled ancestor does not actually die), which would allow the derived genotypes to respond more quickly upon renewal of resource. Third, larger cells, by virtue of their greater surface area, may have a higher rate of resource uptake per cell (rather than per unit volume), which might enable the derived cells to sequester resources that could later be converted into progeny. This explanation is consistent with the difference in average cell size between exponential and stationary phases, which is larger in the derived genotypes than in the common ancestor; this difference may account for some of the increase in relative fitness (see below). However, Luckinbill (1984) observed a similar evolutionary trend toward larger cells, even in populations that never experienced stationary phase or resource limitation, that does not support any of the preceding hypotheses. Finally, cell size may have increased simply because of its allometry with growth rate. Several studies of *E. coli* and related bacteria have documented an allometric relationship between growth rate and cell size, in which growth rate is manipulated *phenotypically* by altering the environment (Schaechter *et al.*, 1958; Neidhardt *et al.*, 1990). Our results indicate a *genetic* relationship between growth rate and cell size. This hypothesis suggests experiments to determine whether the observed genetic relationship is simply an extension of the phenotypic relationship or whether the allometry between growth rate and cell size has been genetically altered.

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Did all 12 independently derived genotypes exhibit the same suite of adaptive changes in their life-histories, or did they find alternative solutions to the challenges imposed by the seasonal environment? At first glance, our results seem to imply substantial divergence of the derived genotypes in the life-history bases of their improved fitness. Two of four fitness components, and all four of the other life-history traits show statistically significant heterogeneity (Table 3). However, it is equally important to emphasize that the *direction* of evolutionary change was highly parallel for most of the fitness components and other life-history traits. Three of the four fitness components and all four of the other life-history traits showed significant directional trends in their mean values (Table 2). In fact, all 12 independently derived genotypes showed the same outcome of higher V_m, higher K_S, lower D', lower Y, higher Z_S, and higher Z_e than their common ancestor. And 11 of the 12 derived genotypes had lower L and higher D than the ancestor. Thus there was near-perfect uniformity in the directional responses exhibited by replicate populations.

An index of the relative extent of divergence versus parallelism is given by the following ratio:

$$I_X = \frac{\sqrt{\mathrm{Var}_G(X)}}{|\Delta X|}$$

where $\mathrm{Var}_G(X)$ is the between-genotype variance for trait X, and ΔX is the average change from the common ancestor. (By using the standard deviation corresponding to the genetic variance, both the numerator and denominator have the same units.) This index therefore provides a measure of the average difference among the independently derived genotypes relative to the average evolutionary change from the ancestral state.

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Table 4. Divergence versus Parallelism in Fitness Components and Other Life-History Traits for the Independently Derived Genotypes

Life-History Trait, X	$\mathrm{Var_G}(X)$	ΔX	$\sqrt{\mathrm{Var_G}(X)}/\lvert\Delta X\rvert$
Relative Fitness:			
W	2.72×10^{-4}	0.349	0.047
Fitness Components:			
L (h)	6.79×10^{-3}	-0.279	0.295
V_m (h^{-1})	4.67×10^{-5}	0.116	0.059
K_S ($\mu g\ ml^{-1}$)	(0)	0.015	0
D (h^{-1})	8.40×10^{-5}	0.016	0.573
Other life-history traits:			
Y (μg^{-1})	1.33×10^{10}	-7.97×10^{5}	0.145
D' (h^{-1})	4.09×10^{-6}	-0.006	0.337
Z_S (fl)	4.07×10^{-3}	0.300	0.213
Z_e (fl)	1.19×10^{-2}	0.568	0.192

NOTE.—$\mathrm{Var_G}(X)$ is the between-genotype variance for trait X (Table 3), and ΔX is the mean change from the common ancestor (Table 2). The ratio $\sqrt{\mathrm{Var_G}(X)}/\lvert\Delta X\rvert$ provides a measure of the average difference among derived genotypes (divergence) relative to the average change from the ancestral state (parallelism).

For all eight fitness components and other life-history traits, this index is much less than one (Table 4), which indicates that the differences among the independently derived genotypes are small relative to the average change in these characters from the

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ancestral state. However, the independently derived genotypes are somewhat more variable in most of these traits than in their relative fitnesses (also based on this index), which implies that they have acquired slightly different adaptations to a seasonal environment. Evidently, the 12 replicate populations underwent similar adaptations in terms of the fitness components and other life-history traits, but these were not identical, as indicated by heterogeneity among the independently derived genotypes for some of the characters.

Adequacy of the Dynamic Model Used to Amalgamate Fitness Components into a Measure of Relative Fitness

The population model that we have used might be regarded as explanatory in two different ways. First, how well does the model predict the outcome of competition between ancestral and derived genotypes, when the relevant demographic parameters for each genotype are estimated independently of the competition experiments? Second, did the model provide any indication of the most likely evolutionary changes in these parameters?

After 2,000 generations of evolution, the mean fitness of the derived genotypes relative to their common ancestor was ~1.35, whereas the predicted mean fitness based on the model and independently estimated parameters was only ~1.21. In other words, the simple model of resource-based competition in a seasonal environment accounts for only about 60% (0.21/0.35) of the observed adaptation. The discrepancy is not simply due to a lack of statistical resolution, because all 12 derived genotypes gave actual and predicted relative fitnesses that differed in the same direction and by amounts greater than can be accounted for by uncertainty in the demographic parameters.

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We see two types of limitation of the simple model that might account for the unexplained portion of the fitness differential between the ancestral and derived genotypes. First, the description of the mechanistic basis for competition may be inadequate. The model used here assumes that competition is mediated entirely by scramble competition for glucose. However, bacteria may excrete metabolites that act as either secondary resources or inhibitors of growth, and these metabolites may differentially affect competing genotypes (Helling *et al.*, 1987; Levin, 1988). If excreted metabolites are involved, then they must work to the advantage of the derived genotypes, because the actual fitness advantages are greater than explained solely on the basis of glucose-mediated competition. However, the fact that the population densities were fairly low (~5 x 10^5 cells ml^{-1} to ~5 x 10^7 cells ml^{-1} over the course of the growth cycle) in the evolution experiment tends to weaken this explanation, because the concentration of metabolites should be proportional to density. However, one can imagine experiments (employing conditioned media or varying the initial frequency of competitions) to test this possibility further.

Second, the description of the bacterial demography may be inadequate to encompass all of the relevant dynamics. For example, the model implicitly assumes that the rate at which bacteria increase in numbers is strictly proportional to the rate at which they remove resources from the medium and, furthermore, that these two processes are synchronous. The numerical yield, Y, scales the conversion of resources into bacterial cells during exponential growth. But Y was estimated from stationary-phase populations, which are composed of much smaller cells than exist during exponential phase (Table 2). It seems likely, therefore, that the bacteria are taking up resources during exponential growth faster than the ratio V_m / Y ($\mu g\ h^{-1}$ per cell) would imply. If so, then a more appropriate measure of maximum growth rate (in terms of accounting for the observed fitness improvement) would be

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$$V_{m,adj} = \frac{V_m Z_e}{Z_S}$$

The product $V_m Z_e$ reflects the rate of increase in biovolume during exponential phase growth, whereas the denominator indicates the eventual (but asynchronous) conversion of this biovolumetric increase into cells of size Z_S. $V_{m,adj}$ therefore takes into account the more rapid accumulation of biomass by the bacteria than would be apparent from their numerical increase during exponential phase. We calculated $V_{m,adj}$ for each genotype and then used these adjusted maximum growth rates (along with the other demographic parameters) to compute the expected fitnesses of the derived genotypes relative to their common ancestor. The mean expected fitness using the adjusted maximum growth rates was 1.36, which was indistinguishable from the mean value of 1.35 measured in the competition experiments ($P > 0.5$ based on a t-test for paired comparisons). Thus, the differential underestimation of resource accumulation for the ancestral and derived genotypes can plausibly explain the "missing" 40% of the improvement in fitness. We recognize that this adjustment will give an incorrect trajectory for cell numbers during growth phase. Our intention here is not to provide a realistic and complex description of population dynamics but rather to suggest a possible explanation for the deviation between relative fitnesses estimated by direct competition experiments and those predicted from the simple demographic model. To develop a more formal model along the lines of this adjustment, one might model population biomass (rather than cell numbers) or otherwise incorporate more complex functional relationships between substrate concentration, growth rate and numerical yield.

The other application of the demographic model was to infer the relative intensities of selection acting on the several fitness components. According to the model, selection to increase maximum growth rate (V_m) and to reduce the duration of lag prior to growth in fresh medium (L) was much stronger than selection to reduce the concentration

of resource required to support half of the maximum growth rate (K_S) and to reduce death rate during stationary phase (D). Indeed, we observed a good correspondence between these theoretical selection gradients and the actual responses to selection: both V_m and L improved substantially during the experimental evolution, whereas neither K_S or D showed any improvement.

We note also, that V_m and L promote exploitation of the resource when it is abundant, whereas K_S and D are relevant only when the resource has become depleted. Therefore, it is fair to say that the bacteria in this study have adapted to the "feast," but not the "famine," aspect of the seasonal environment. But it is also important to emphasize that the opportunity for selection to act on the various demographic parameters (Table 1) depends on the initial state of the life-history, as well as on the environment. Had either K_S or D been much higher (worse) initially, their corresponding selection gradients would have been steeper and there would have been more room for improvement in these fitness components. As it was, however, K_S and D were both already so low for the ancestral genotype that selection to improve them was very weak. The fact that our ancestral bacterium was already so well adapted to famine conditions may suggest strong selection in the past for the corresponding fitness components (see, e.g., Koch, 1971; Koch, 1985; Mikkola & Kurland, 1992).

Of course, a genetic response depends not only on the intensity of selection but also on the availability of genetic variation for that trait and on genetic correlations between traits. Had we not observed significant improvements in either strongly selected fitness component (V_m and L), this hypothetical outcome might have been due to either a lack of genetic variation or a tradeoff with another fitness component. The fact that there was substantial genetic variation in these fitness components (and no evidence for intractable tradeoffs) is presumably because the experimental conditions represent a novel environment for these bacteria, whereas in the ancestral environment one would

expect those substitutions that improve fitness to have been largely exhausted by prior selection (Service & Rose, 1985; Lenski *et al.*, 1991; Bennett *et al.*, 1992).

In contrast to the results reported here, (Dykhuizen & Hartl, 1981) observed significant improvements in both V_m and K_S for *Escherichia coli* that evolved in glucose-limited continuous culture (chemostats). This difference in outcomes is not unexpected, however, in light of the difference in experimental environments imposed. In continuous culture, bacteria hold the equilibrium concentration of limiting resource, S^*, to a value that is exactly sufficient to offset washout from the vessel (Stewart & Levin, 1973; Hansen & Hubbell, 1980). In the study by Dykhuizen and Hartl (1981), the flow rates through the culture vessels, f, were 20% or 40% of the founding bacterium's maximal growth rate, V_m. Given that $V = V_m S^* / (S^* + K_S) = f$, then S^* would have been either 0.25 K_S or 0.67 K_S according to the Monod model. Here V is the actual Malthusian parameter in continuous culture, and so the proportional selection gradients for V_m and K_S differ by only a factor of

$$\frac{\left[\left(\frac{V_m}{V}\right)\left(\frac{\partial V}{\partial V_m}\right)\right]}{\left[\left(\frac{K_S}{V}\right)\left|\frac{\partial V}{\partial K_S}\right|\right]} = \frac{\left(S^* + K_S\right)}{K_S} = \frac{S^*}{K_S} + 1 .$$

Thus, the proportional selection gradients for V_m and K_S in the study by Dykhuizen and Hartl (1991) differed by only a factor of 1.25 to 1.67 (depending on flow rate), in contrast to the >100-fold difference in the corresponding gradients in the seasonal environment used in this study (Table 1).

Also using *E. coli* in a glucose-limited medium as a model system, Luckinbill (1978, 1984) employed two serial transfer environments to test for specific adaptations to *r*- and *K*-selection regimes. In the *r*-selection regime, bacteria were kept in a state of

perpetual exponential growth by repeated transfers well before the populations had reached stationary phase; in the *K*-selection regime, bacteria were allowed to exhaust the glucose and enter stationary phase before they were transferred into fresh medium. Luckinbill demonstrated improvements in relative fitness in both environments, but he found that the adaptations were non-specific with respect to the selection regime. That is, bacteria that had evolved in the *K*-selection environment did as well, on average, in the *r*-selection environment as bacteria that had evolved in the *r*-selection environment, and vice versa. But the *K*-selection regime employed by Luckinbill was, in fact, quite similar to the seasonal environment used in this study. And as we have discussed, this seasonal environment selects most strongly for a higher maximal growth rate, with much weaker selection for any traits that might be construed as *K*-selected (at least when the ancestral genotype has life-history components similar to the strain used in this study). So Luckinbill's intended *K*-selection regime may, in fact, have been another strongly *r*-selecting regime. The changes in bacterial fitness components reported by Dykhuizen and Hartl (1981) and by Luckinbill (1978, 1984), as well as those seen in this study, appear to be consistent with the relative intensities of selection acting on those traits. These analyses indicate to us the utility of an explicit demographic model for interpreting evolutionary changes in life-history traits.

In conclusion, 12 bacterial populations that evolved independently for 2,000 generations in replicate seasonal environments underwent a similar suite of changes in their life histories. The derived genotypes responded more quickly to resource renewal and they had higher maximum growth rates than their common ancestor. Both of these adaptations facilitate resource exploitation during the periods of feast in the seasonal environment. But the derived genotypes showed no corresponding improvement in those fitness components that would allow them to better tolerate the periods of famine that they also experienced. The differential adaptation to periods of feast and famine can be

understood in terms of the relative opportunities for selection to act on the different fitness components. In particular, the ancestral genotype used in this study was already so well adapted to the periods of resource deprivation imposed by the experimental regime that there was almost no room for improvement in the corresponding fitness components, whereas there was substantial room for improvement in the fitness components that mattered during the periods of resource abundance. Accompanying these demographic adaptations was a dramatic change in the morphology of the bacteria, with the derived genotypes having much larger cells than their common ancestor. The significance of this morphological change with respect to their newly evolved life-history is unclear.

Chapter 2

THE LAG PHASE OF *ESCHERICHIA COLI* IN A PERIODIC ENVIRONMENT: PREDICTIONS AND TESTS OF A MATHEMATICAL MODEL.

INTRODUCTION

The lag phase of the bacterial growth cycle describes the initial delay in the start of exponential growth when bacteria encounter a fresh source of nutrient after a period of starvation (Neidhardt *et al.*, 1990). The lag is an intrinsic characteristic of a microbial population, and it introduces a time delay in the response of individual bacteria to environmental change. This time delay is caused by physiological changes necessary before the bacteria are able to start growing and dividing. These changes include a dismantling of the stationary phase apparatus (Huisman *et al.*, 1996), as well as gearing up the nutrient transport functions and metabolic systems involved in growth and cell division (Cooper, 1991; Huisman *et al.*, 1996). The lag phase has also been recently shown to be dependent on the population density of the bacterial culture (Fuqua *et al.*, 1994; Fuqua *et al.*, 1996; Fuqua & Green, 1998).

Time delays have long been of considerable interest to population ecologists, as a potential explanation for complex fluctuations observed in natural and laboratory populations. For example, blowflies (*Lucilia cuprina*) exhibit oscillations produced by a time lag between the response of life-history traits, such as fecundity and mortality, to the population density (Nicholson, 1958). When food is abundant and adults are numerous,

plenty of eggs are laid. However, when food runs out the eggs fail to develop, resulting in a population crash. Time delays in reproduction may also result from the storage of nutrients when resources are abundant. *Daphnia* species exhibit population cycles as energy is stored as lipid, which is then used when food supplies become scarce due to high population densities (Goulden & Hornig, 1980; Goulden *et al.*, 1982; Tessier *et al.*, 1983).

Such intrinsic or density dependent causes may interact with extrinsic or environmental causes of periodicity to result in complex dynamics. Simple theoretical treatment of populations has shown that time delays may interact with periodic environments to produce a range of complicated dynamics from limit cycles to chaos (May, 1974; May, 1981; Nisbet & Gurney, 1982). The range of dynamical behaviors produced by the interaction of intrinsic and extrinsic causes of periodicity is most pronounced in models which include population structure (Caswell, 1989b; Constantino *et al.*, 1997; Tuljapurkar & Caswell, 1997). Cell cycle based demographic models for phytoplankton growing in a periodic environment demonstrated non-periodic cycling at frequencies different from that of the forcing variable (Pascual & Caswell, 1997). Adding a nutrient storage based time delay term to the model also produced similar results.

Laboratory populations of *Escherichia coli* provide a useful system in which to test the interaction of intrinsic and extrinsic causes of periodicity, due to their short generation times and relative ease of replication within treatment populations. *Escherichia coli* populations also exhibit population structure, as the probability of division in individual cells may vary with the time since last division (age-structure) or the cell-size distribution (stage-structure) (Bramhill, 1997). A number of hypotheses have been proposed for the mechanistic basis for this time dependence. These range

from internal causes, such as the DNA replication cycle and cell division system, to external factors, such as population density and mineral or nutrient abundance. Recent advances in the understanding of the regulation of bacterial cell division suggests that these mechanistic bases may be related through regulatory feedbacks (Rothfield *et al.*, 1999).

In this chapter, I present a simple mathematical model for the growth of bacterial populations in a periodic environment. In this model, the limiting resource is depleted by the organisms and then replenished at some later time, where replenishment is driven by some external periodicity. I assume that there is a lag phase of constant duration, L time units, such that any population that exhausts the limiting resource requires L time units after resource renewal to begin growing again. I show by numerical simulations that the existence of this lag phase may interact with the environmental periodicity to generate dynamics ranging from simple equilibria to high order periodic cycles. Finally, I present and analyze the results of some experiments with populations of *E. coli* that were designed to determine if such complex dynamics, could, in fact, be seen in a biological system.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions.

The *E. coli* B (REL606) strain used in this study has been used in several other ecological and evolutionary studies in our laboratory (Lenski *et al.*, 1991; Lenski & Travisano, 1994; deVisser *et al.*, 1999). Its growth parameters have been characterized in

previous work (Vasi *et al.*, 1994). All experiments were started from a single clone isolated from a culture stored indefinitely at -80° C.

Bacteria were grown in Davis minimal medium (DM) (Carlton & Brown, 1981) supplemented with 25 μg ml^{-1} glucose in a shaking incubator at 37°C. The populations were diluted 100-fold into fresh medium, where they typically went through an entire growth cycle consisting of lag, exponential and stationary phases, and exhausted the available resources; when transfers were very frequent, the populations might still been growing when diluted into fresh medium. The populations typically grew from an initial population density of ~5 x 10^5 cells ml^{-1} to a final density of ~5 x 10^7 cells ml^{-1}. The 100-fold daily growth corresponds to ~6.6 ($\log_2$ 100) generations of binary fission. To ensure acclimation to the experimental regime, bacteria were cultured for one day in DM supplemented with 1000 μg ml^{-1} glucose after removal from the freezer. They were then conditioned for one day in DM supplemented with 25 μg ml^{-1} glucose.

Model of Bacterial Population Growth in a Periodic Environment.

The model used in this study is one of resource-based population growth (Monod, 1949; Kubitschek, 1970; Stewart & Levin, 1973; Hansen & Hubbell, 1980; Tilman, 1982), modified to take into account a lag phase prior to exponential growth. This model has been used in previous work with this strain (Vasi *et al.*, 1994). The density of bacteria is denoted N (cells ml^{-1}) and the concentration of the limiting resource S (μg glucose ml^{-1}).

The bacterial growth cycle is integrated in three discrete parts, as illustrated in Figure 2. The first part consists of the lag phase, during which a population neither grows nor consumes resource:

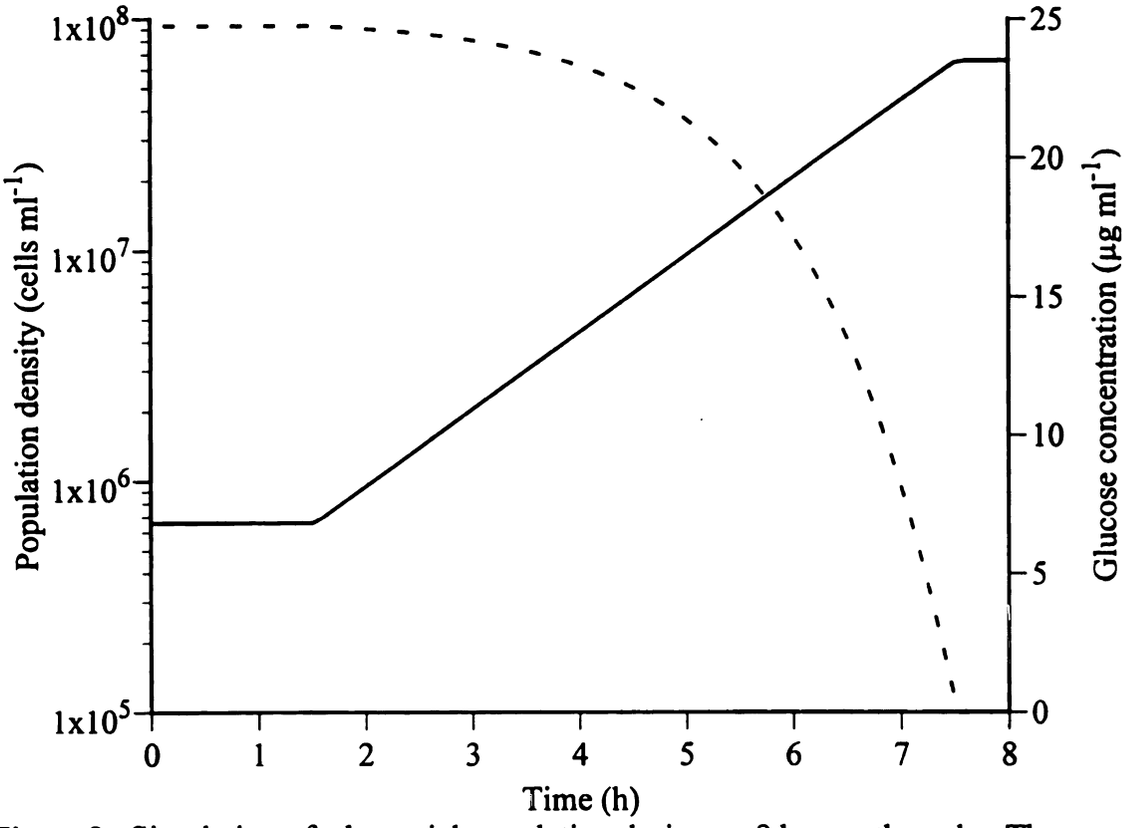

Figure 2. Simulation of a bacterial population during an 8 h growth cycle. The curves represent bacterial density plotted on a logarithmic scale (solid line) and the resource concentration plotted on a linear scale (dashed line).

$$\frac{dN}{dt} = \frac{dS}{dt} = 0.$$

The second part consists of the exponential phase, during which the population increases and resources are consumed at rates depending on the parameters of the Monod model:

$$\frac{dN}{dt} = \left[V_m \frac{S}{(S + K_S)} \right] N,$$

and

$$\frac{dS}{dt} = -\left(\frac{1}{Y}\right)\left(\frac{dN}{dt}\right).$$

V_m (h^{-1}) is the maximal growth rate, K_S (μg glucose ml^{-1}) is the resource concentration at which the growth rate is half of maximum and Y (cells μg^{-1} glucose) is the numerical yield. The numerical yield is the number of viable cells produced by the population per unit of limiting resource utilized during a growth cycle, when all resources are depleted. The third part consists of the stationary phase, during which time the cell population may decline due to death, while the resource concentration remains constant:

$$\frac{dN}{dt} = -DN$$

and

$$\frac{dS}{dt} = 0.$$

Model parameters were estimated in previous work (Vasi *et al.*, 1994); the estimates of the parameter values used for the simulations are repeated in Table 5. In numerical analyses, I duplicated the experimental serial transfer conditions so that direct comparisons with experiments could be made. In experimental tests of the periodic model, populations were started by inoculating $1/100^{th}$ volume of a 10 ml stationary phase culture into 9.9 ml of medium containing 25 μg ml^{-1} glucose. Therefore, each nutrient cycle in each simulation was begun with medium containing 99% of 25 μg glucose ml^{-1} ($S = 0.99(25) = 24.95$ μg ml^{-1} glucose) to which 1/100th of the density of a stationary phase culture was added. The initial population density was given by $N = 0.01\ Y(25)$. The equations were simulated with time steps of 0.001 h, using the Euler method.

Table 5. Model parameter values, using parameter estimates from Vasi *et al.*

Parameter	Mean
L	1.5264 h
V_m	0.7726 h^{-1}
K_S	0.0727 μg glucose ml^{-1}
Y	2.6352 x 10^6cells μg^{-1} glucose
D^*	(0)

* The actual estimate of D was negative but its value was not significantly different from zero. D was set to zero in this model.

The first part runs from $t = 0$ to $t = L$, where L (h) is the lag time and t is the time after transfer into fresh medium. The second part runs until one of the following conditions are met:

$t = t_m$ where t_m is the length of the transfer cycle. In this case, the population does not go through the third part.

$S \sim 0$ (or the arbitrarily low threshold of 10^{-6} μg ml^{-1} glucose).

The third part runs from the end of the second until $t = t_m$. After each nutrient cycle, the populations are diluted into fresh medium once again.

The simulations used nutrient cycles that ranged in duration from t_m = 5 h to 8 h in increments of 0.01 h. Each population was simulated for 1000 transfer cycles (6.00 –

6.4 h), or 50 cycles (5.00 – 5.99 h and 6.40 – 8.00 h). All simulations ran long enough to give a complete picture of the population dynamics for each nutrient cycle.

Experimental Tests of the Periodic Model.

Thirty replicate bacterial populations were maintained in serial transfer culture for approximately 100 hours, with six populations at each of five transfer cycles: 5, 6, 7, 8 or 9 h. Pairs of replicate populations were maintained in a staggered fashion over the nutrient cycles, to minimize correlated errors due to uncontrolled environmental variation (e.g., slight fluctuation in temperature). For example, in the 5 h treatment one pair of replicates was initiated at $t = 0$ h. The second pair was started at $t = 5$ h, and the final pair was begun at $t = 10$ h. Bacterial densities were estimated prior to transfer into fresh media every cycle using an electronic particle counter. Cultures were diluted 100-fold into an isotonic medium. Bacteria in 0.05 ml of the resulting dilution were counted using a Coulter particle counter, and the average cell size was measured twice. The frequency distribution for particle sizes exhibited a distinct trough between background particles and bacterial cells. The distributions were edited to eliminate the background counts. Each edited distribution provided estimates of the population density and total biovolume. The two estimates were averaged to get final estimates of the population density and total biovolume.

Autocorrelation analyses

Autocorrelation analyses were carried out on the populations growing in 7, 8 and 9 h nutrient cycles. The beginning of each series was removed for all autocorrelation analyses to remove any transient effects. The autocorrelation at time interval k is simply

the Pearson correlation between a series of data at time t, and the same series at time $t+k$ time units. It is calculated as the ratio of covariance between data at time t and $t+k$ and the variance of the data at time t (Box, 1994). Autocorrelation plots showing the autocorrelation at time intervals 1 - 8 h were computed separately for each population. The autocorrelation coefficients were averaged across the 6 replicate populations for each time interval. Each autocorrelation coefficient was compared with 0 using one tailed t-tests; I used one-tailed tests because I was specifically looking for negative autocorrelations indicative of cycling. Bonferroni corrections for multiple tests were applied to the significance probabilities to correct for 8 tests applied within each treatment (Rice, 1989).

RESULTS

Simulations of the Periodic Model for Bacterial Population Growth.

Figure 3(a) shows the results of simulations for populations that are transferred at 5 h intervals. The first cycle exhibits a lag phase, in which no growth occurs. After 5 h, the population is in mid-exponential phase. The population goes through a 100-fold dilution and is transferred to fresh medium. As the population has not entered stationary phase in the previous cycle, there is no lag phase. The population continues to grow exponentially throughout the second batch, but at the end of 10 h the population is less dense than at 5 h. It is once again diluted into fresh medium and this cycle continues. The population is unable to keep up with successive dilutions every 5 h and eventually goes extinct.

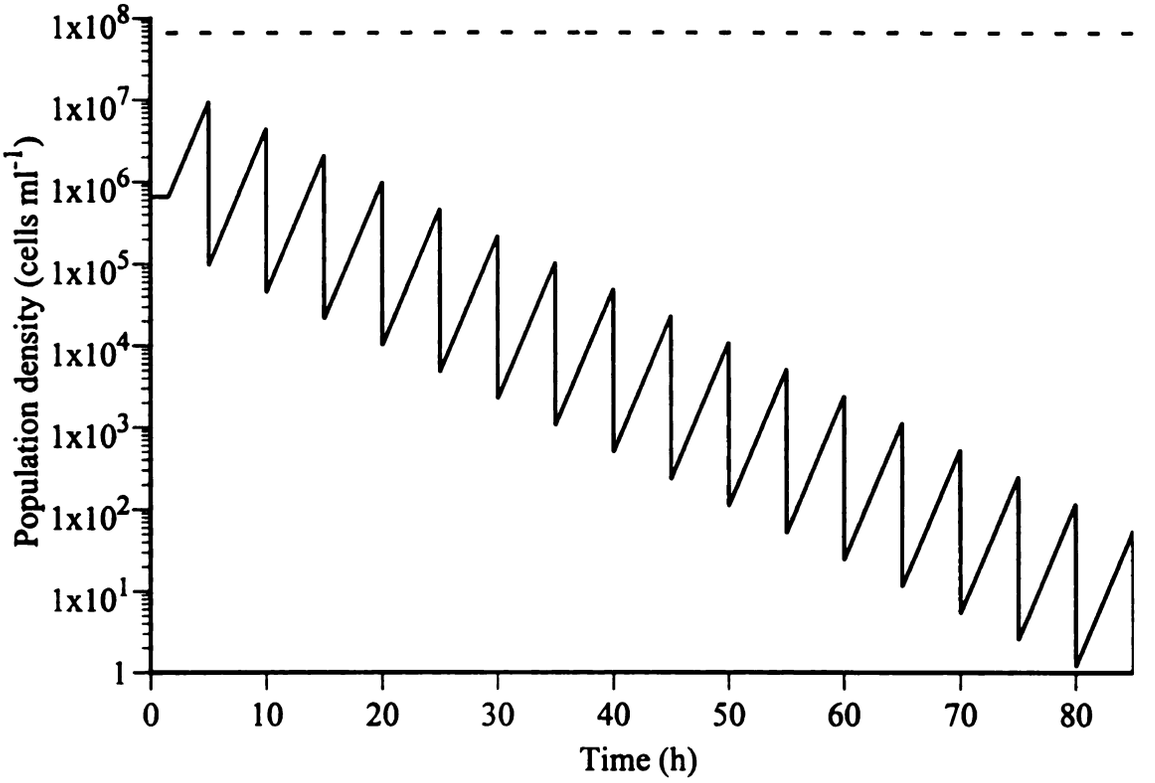

Figure 3(a). Simulation results of a bacterial population growing in batch culture with transfers every 5 h (solid line). The population does not grow enough in each batch cycle to make up for the dilution, and goes extinct. The expected endpoint stationary phase density is also shown (dashed line).

When populations are transferred at 6 h intervals, the dynamics are different. Figures 3(b) and (c) show the early and late portion of the trajectory of a population transferred at 6 h intervals. In Figure 3(b) the first cycle has a lag phase, and again at the end of 6 h the population is in mid-exponential phase. Following dilution, the population continues to grow exponentially, and at 12 h, it reaches a density that is slightly higher than at 6 h. The population is diluted into fresh medium, and continues to grow exponentially, reaching a density at 18h which is higher than at 12 h. At the end of each cycle, the population density is slightly greater than at the end of the previous cycle. The expected end-point stationary density (25* $Y = 6.59 \times 10^7$ cells ml^{-1}) is shown with a dashed line on both graphs. The density of the population growing in 6 h cycles

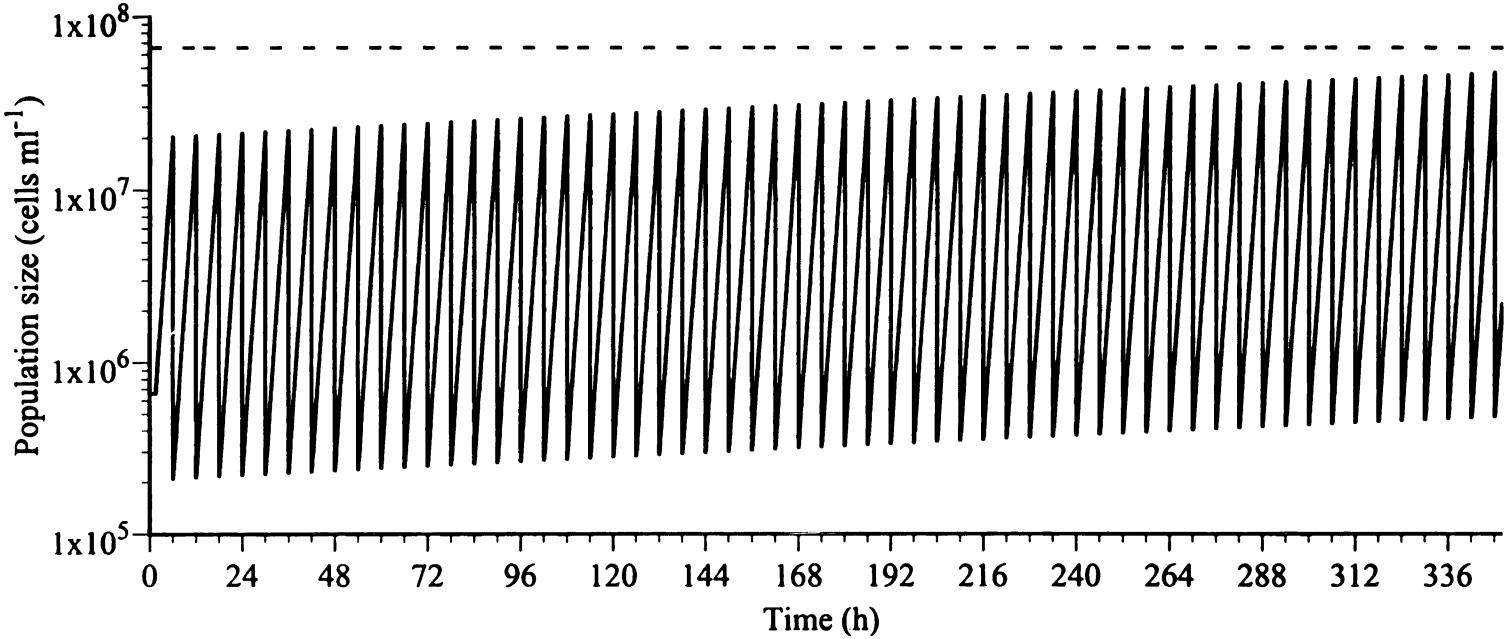

Figure 3(b). Simulation results of a bacterial population (solid line) growing in batch culture with transfers every 6 h from 0 – 350 h. The expected endpoint stationary phase population density is also shown (dashed line).

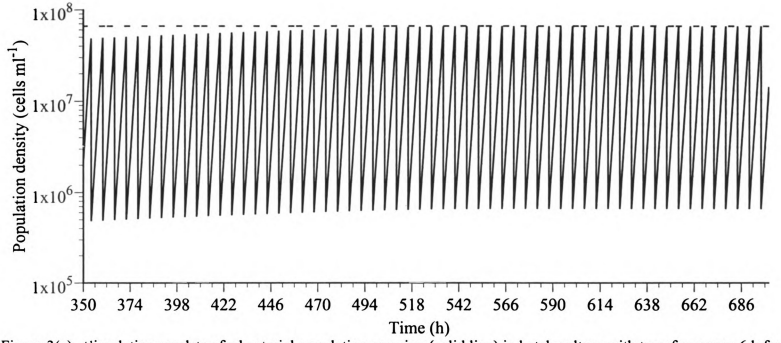

Figure 3(c). Simulation resulsts of a bacterial population growing (solid line) in batch culture with transfers every 6 h from 350 – 700 h. The expected endpoint stationary phase population density is also shown (dashed line).

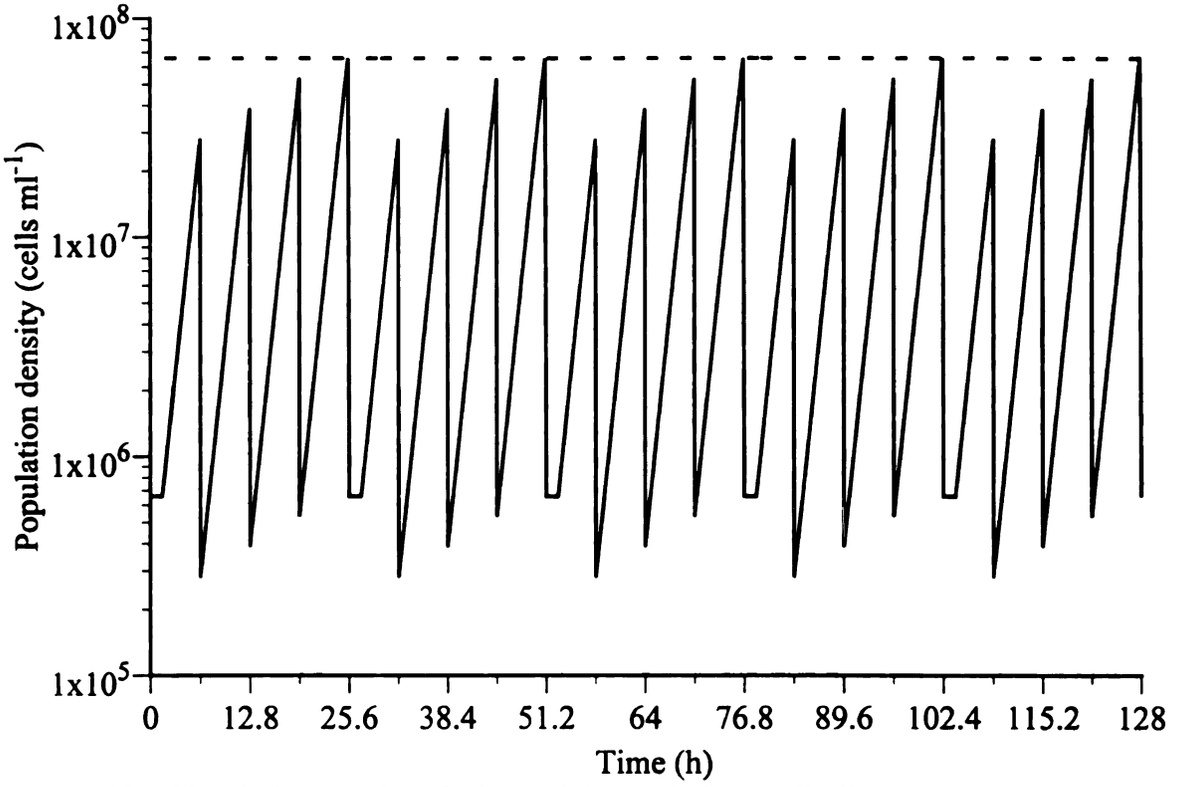

Figure 3(d). Simulation results of a bacterial population (solid line) growing in batch culture with transfers every 6.4 h. The population dynamics repeat themselves every 4 cycles. The expected endpoint stationary phase population density is also shown (dashed line).

approaches the expected stationary density asymptotically but does not ever quite reach this density (Figure 3(c)). Instead it stabilizes at a slightly lower density of 6.57×10^7 cells ml^{-1}, where it remains in a state of perpetual near-exponential growth, neither increasing nor decreasing at the end of each cycle. Glucose is never quite depleted at the end of any transfer cycle.

Figure 3(d) shows the dynamics of a bacterial population growing in batches transferred every 6.4 h. The population experiences a lag phase in the first cycle, and is diluted into fresh medium after 6.4 h while it is still growing. The population grows for the next three cycles, without a lag phase, finally reaching the end-point stationary density of 6.59×10^7 cells ml^{-1}. The population dynamics then follow a periodic cycle that repeats every four nutrient cycles.

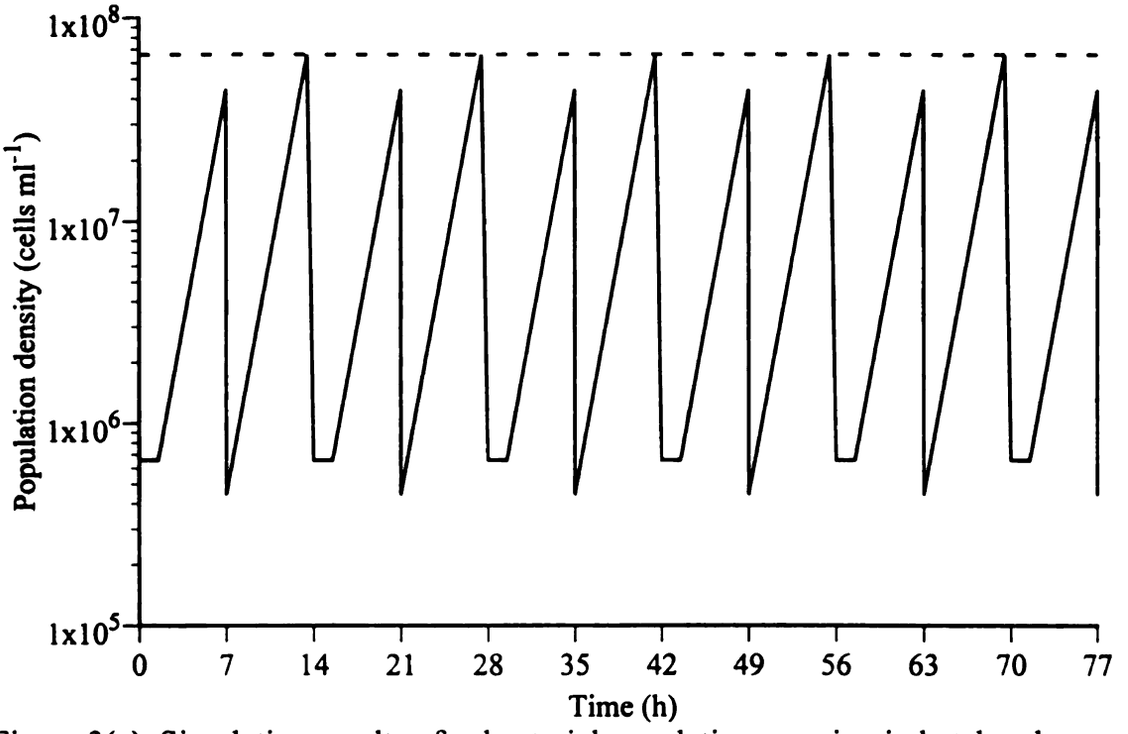

Figure 3(e). Simulation results of a bacterial population growing in batch culture with transfers every 7 h (solid line). The population exhibits a two point limit cycle. The expected endpoint stationary phase population density is shown (dashed line).

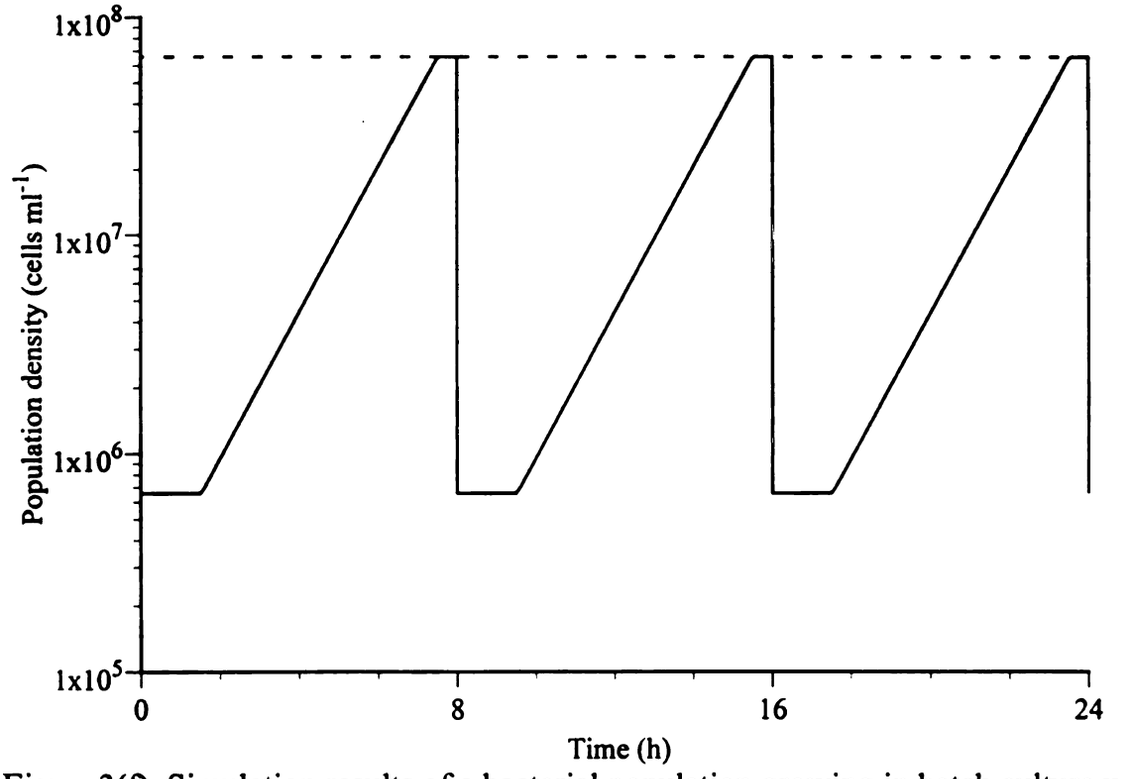

Figure 3(f). Simulation results of a bacterial population growing in batch culture with transfers every 8 h (solid line). The population exhibits a single equilibrium at the endpoint stationary phase density. The expected endpoint stationary phase population density is also shown (dashed line).

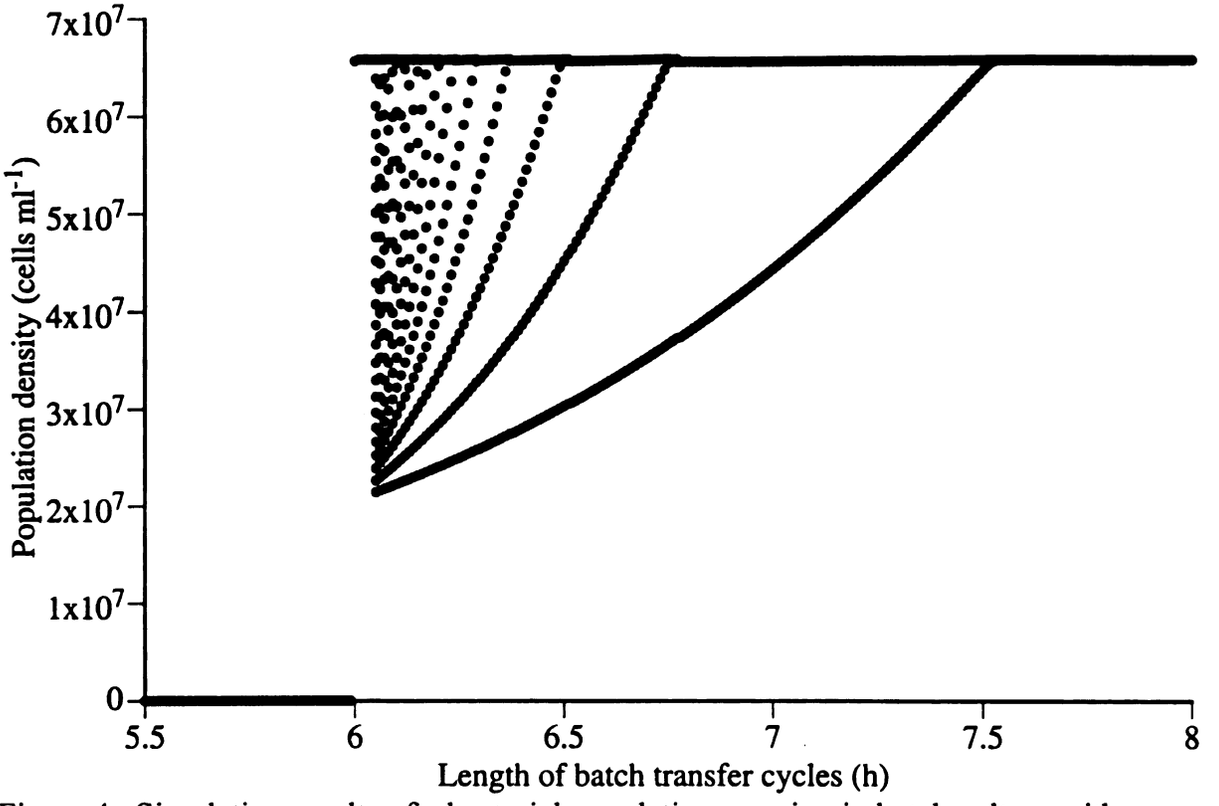

Figure 4. Simulation results of a bacterial population growing in batch culture with transfers ranging form 5.5 – 9 h. Data points represent equilibrium population densities at each period length. Multiple data points for the same period represent limit cycles.

Figure 3(e) shows the population density dynamics of a population growing in batches that are transferred at 7 h intervals. The populations cycle between the expected endpoint stationary density and a lower value at the end of each cycle, exhibiting a 2 point limit cycle.

Figure 3(f) shows the population density dynamics of a population growing in batches transferred at intervals of 8 h. There is enough time in each interval for the population to complete the full growth cycle from lag to stationary phase, and utilize all available resources.

The results of all the simulations are shown combined in Figure 4. The full range of dynamics predicted by the model can be observed. From 5 – 5.99 h, there is a single

stable equilibrium, that of extinction. From 6.00 – 6.04 h the population has a single equilibrium which is slightly lower than the expected endpoint stationary phase density. From 6.05 – 6.37 h the population cycles stably between 22 and 5 values. When grown in cycles from 6.38 to 6.51 h the population cycles between four values, from 6.52 h to 6.77 h between three values, and from 6.78 h to 7.57 h between two values. For batch transfer cycles 7.58 h and longer, the population has a single equilibrium, at the expected endpoint stationary phase density.

Empirical Tests of the Periodic Model

Population density, mean cell volume and total biovolume dynamics. Figure 5 shows the density dynamics of all the treatment populations. Only the population density at the end of each transfer cycle is plotted. In the cultures that were transferred every 5 h, the population declined exponentially during the first several cycles, as expected, but then stabilized unexpectedly at a low density of ~3.09 x 10^5 cells ml^{-1}. Populations in the 6 h transfer cycle treatment group declined exponentially until they stabilized at ~4 x 10^5 cells ml^{-1}. There was an error in the experimental procedures for the 6 h treatment, in which some populations were transferred into fresh medium 1 h too early (i.e., in 5 h instead of 6 h). The transfer occurred at 35 h (instead of 36 h) in the first two replicates, at 29 h (instead of 30 h) in the third and fourth replicate and at 23 h (instead of 24 h) in the fifth and sixth replicate. The missing hour of growth was offset by letting the population grow for an extra hour in the next transfer cycle (i.e., 7 h instead of 6 h).

Populations in the 7 h transfer group exhibited considerable noise during the first part of the experiment. This was in part due to another error in experimental procedures. Replicates one and two were transferred at 22 h instead of 21 h, replicates three and four were transferred at 29 h instead of 28 h and replicates five and size were transferred at 36

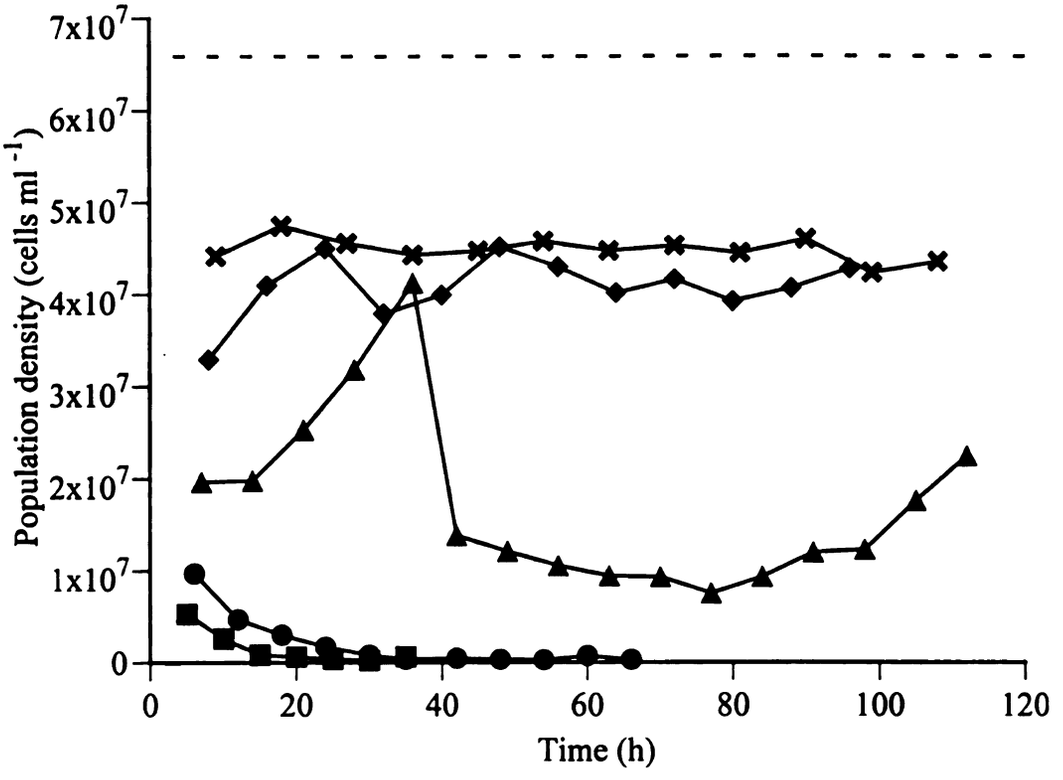

Figure 5. Population density dynamics of experimental population grown in batch culture with transfers every 5 h (squares), 6 h (circles), 7 h (triangles), 8 h (diamonds) and 9 h (crosses). Data points represent densities for a representative population from each series. The expected endpoint stationary phase population density is also shown (dashed line).

h instead of 35 h. Aside from this perturbation (evident in Figure 5), the population density also drifted slowly upwards by the end of the experiment. Populations grown in batches transferred every 8 h increased during the early part of the experiment, then stabilized at approximately 4 x 10^5 cells ml^{-1}. There was considerable noise in the data. Populations growing in batches that were transferred every 9 h also exhibited a lot of noise throughout the experiment. The bacteria stabilized at a density of approximately 4.5 x 10^7 cells ml^{-1}.

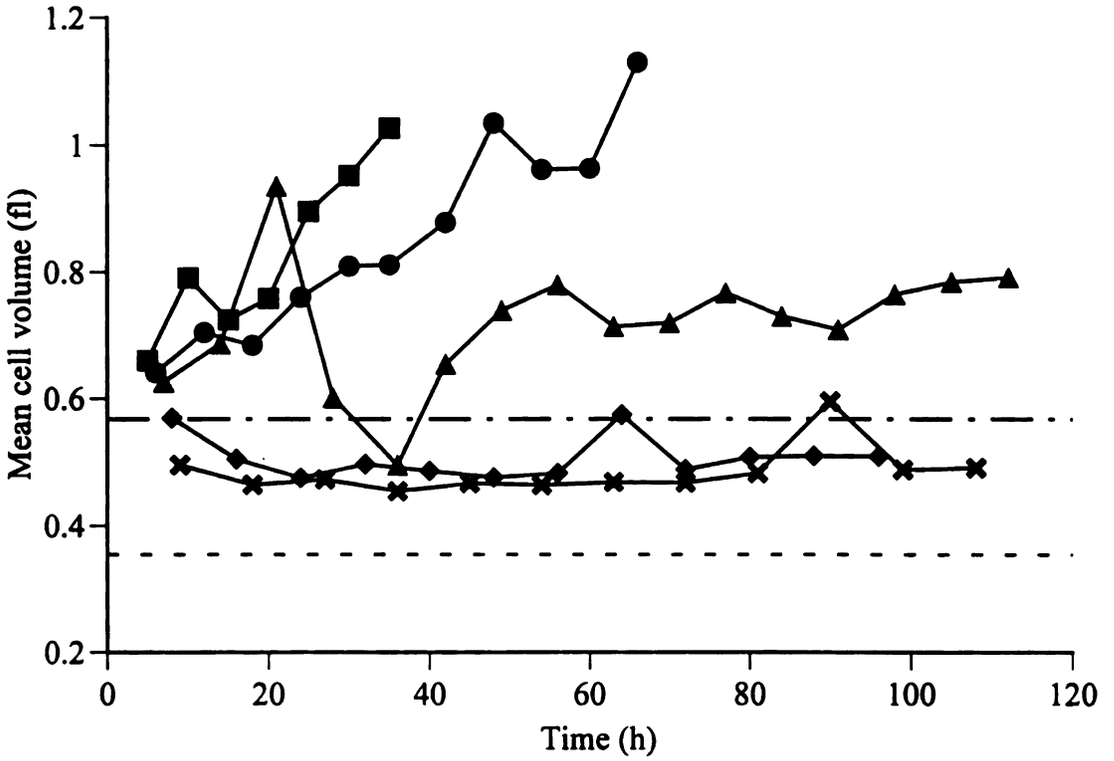

Figure 6. Mean cell volume dynamics of experimental populations grown in batch culture with transfers every 5 h (squares), 6 h (circles), 7 h (triangles), 8 h (diamonds) and 9 h (crosses). Data points represent densities for a representative population. The expected mean cell volume for exponentially growing populations (dashed-dotted line) and for stationary phase populations is also shown (dashed line).

The 7 – 9 h treatment populations all stabilized at densities below the stationary phase density expected after a 24 h growth cycle from previous data (Vasi *et al.*, 1994). The reason for this discrepancy can be seen from the mean cell volume dynamics (Figure 6). All the treatment populations showed larger cells than expected from previous data (Lenski & Travisano, 1994; Vasi *et al.*, 1994). The cells in 5 and 6 h cycles are larger than the 7 h treatment populations. The 5, 6 and 7 h populations were all larger than expected from previous data on exponentially growing cells (~0.57 fl). The 8 and 9 h

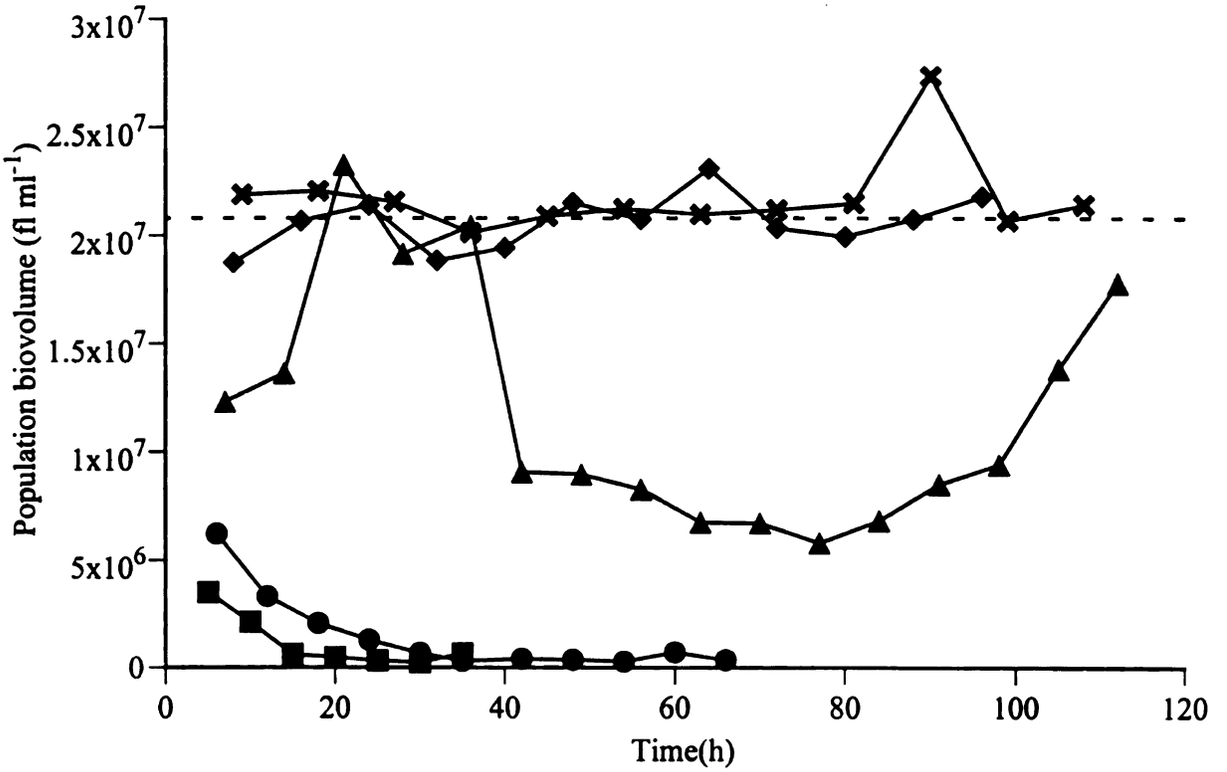

Figure 7. The total population biovolume dynamics of experimental populations grown in batch culture with transfers every 5 h (squares), 6 h (circles), 7 h (triangles), 8 h (diamonds) and 9 h (crosses). Data points represent densities for a representative population from each series. The expected endpoint stationary phase total population biovolume is also shown (dashed line).

treatment populations are smaller than the 5 – 7 h treatments. These long-cycle populations are still slightly larger than expected for 24 h stationary phase populations (~0.37 fl), but they are smaller than exponentially growing cells. There is a decreasing trend in cell volumes from 5 – 9 h (5 – 6 h > 7 h > 8 – 9 h) in the treatment populations. Evidently, the cells continue to divide without actually growing after the glucose is depleted, leading to cells that are more numerous but also smaller the longer time they spend in stationary phase.

The data for the total population biovolume (product of cell number and average cell volume) showed similar trends to the population density data (Figure 7). The 5 h treatment populations decline and stabilize at a low total biovolume of ~ 3 x 10^5 fl ml^{-1}.

The 6 h treatment populations declined and stabilized at a biovolume of $\sim 3.5 \times 10^5$ fl ml^{-1}. The 7 h treatment populations had an intermediate total biovolumes ranging from $\sim 0.5 \times 10^7$ to $\sim 1.5 \times 10^7$ fl ml^{-1}. The total biovolume of the 8 - 9 h treatment cultures was approximately 2.1×10^7 fl ml^{-1}, similar to that expected from previous data of 24 h stationary phase populations, and thus confirming the explanation (above) that there is continued cell division without net growth after glucose is depleted.

Autocorrelation analysis. Autocorrelation analyses were performed on the series of biovolume dynamics, as these combined both the density and cell volume effects. They were also less noisy than the population density dynamics. The biovolume data were first transformed by "differencing" (Box *et al.*, 1978; Box *et al.*, 1994). Each point was subtracted from the next point, thus removing the curvilinear trend in the data. Each biovolume series was then "detrended" to remove the linear decline in the data. The transformed series showed no curved or linear trend. The autocorrelation coefficients of order ranging from lag 1 to lag 8 were then estimated. The autocorrelation coefficients of each replicate population were treated as one "observation"; thus the six replicate populations provided 6 independent estimates of the autocorrelation coefficient at each time lag. Figures 8(a)-(c) show the autocorrelation functions of the 7, 8 and 9 h cycle populations. The autocorrelation coefficients were tested against a null value of zero using a one-tailed *t*-test. Qualitative assessment of the curves fitted to the data suggest that the 7 and 8 h cycle populations show autocorrelation functions that exhibit cyclical tendencies as illustrated by the alternation of positive and negative values. However, a Bonferroni correction was applied to the *P*-values, to correct for the multiplicity of *t*-tests (Rice, 1989). No significant autocorrelations were found after applying the Bonferroni criterion.

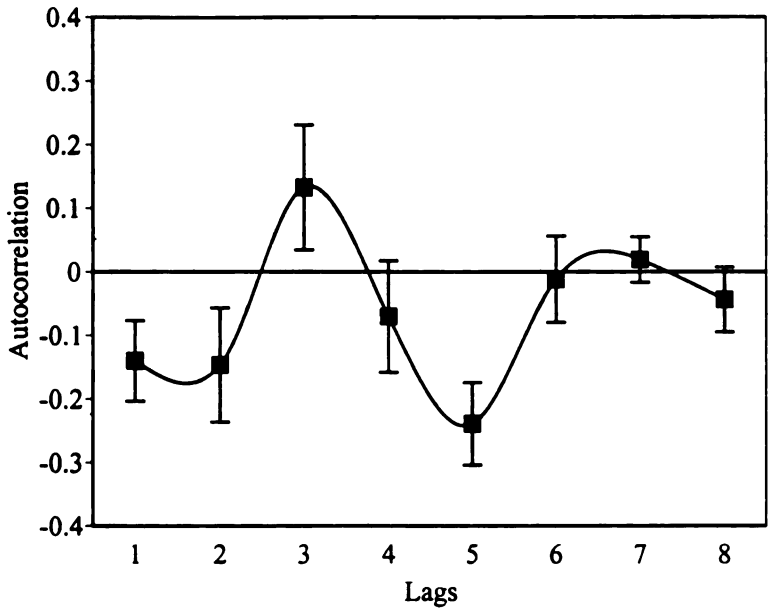

Figure 8(a). Autocorrelation function for the total biovolume dynamics of bacterial populations grown in batch culture with transfers every 7 h. Symbols represent means and standard errors. A spline curve is fitted to the autocorrelation function using least squares (solid line).

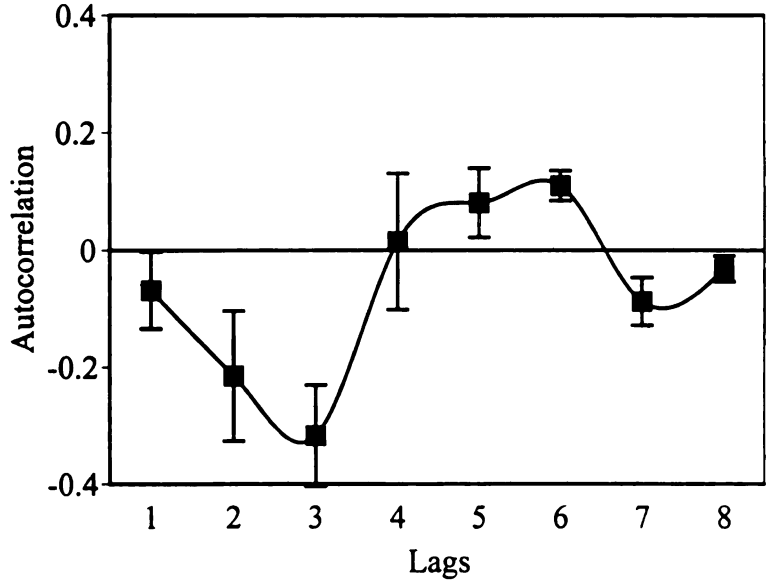

Figure 8(b). Autocorrelation functions for the total biovolume dynamics of bacterial populations grown in batch culture with transfers every 8 h. Symbols represent means and standard errors. A spline curve is fitted to the autocorrelation function using least squares (solid line).

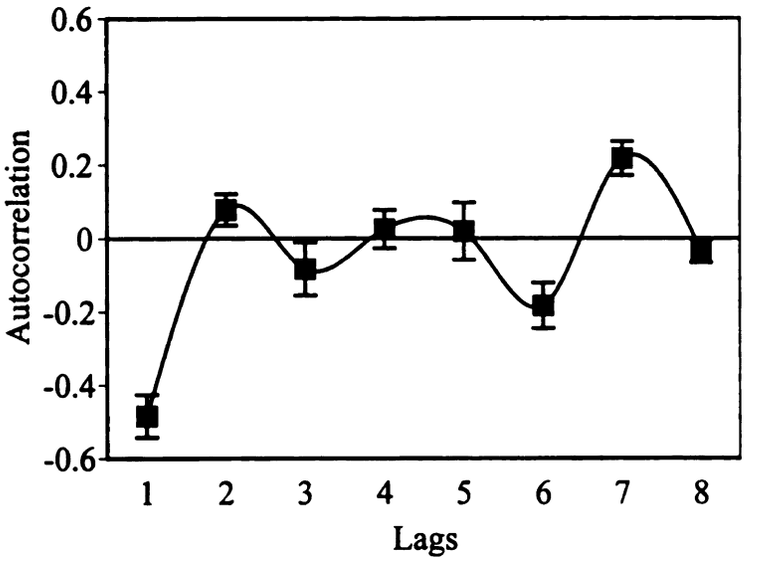

Figure 8(c). Autocorrelation functions for the total biovolume dynamics of bacterial populations grown in batch culture with transfers every 9 h. Symbols represent means and standard errors. A spline curve is fitted to the autocorrelation function using least squares (solid line).

DISCUSSION

In this Chapter, I have presented a simple model for bacterial growth in a periodic environment, in which there is a lag phase of constant duration during which no growth occurs. The lag phase occurs only when the population has previously undergone a period of resource depletion. Numerical simulations showed that for relatively short transfer cycles the populations are diluted to extinction. For longer cycles, the population has a single equilibrium where it remains in a perpetual state of near-exponential growth. For still longer transfer cycles, the population exhibits limit cycles ranging from very high to low periodicity. For the longest transfer cycles, there was a single equilibrium.

However, experiments to test this model did not reveal the striking periodic behavior predicted. The experiments showed discrepancies from the model predictions in a number of ways. Firstly, the populations that exhibited declining density dynamics did not decline to extinction but stabilized at some low density (5 and 6 h transfer

treatments). Also, populations that reached and maintained intermediate densities did so at densities lower than expected (7 h treatment). Finally populations that reached the expected endpoint biovolumes had cells that were considerably larger than expected, and had a considerably lower population density than expected (8 and 9 h treatments).

There are two classes of possible reasons for the discrepancies between the predictions of the lag phase model and the experimental results. The first of these assumes that the basic model is correct, but there were problems with experimental design and noise. It is possible that the model for the lag phase is generally correct, but the time frame chosen for the experimental test was incorrect. The predicted periodicity in the population dynamics may occur in a small portion of the range of serial transfer intervals chosen. Thus, the experiments did not have the resolution in the necessary range to reveal the predicted dynamics. It is also possible that the lag phase model is generally correct, but the data are too messy due to measurement errors, or there is too much environmental noise. There has been considerable research on the use of time series analysis (Box *et al.*, 1994) to elucidate periodicity in data series. However, the length of the time series necessary to reveal such patterns of non-linear temporal dependencies is typically longer than measured in these experiments (Box *et al.*, 1994). The length of the series required to generate a useful autocorrelation function is a minimum of 4 times the length of the periodicity expected in the series. Thus, in order to reveal a periodic cycle of 4, population density data are required for at least 16 serial transfer cycles. The population densities were measured in these experiments for only about 12 serial transfer cycles. The lack of suitable length for the population series was one of the reasons I chose to treat replicate populations as single "observations" for the time-series analysis. The experiments were logistically difficult as a result of the replication and suitable randomization of treatments. Such long series of population data are generally unfeasible for actual populations. Also, for long series, the dynamics may

change over the course of the experiment due to evolutionary changes occurring in the population.

Finally, it is possible that the simple model for lag phase described in this Chapter is incorrect. The assumption of a discrete "switch" for the all or none occurrence of the lag phase is probably incorrect. The lag phase may change over the course of the experiments due to physiological changes within the populations. The lag phase is a relatively poorly understood part of the bacterial growth cycle, because the bacterial cells in lag phase do not remain uniform in internal physiology or age-structure and have therefore proven difficult to study (Campbell, 1957; Cooper, 1991). In order to construct a better model for bacterial population dynamics, it is essential to better understand the physiology of the lag phase, and whether it may be affected by other demographic factors such as the initial population density or the time spent in stationary phase. Empirical evaluation of whether these factors affect the lag phase is presented in the next Chapter.

Chapter 3

EXPERIMENTAL TESTS OF TWO FACTORS THAT MAY INFLUENCE THE DURATION OF LAG PHASE.

INTRODUCTION

In the previous chapter, I presented a mathematical model of population growth in a periodic environment. In this model, the limiting resource is depleted by the organisms and then replenished only at some later time; this resource renewal is driven by some external periodicity. I showed that the existence of a discrete lag phase, associated with a switch from non-growing to growing organisms, may interact with the environmental periodicity to generate complex dynamics, including limit cycles, etc. However, experiments to test the predictions of this model largely failed, presumably because some assumption of the model was not fulfilled. One assumption used in the model is that the lag phase has a constant length, which is independent of such factors as the time the organisms spent in a non-growing state (prior to resource renewal) or the population's initial density (after resource renewal). In this chapter, I examine the validity of this assumption experimentally by manipulating these two factors.

The lag phase is the early portion of the bacterial growth cycle, during which the population gears up its metabolic machinery for exponential growth. For the purposes of empirical evaluation, the time that elapses between the provision of fresh medium and the initiation of exponential growth is the lag time. During the lag phase, bacterial populations presumably shut down systems used during stationary phase and induce

processes necessary for cell growth and division. One can imagine that if either of these processes could be sensitive to the amount of time a population has starved; and the latter process could be sensitive to initial population density if cells modify the medium by excreting enzymes or metabolites.

There has been considerable research on the biochemical and physiological changes that occur in bacterial cells during stationary phase. Many of these changes are activated by σ^S, a transcription factor encoded by the *rpoS* gene. σ^S is a master regulator of the expression of a large number of other genes involved in starvation survival (Lange & Hengge-Aronis, 1991). σ^S has also been shown to activate regulatory systems involved in resistance to other environmental stresses, such as nitrogen starvation and osmotic, oxidative and temperature stresses (Groat *et al.*, 1986). This research has been the subject of reviews detailing how σ^S activates specific regulatory systems and genes activated by these regulatory units (Hengge-Aronis, 1996; Huisman *et al.*, 1996).

Escherichia coli populations recovering from starvation have been analyzed for global protein synthesis using two-dimensional gel electrophoresis. Siegele & Guynn (1996) isolated nine proteins specific to the lag phase, which they called outgrowth or recovery from starvation. Five other proteins whose rate of synthesis was greatly accelerated during lag phase were also identified. Another study (Kusj *et al.*, 1998) looked at the functional stability of total mRNA during the culture cycle of *E. coli* populations. They found that mRNA half-lives are long during lag phase, decrease during exponential phase and are long again during stationary phase. Further investigation showed that the amount of a specific type of mRNA (for chloramphenicol acetyltransferase) does not change over the growth cycle. This suggests that the transcription rate is compensated to offset the long half-life of mRNA. The long half-life

of mRNA during stationary phase means that the mRNA is immediately available for transcription during recovery from starvation when nutrients become available.

Staphylococcus aureus populations starved for 7 days in a glucose-limited medium exhibited a lag time of 120 – 150 minutes when medium containing glucose and amino acids was added. The lag time was found to be independent of the length of starvation survival (Clements & Foster, 1998). Starvation specific proteins were down-regulated within 30 minutes. RNA synthesis increased immediately, followed by protein synthesis within 5 minutes, cell enlargement within 30 minutes and initiation of chromosome replication within 90 minutes.

Recently, *E. coli* populations have been shown to use small extra-cellular molecules released into the medium to "communicate" the cell density and the metabolic potential of the environment (Surette & Bassler, 1998). These extra-cellular molecules were produced at maximum concentration during late exponential and early stationary phase in glucose containing medium. By late stationary phase, the signal was no longer detectable. These molecules have not as yet been characterized, but have been shown to interact with the Type II signaling system of *Vibrio* (Fuqua *et al.*, 1994; Fuqua *et al.*, 1996; Fuqua & Green, 1998). This is one of the two well studied types of signaling systems used by *Vibrio* species for "quorum-sensing", which uses an acyl-homoserine lactone (HSL) molecule for inter-cellular signaling. Typically, HSL's are used by *Vibrio* populations to signal induction of luminescence when the population reaches a certain critical density.

An extra-cellular factor has also been shown to inhibit the initiation of chromosomal DNA replication in *E. coli* (Withers & Nordstrom, 1998) via a "quorum-sensing" mechanism. The factor has a transient effect and is produced maximally at high population densities. HSL's have also been proposed for involvement in the activation

of the stationary phase systems in *E. coli* (Huisman & Kolter, 1994). Exponentially growing *E. coli* cells were found to release an extra-cellular factor into the culture medium, which reduced the lag phase of bacteria in minimal glucose medium by several hours (Nikolaev, 1997).

In this Chapter, I describe experiments that test whether the lag phase is a function of either starvation time or initial population density. A positive answer to either of these questions would demonstrate that the assumption that the lag phase has a fixed length is not valid. Such a finding might help explain discrepancies between experimental results and model predictions, which were based on that assumption, in Chapter 2.

MATERIALS AND METHODS

Bacterial strain and Culture Conditions

The *E. coli* B (REL 606) strain used in this study has been used in several other ecological and evolutionary studies in our laboratory (Lenski *et al.*, 1991; Lenski & Travisano, 1994; de Visser *et al.*, 1999). Its growth parameters have been characterized previously (Vasi *et al.*, 1994). All experiments were started from a single clone isolated from a culture stored indefinitely at –80° C.

Bacteria were grown in Davis minimal medium (DM) (Carlton & Brown, 1981) supplemented with 25 μg ml^{-1} glucose in a shaking incubator at 37°C. Under the standard conditions, the populations were diluted 100-fold into fresh medium, where they

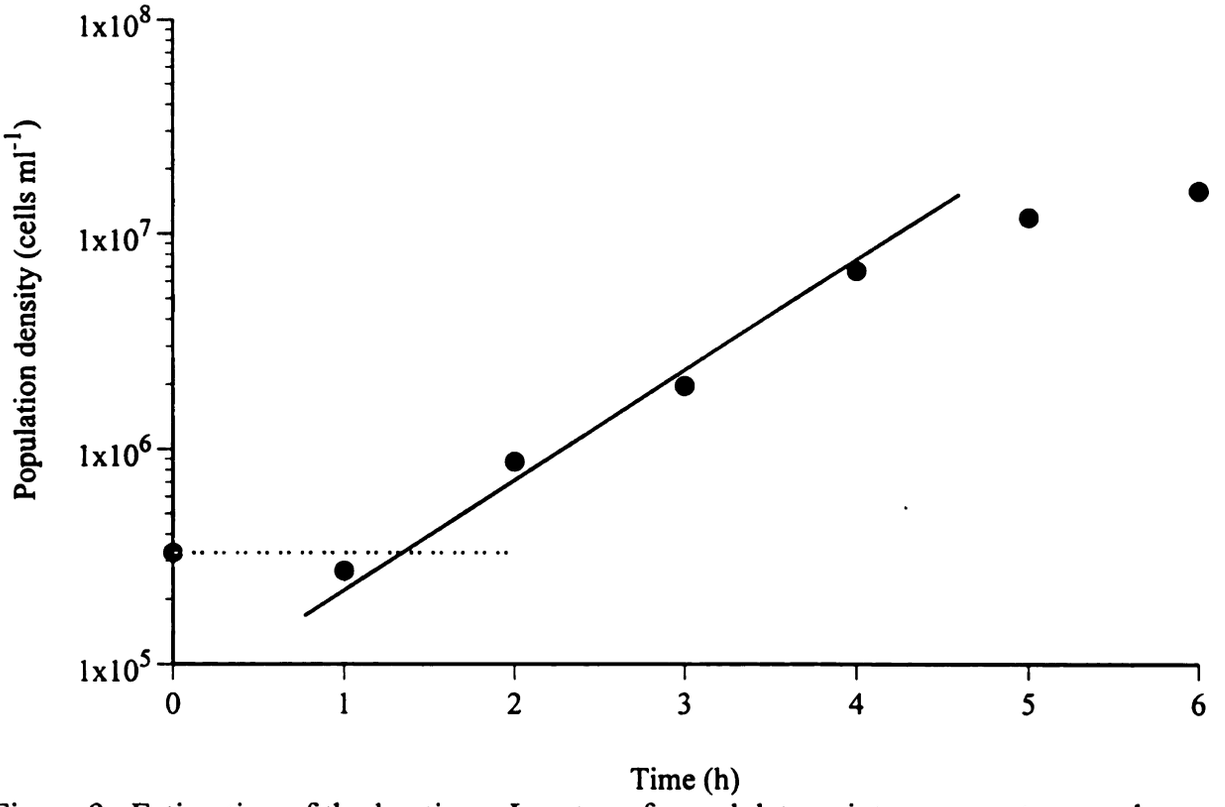

Figure 9. Estimation of the lag time. Log-transformed data points represent a sample growth curve in medium with 25 μg ml^{-1} glucose. A regression line was fit through the exponential part (2 - 6 h) of the growth cycle (solid line). The point at which this line intersects the initial population density (dashed line) is the estimated lag time (*L*).

grew until they reached stationary phase and had exhausted the available resources. The populations grew from an initial population density of ~5 x 10^5 cells ml^{-1} to a final density of ~5 x 10^7 cells ml^{-1}. The 100-fold daily growth corresponds to ~6.6 (log_2 100) generations of binary fission.

To ensure acclimation to the experimental regime, bacteria were cultured for one day in DM supplemented with 1000 μg ml^{-1} glucose after removal from the freezer. They were then conditioned for one day in DM supplemented with 25 μg ml^{-1} glucose. 24 h represents a complete cycle of lag phase, exponential phase and stationary phase in these strains.

Estimation of the lag time

Densities were estimated approximately every 1 – 2 h by viable cell counts on tetrazolium arabinose plates (TA) indicator plates (Levin *et al.*, 1977). The lag time was estimated by regression as follows (see Figure 9). The densities were log-transformed and a regression line was fit to the exponential (log-linear) portion of the growth curve. This line was then extrapolated back to where it intersected the initial population density. The time at the point of intersection was the estimated lag time. Although the actual transition may be more gradual than this procedure implies, it effectively calculates the time that has been "lost" by a population prior to its achieving exponential growth.

Effects of Starvation

The populations were conditioned for 2 days in standard medium to ensure that all treatment populations went through one complete growth cycle (lag, exponential and stationary phases) during the conditioning step. Earlier data (Vasi *et al.*, 1994) showed that the bacterial populations enter stationary phase at approximately 8 h after 1:100 dilution into fresh medium. Populations were transferred into fresh media 8, 24 and 48 h after they began this second conditioning cycle. This resulted in treatment populations that had starved for approximately 0 h, 16 h and 40 h. Six independent estimates of the lag time were obtained for each treatment, in sets of complete blocks. The exponential phase growth regression was computed for population densities between 7×10^5 cells ml^{-1} and 3×10^6 cells ml^{-1}. One replicate from the 40 h treatment had only a single sample between these densities; this replicate was not used in this analysis.

Effects of Initial Population Density

These experiments were carried out in DM supplemented with 1000 μg ml^{-1} glucose, in order that a greater range of initial population densities might be compared. The conditioning step was carried out in DM + 1000 μg ml^{-1} glucose, which supports population densities of ~3 x 10^9 cells ml^{-1}. The experimental populations were started using dilutions of 1:10^6, 1:10^4 and 1:10^2 from the conditioned populations, with expected initial densities of ~3 x 10^3 cells ml^{-1}, ~3 x 10^5 cells ml^{-1} and ~3 x 10^7 cells ml^{-1}, respectively. Six independent estimates of the lag time were obtained for each treatment, in sets of complete blocks. The regressions were carried out from 4 h to 10 h after culture inoculation in the ~3 x 10^3 cells ml^{-1} and ~3 x 10^5cells ml^{-1} treatment populations, and from 2 h to 7 h in the ~3 x 10^7 cells ml^{-1} initial inoculum treatment populations. Different intervals had to be chosen since the duration of exponential phase was considerably different for the different treatment populations. The ~3 x 10^3 cells ml^{-1} treatment populations had to go through 6.6 (log_2100) generations more growth than the ~3 x 10^5cells ml^{-1} treatment, which had to go through 6.6 generations (log_2100) more growth than the ~3 x 10^5 cells ml^{-1} treatment populations.

RESULTS

The mean lag time varied among starvation treatments (Table 6, Figure 10). The 0 h treatment populations had a lag that was shorter than the 16 h population, and the 16 h population had a lag that was shorter than the 40 h population. Planned comparisons showed that the 0 h treatment populations were different from the 16 h treatment populations, but that 16 h populations were not significantly different from the 40 h treatment populations. Since these planned comparisons were not orthogonal, the error

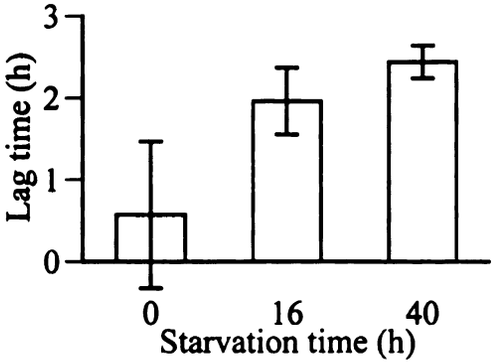

Figure 10. The effect of starvation time on the lag phase. Data points represent means and standard errors for six replicate populations in the 0 and 16 h treatments, and five replicate populations in the 40 h treatment.

rates for each were adjusted using the Sidak multiplicative inequality so that the experimentwise error rate remained 0.05 (Sokal & Rohlf, 1982, pp. 242).

A closer look at the actual growth curves showed that the 0 h treatment populations started the growth cycle at densities of ~ 2 x 10^5 cells ml^{-1}, which is lower than expected from previous data (Figure 11a-c). These cultures were started 8 h after the beginning of the conditioning cycle. 8 h may be an underestimate of the time required to reach the expected endpoint stationary phase density. Thus it is likely that the 0 h populations were started during late exponential or transitional phase, not stationary

Table 6. Analysis of variance of the effects of starvation time on the lag time.

Source	Sum of Squares	df	Mean Squares	F	Sig
Among treatments	10.66	2	5.33	13.21	***
Treatment 0 h vs 16 h	5.79	1	5.79	14.35	**
Treatment 16 h vs 40 h	0.62	1	0.62	1.56	ns
Within treatments	5.64	14	0.40		
Total	16.30	16			

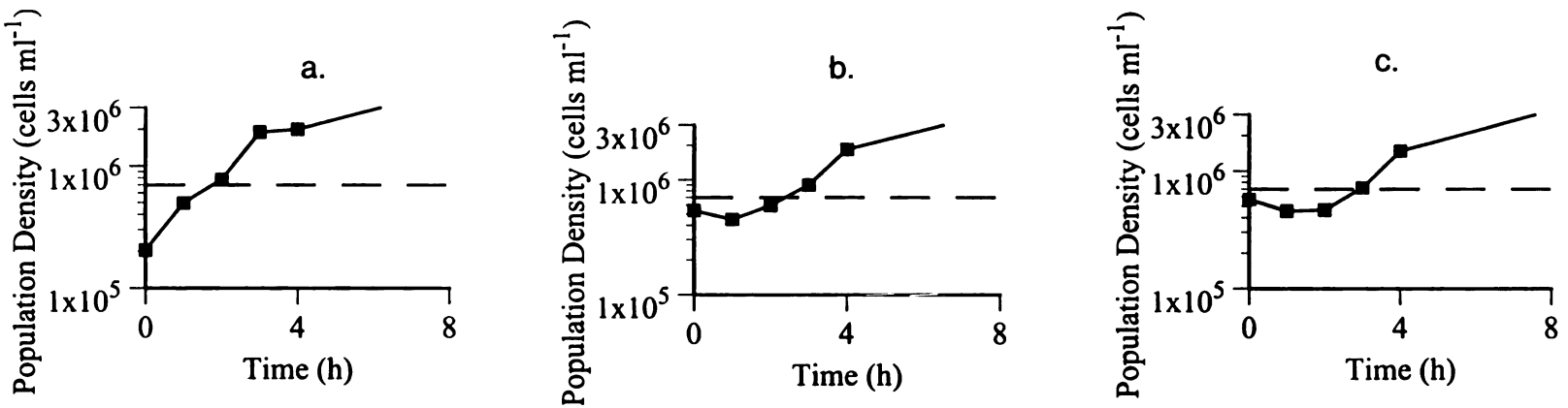

Figure 11. Population density dynamics of a single replicate population treated with starvation times of (a) 0 h (b) 16 h (c) 40 h. The lower limit used for the regression (dashed line) is also shown.

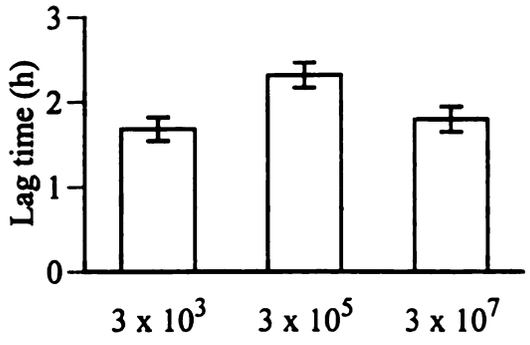

Figure 12. The effects of initial population densities on the lag time. Data points represent the means of six replicate populations. Error bars show standard errors.

phase itself. This could also explain the rather large error bars in the estimates for lag phase for the 0 h treatments, as the initial inoculum was probably in a transitional phase between exponential and stationary phase.

There was a significant effect of the initial inoculum on the lag time (Figure 12, Table 7). Planned comparisons showed that the ~3 x 10^3 cells ml^{-1} initial inoculum treatment populations were significantly different from the ~3 x 10^5 initial inocolum treatment populations which were in turn different from the ~3 x 10^7 initial inoculum treatment. Since these planned comparisons were not orthogonal, the error rates for each were adjusted using the Sidak multiplicative inequality so that the experimentwise error rate remained 0.05 (Sokal & Rohlf, 1982, pp. 242).

Table 7: Analyses of variance of the effects of initial inoculum size on the lag time.

Source	Sum of Squares	df	Mean Squares	F	Sig
Among treatments	1.39	2	0.69	5.46	*
10^3 vs 10^5 treatment	1.22	1	1.22	9.61	*
10^5 vs 10^7 treatment	0.82	1	0.82	6.44	*
Within treatments	1.90	15	0.13		
Total	3.29	17			

DISCUSSION

In this chapter, I sought to test the assumption made in the lag phase model, that the lag phase is a discrete function ("on" or "off" depending on whether the population entered stationary phase) that is independent of other physiological factors. Experimental tests of two factors, namely starvation time and initial population density, showed that lag phase is prolonged by the former, and affected by the latter in a non-linear way. Extending the periodic model by adding the effects of the starvation time and initial inoculum size may help to explain the discrepancies of the model with the experiments reported in Chapter 2. Simulations of a lag phase model extended to incorporate the lag phase as a linear function of starvation time did not exhibit the complicated dynamics predicted by the "discrete" lag phase model. (The lag-starvation model was based on a linear regression fit to the data in Figure 10 in which L=0.0061*Starvation time (Vasi, unpublished data).) Instead, populations either went extinct at very short transfer cycles (less than 6 h) or had a single equilibrium at longer transfer cycles (greater than 6 h). Thus the incorporation of factors affecting the lag phase into a mathematical model of bacterial growth may help to explain the lack of periodicity in the experiments presented in Chapter 2.

Natural populations of bacteria live in a "feast or famine" (Koch, 1971) environment, in which fluxes of nutrients become available for short periods of time, with long periods of intervening starvation. Thus, bacterial populations living in soil, water or as parasites must spend long periods of time recovering from starvation, going through the lag phase. Also microbial populations in nature occur at variable densities, also affecting the duration of lag phase when nutrients become available. These factors may produce considerable variability in population dynamics. The potential destabilizing

influence of lag phase on microbial population dynamics may be an important consideration in the understanding of fluctuations of microbial populations.

Chapter 4

ECOLOGICAL STRATEGIES AND FITNESS TRADEOFFS IN *ESCHERICHIA COLI* MUTANTS ADAPTED TO PROLONGED STARVATION*

INTRODUCTION

In nature, most bacterial populations are thought to experience periods of famine. Yet, until recently, studies of bacterial physiology and genetics have focused 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 & Mittler, 1993; Sneigowski, 1995; Foster, 1998) and has examined the rate and nature of mutations during starvation (Stahl, 1988; Boe, 1990; Hall, 1990; Mittler & Lenski, 1990; Foster & Cairns, 1992; Mittler & Lenski, 1992; MacPhee, 1993; Foster & Cairns, 1994; Maenhaut-Michel & Shapiro, 1994; Rosenberg *et al.*, 1994; Jayaraman, 1995; Sneigowski, 1995; Foster, 1997; Harris *et al.*; Bhattacharjee & Mahajan, 1998). Another interesting line of research seeks to understand the physiological and genetic bases of adaptation to prolonged starvation (Koch, 1971; Lange & Hengge-Aronis, 1991; Siegele & Kolter, 1992; Hengge-Aronis, 1993; Zambrano *et al.*, 1993; Huisman & Kolter, 1994; Hengge-Aronis, 1996; Huisman *et al.*, 1996; Zambrano & Kolter, 1996; Cotter & Miller, 1997; Joux *et al* , 1997; Thorne & Williams, 1997; Uhde *et al.*, 1997; Lazar *et al.*, 1998; Pratt & Silhavy, 1998; You *et al.*, 1998). Our paper builds upon this second line of research.

* This chapter is presented verbatim from Vasi & Lenski (1999)

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 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 & 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 & Hengge-Aronis, 1991; Hengge-Aronis, 1993; Hengge-Aronis, 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 have previous studies, 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 four other 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 & 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 onto tetrazolium arabinose (TA) agar plates (Lenski, 1988). During 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 again without adding any nutrients. (The use of screw-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 ~5 x 10^7 cells per ml.)

Figure 13 shows the overall population dynamics of the second-stage starvation-

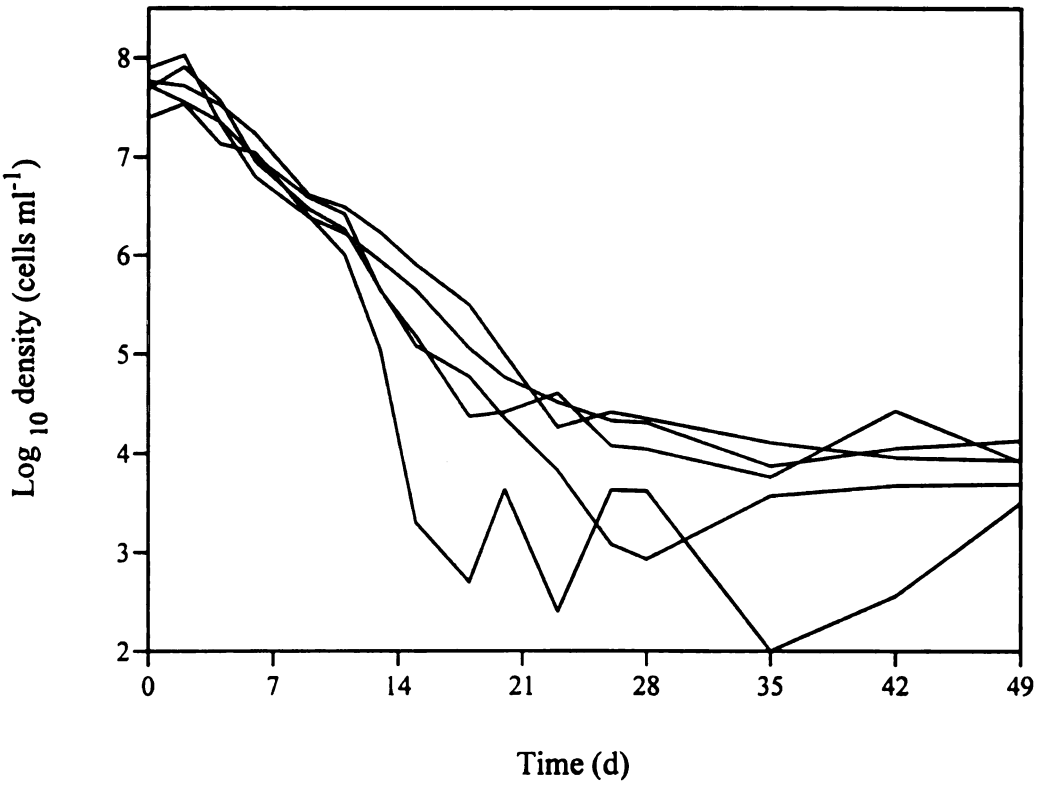

Figure 13. Dynamics of the five populations during the starvation-selection experiment. A separate line indicates each population; the population densities have been $\log_{10}$-transformed. The populations were founded with five different strains of *E. coli* B, which had already diverged from one another 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.

selection experiment for the five populations that gave rise to the mutants S1-S5, based on 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 leveling off at a density of ~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 re-streaked 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).

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 decline (Fig. 13), 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 & 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 14 shows that two

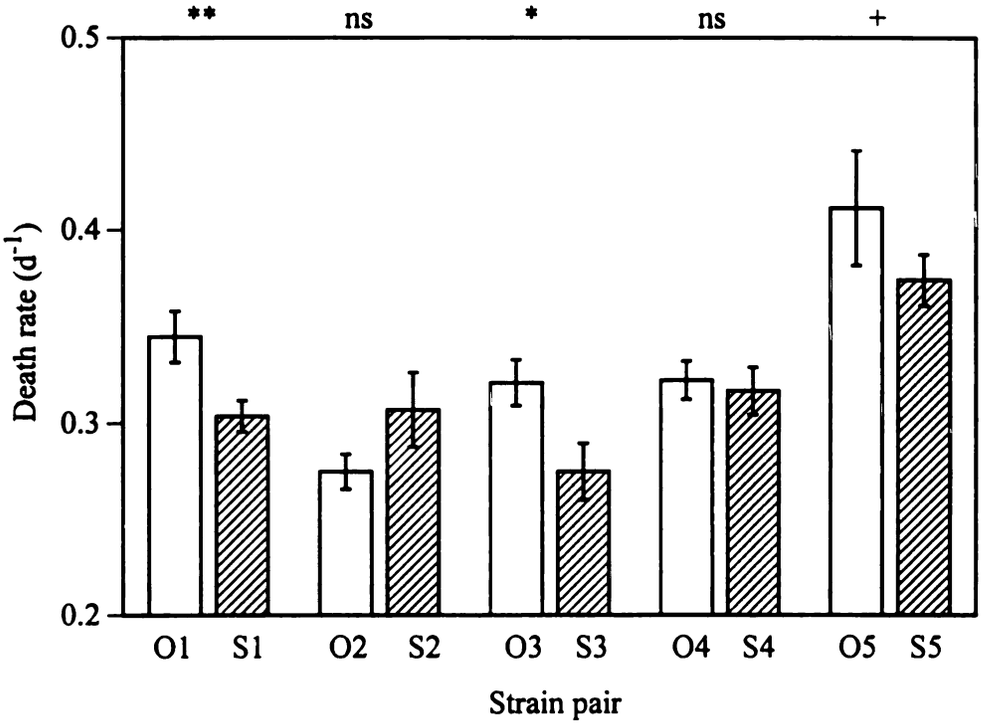

Figure 14. Death rate in pure culture of starvation-selected mutants (S1-S5) and their progenitors (O1-O5). Rates were measured over 15 d. Error bars represent one standard error. For each mutant and progenitor, a one-tailed paired *t*-test was done to test the hypothesis that the mutant had a lower death rate in pure culture than did its progenitor. Results are summarized above each strain pair by: ns $0.1 < P$; + $0.05 < P < 0.1$; * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$.

mutants, S1 and S3, had significantly lower death rates in pure culture than did their progenitors; a third mutant, S5, had a marginally significant 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 15 shows that two of the mutants, S3 and S4, made

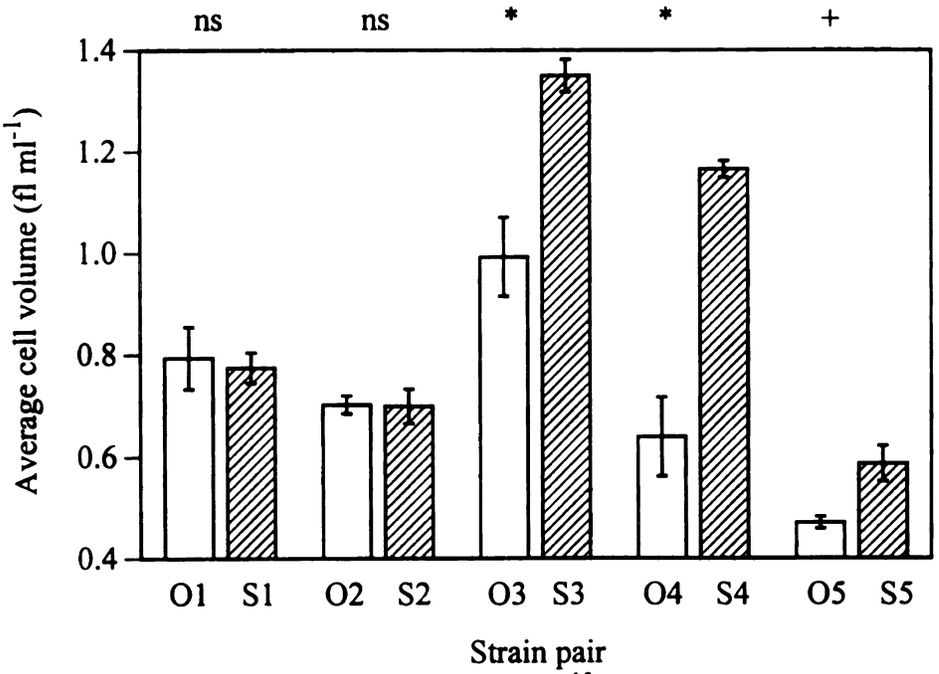

Figure 15. Average cell volume (1 fl = 10^{-15} l) of starvation-selected mutants (S1-S5) and their progenitors (O1-O5) in early stationary phase. Error bars indicate one standard error. For each mutant and progenitor, a one-tailed paired *t*-test was done to test the hypothesis that the mutant produced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

significantly larger cells 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 16 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 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 obtained in the first experiment above). Figure 17 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 14, 16, and 17 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.

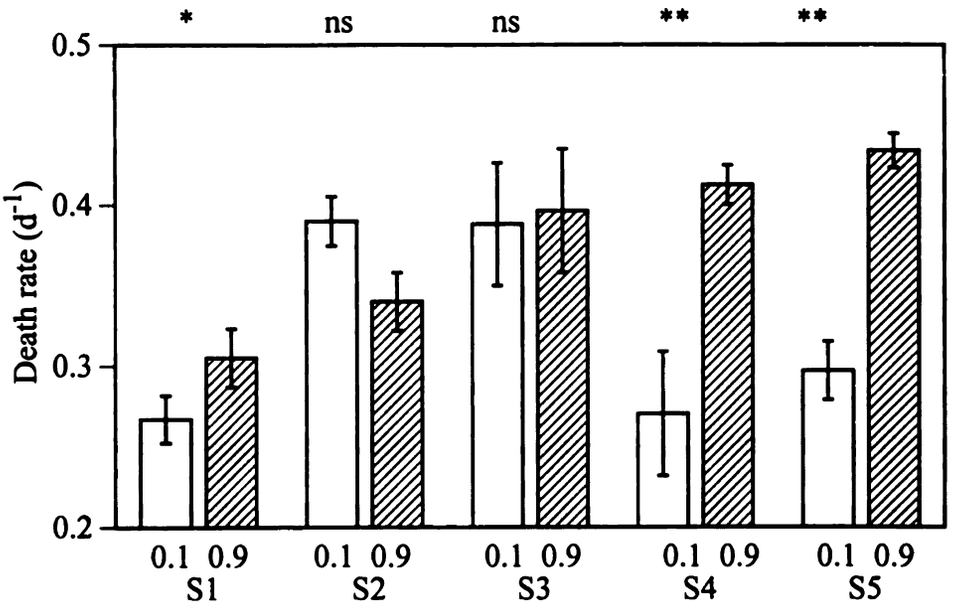

Figure 16. Death rates in mixed culture of starvation-selected mutants (S1-S5) at two different initial frequencies (10% and 90%) with their progenitors. Rates were measured over 15 days. Error bars indicate one standard error. For each mutant, a one-tailed paired t-test was done to test the hypothesis 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 $0.1 < P$; * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$.

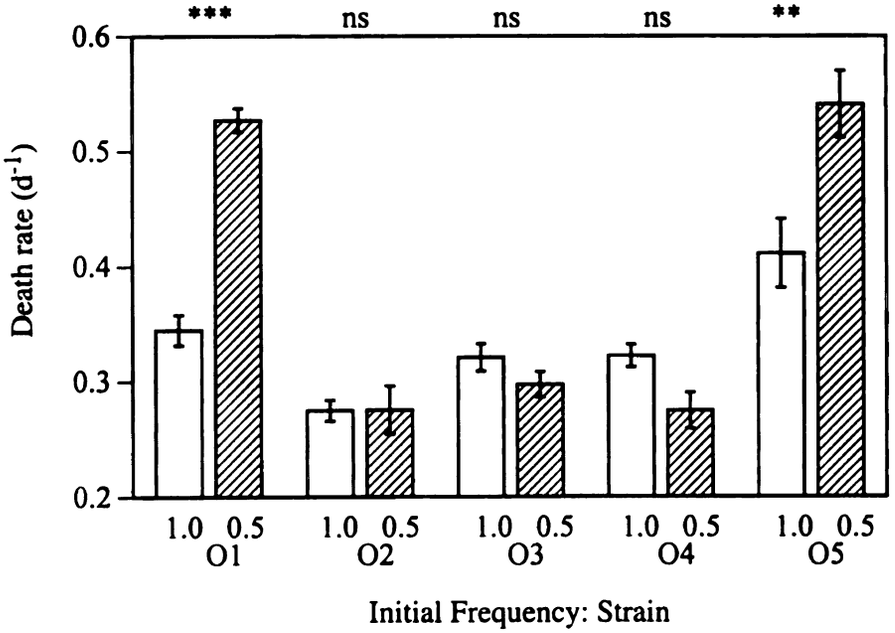

Figure 17. Death rates of progenitors (O1-O5) in pure culture (100%) and mixed (50%) with their starvation-selected mutants. Rates were measured over 15 days. Error bars are standard errors. For each progenitor, a one-tailed *t*-test was done to test the hypothesis that its death rate was lower when in pure culture (100%) than when mixed (50%) with its derived mutant. Results are summarized above each progenitor by: ns $0.1 < P$; ** $0.001 < P < 0.01$; *** $P < 0.001$.

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 18 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

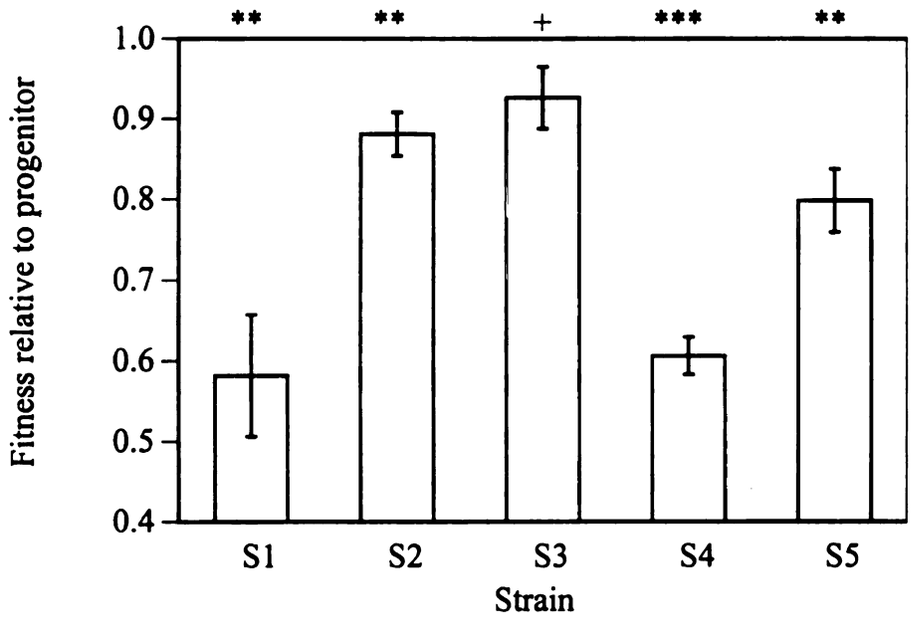

Figure 18. Fitness of each starvation-selected mutant (S1-S5) relative to its progenitor during a one-day competition in fresh DM25 medium. Error bars correspond to one standard error. For each mutant strain, a one-tailed *t*-test was done to test the hypothesis that its relative fitness in competition for fresh medium was less than 1.0. Results are summarized above each mutant by: +, $0.05 < P < 0.1$; **, $0.001 < P < 0.01$; *** $P < 0.001$.

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, 4 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 favorable growth conditions.

DISCUSSION

Previous research on bacterial adaptation to prolonged starvation has focused 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 8). 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 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 & Kolter, 1992; Zambrano *et al.*, 1993; Zambrano & Kolter, 1996) that *E. coli* can evolve enhanced

Table 8. 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.

survival capacity under prolonged starvation, and they extend these 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 (Fig. 18). 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 (Levins, 1968; Slatkin & 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 & Ayala, 1981; Rose & Charlesworth, 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 & Levin, 1985; Lenski, 1988) and antibiotics (Schrag & 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 & 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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