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EXPERIMENTAL EVIDENCE FOR SYMPATRIC ECOLOGICAL DIVERSIFICATION DUE TO FREQUENCY-DEPENDENT COMPETITION IN *ESCHERICHIA COLI*

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Abstract.—We investigate adaptive diversification in experimental *Escherichia coli* populations grown in serial batch cultures on a mixture of glucose and acetate. All 12 experimental lines were started from the same genetically uniform ancestral strain but became highly polymorphic for colony size after 1000 generations. Five populations were clearly dimorphic and thus serve as a model for an adaptive lineage split. We analyzed the ecological basis for this dimorphism by studying bacterial growth curves. All strains exhibit diauxie, that is, sequential growth on the two resources. Thus, they exhibit phenotypic plasticity, using mostly glucose when glucose is abundant, then switching to acetate when glucose concentration is low. However, the coexisting strains differ in their diauxie pattern, with one cluster in the dimorphic populations growing better in the glucose phase, and the other cluster having a much shorter lag when switching to the acetate phase. Using invasion experiments, we show that the dimorphism of these two ecological types is maintained by frequency-dependent selection. Using a mathematical model for the adaptive dynamics of diauxie behavior, we show that evolutionary branching in diauxie behavior is a plausible theoretical scenario. Our results support the hypothesis that, in our experiments, adaptive diversification from a genetically uniform ancestor occurred due to frequency-dependent ecological interactions. Our results have implications for understanding the evolution of cross-feeding polymorphism in microorganisms, as well as adaptive speciation due to frequency-dependent selection on phenotypic plasticity.

Key words.—Adaptive dynamics, diauxie, experimental evolution, phenotypic plasticity, sympatric speciation.

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Understanding the origin and maintenance of biological diversity is a fundamental problem in ecology and evolution. Until recently, a central tenet of the modern synthesis was that generation of diversity through speciation occurs most often in allopatry, that is, as a by-product of geographical isolation between subpopulations of an ancestral species (Mayr 1963; Dobzhansky 1970). It is somewhat ironic that in this theory, the initial splitting itself is not adaptive and instead occurs due to geographical factors that are outside the realm of Darwinism. In contrast, recent years have seen a shift in focus toward processes of adaptive speciation, in which the splitting is itself an adaptive response to ecological interactions within an ancestral population (Dieckmann et al. 2003). Typically, processes of adaptive speciation require some degree of sympatry between the diverging lineages. Much empirical and theoretical support has been gathered for sympatric speciation (e.g., reviews by Via 2001; Turelli et al. 2001). In particular, the theory of adaptive dynamics (Metz et al. 1996; Geritz et al. 1998) has provided a mathematical framework for studying processes of adaptive, sympatric diversification. Within this framework, the phenomenon of evolutionary branching (Geritz et al. 1998) has emerged as a basic theoretical metaphor of adaptive splitting due to frequency-dependent interactions in many different ecological scenarios (Doebeli and Dieckmann 2000).

Having a suitable theoretical framework for adaptive speciation has removed some of the main reservations against sympatric speciation as a plausible evolutionary process. Nevertheless, direct experimental evidence for adaptive diversification under fully sympatric conditions is still largely lacking. In the case of strictly asexual microorganisms, species can be classified only by genetic or functional divergence, so the appearance of polymorphism and sympatric speciation are equivalent processes. Evolutionary studies of microorganisms are a useful tool for studying evolutionary questions because of their short generation time, large population size, and controllable environments (Elena and Lenski 2003) and thus far these systems have yielded many examples of diversity arising from a genetically uniform ancestral population (for a review see Rainey et al. 2000). Spatial heterogeneity has been shown to increase both the emergence of diverse colony morphologies of *Pseudomonas fluorescens* and the subsequent maintenance of this diversity via negative frequency-dependent selection (Rainey and Travisano 1998; Kassen et al. 2000; Buckling et al. 2000). In these experiments, the structure of the environment affords niches corresponding to different parts of the culture vessel that are filled by specialists that regulate their own population sizes in a density-dependent manner. In a recent review, Kassen (2002) argues that nutrient mixtures are also a form of spatial heterogeneity with very fine-scale variation.

A fully sympatric example comes from the evolution of cross-feeding polymorphisms, during which an ancestral *Escherichia coli* strain growing on glucose as the limiting resource diversifies into an apparent glucose specialist and specialist cross-feeders that scavenge waste products, such as acetate, arising from glucose metabolism (Helling et al. 1987; Rosenzweig et al. 1994; Treves et al. 1998; Rozen and Lenski 2000). Rosenzweig et al. (1994) elucidated some of the genetic and physiological mechanisms by which cross-feeding polymorphisms can be maintained in continuous chemostat cultures, but many open questions remain, especially for populations maintained in serial batch cultures (Rozen

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and Lenski 2000). For polymorphic populations evolving in the seasonal environments generated by such serial transfer, Turner et al. (1996) showed that a trade-off between growth rates early and late in the season—that is, growth rates when glucose is abundant as opposed to when glucose is scarce—is unlikely to be the mechanism maintaining coexistence. Rozen and Lenski (2000) demonstrated that cross-feeding interactions, effectively involving growth on a mixture of resources, do occur between the two strains that are present after 20,000 generations and that the strains differ in their maximum growth rates in the glucose-only medium that they evolved in. However, insights into the details of the ecological mechanisms underlying diversification on resource mixtures remain elusive.

In this paper we report results from evolution experiments in which *E. coli* populations were grown over many generations in batch cultures containing a mixture of glucose and acetate. This setup represents a very simple version of the scenarios envisaged in salient models of evolutionary branching due to competition for a continuous spectrum of resources (Dieckmann and Doebeli 1999). With two resources, it might be expected that two strains will arise that partition the resources, but how this diversification occurs will depend on the physiological constraints of the system and the resulting ecological dynamics. Our study focuses on the latter connection between ecological and evolutionary dynamics. The details of the evolutionary trajectories of these populations will be reported elsewhere (G. Saxer, M. Friesen, M. Doebeli, and M. Travisano, unpubl. ms.), but for the present purposes we take as a starting point the fact that after 1000 generations, all 12 propagated populations showed marked diversification in terms of colony morphology relative to the ancestor. Five of the 12 populations were clearly bimodal for colony size after 1000 generations and consisted of one cluster of strains that form large colonies (L strains) on agar plates, and one cluster forming small colonies (S strains). By analyzing growth curve data for many strains from each derived population, we investigate the ecological underpinning of this diversification by associating colony morphology with ecological phenotypes, which are under natural selection due to limiting resources.

We find that in three of the dimorphic populations, colony size corresponds with ecological diversification. All derived strains have diauxic growth curves when grown on a mixture of glucose and acetate, with a steep first phase when glucose is presumably the main substrate being used followed by a second phase that is less steep and likely corresponds to the use of acetate. The transition between the two phases of growth occurs when glucose runs out and the cells shift their metabolism from glucose catabolism to acetate catabolism. Diauxic growth is thus a form of phenotypic plasticity that requires changes in metabolic pathways. Diauxie is a well-studied phenomenon and is known to occur when *E. coli* is grown on certain combinations of carbon sources—in particular, glucose is known to inhibit the catabolism of acetate (Saier et al. 1996). We find that ecological diversification is based on differentiation in diauxie behavior. The L strains grow better in the first phase of diauxie but have a longer lag in switching their metabolism to acetate consumption, as well as a lower growth rate in the second phase of diauxie. In contrast, the S strains grow more slowly in the glucose phase but switch faster and grow better on acetate. Thus, the two emerging and coexisting phenotypic clusters exhibit two different types of phenotypic plasticity during sequential consumption of a mixture of resources.

It is intuitively appealing that such diversification occurs due to frequency-dependent selection: whether it is advantageous to grow faster on glucose at the cost of having a longer switching lag depends on the diauxie types currently present in the population. Using a model for the adaptive dynamics of diauxie behavior, we show that such frequency dependence based on a trade-off between growth rate on glucose and switching lag can, in principle, lead to evolutionary branching, and hence to the emergence and coexistence of different diauxie types.

Our theoretical results thus corroborate our empirical findings, and taken together this work arguably represent the first experimental confirmation of evolutionary branching. Overall, our results help to unravel the details of how ecological interactions can induce adaptive splitting of a single ancestral type into coexisting descendent types under fully sympatric conditions.

Materials and Methods

Strains and Media

We grew 12 populations of the strictly asexual *E. coli* B (Lenski 1988; Lenski et al. 1991) in a seasonal environment with two carbon sources, glucose and acetate, for 150 days. Every day, 100 μl of each population was transferred to 10 ml of fresh media. This 1:101 dilution resulted in about 6.65 doublings (i.e. generations) per day, giving a total of just under 1000 generations. Even-numbered populations were initiated with an ancestral strain that can use L(+)-arabinose and forms pale colonies on tetrazolium-arabinose (TA) indicator plates (Levin et al. 1977), and odd-numbered populations were initiated with an ancestral strain that differs only in its ability to use arabinose and hence forms dark red colonies on TA plates. The two ancestral strains have the same fitness in a range of environments (Lenski 1988; Travisano et al. 1995).

Populations were propagated and assayed in Davis minimal (DM) medium, a minimal salts medium supplemented with thiamine hydrochloride, magnesium sulfate, and a carbon source (Carlton and Brown 1981). Our 12 populations, numbered 25 through 36, evolved in DM with 205 mg/L acetate and 205 mg/L glucose (DM50:50). Another set of 12 populations evolved in DM with 410 mg/L glucose (DMglucose) and 12 populations evolved in DM with 410 mg/L acetate (DMacetate). These populations, numbered 1 through 24, will be discussed elsewhere. To better understand the ecological characteristics of derived strains, we assayed growth curves in DMglucose, DMacetate, DM50:50, and DM90:10 (369 mg/L acetate and 41 mg/L glucose). We used tetrazolium-arabinose (TA) indicator plates to distinguish between Ara+ and Ara− strains and for all quantitative measurements of colony size and morphology (Levin et al. 1977).

Protocols

Twelve populations evolved in DM50:50 for 1000 generations were plated on TA plates and measured with a BioCount Colony Counter (Parrett Enterprises, Ltd., London, U.K.). We measured at least four plates for each population and used only plates with isolated colonies to minimize the effect of crowding on colony sizes. Resulting digital images were analyzed with ImageJ (http://rsb.info.nih.gov/ij/) to yield area measurements of each colony in pixels.

TA plates for each population were assessed visually for variation in colony diameter, shape, size, and color, as well as the presence of abnormal colonies. Colonies grown directly from frozen population samples were picked and stored in 25% glycerol suspensions at −80°C. To test heritability, colony samples were streaked onto fresh TA agar. For each of the three intensively studied populations, populations 29, 31, and 33, we grew a single S and L colony alone in DM50:50 and plated every 24 h. L colonies give rise to L colonies that appear after 24 h of growth on plates, whereas S colonies give rise to a variety of colony sizes that are smaller than the corresponding L colonies and do not appear until after 48 h of growth on plates. To test the heritability of ecological phenotypes in populations 29, 31, and 33, we grew three L and three S colonies of each population from frozen stocks (parents) in DM50:50 and plated the cultures after 24 h of growth to obtain offspring after about 6.65 generations. For each parent frozen colony, we then picked three offspring colonies and assayed their growth curves in DM50:50. S colonies' offspring have a rapid switch to the second phase of diauxie, whereas L colonies' offspring have slow switching behavior.

We measured growth curves in different media with a BioScreen C (ThermoLabsystems, Vantaa, Finland). For each BioScreen run, we first added a scraping from frozen samples of isolated colonies and the ancestral strain to 5 ml of LB broth and grew for 24 h at 37°C at 10 rpm. Next, 50 μl of this stationary culture was added to 5 ml of fresh DM with the carbon source that comprised the measurement environment. This was grown at the same temperature and rpm for 24 h. Finally, 2.5 μl of this stationary culture was added to 250 μl of fresh media in at least three replicate wells of a 100-well BioScreen plate to give the standard 1:101 dilution of culture to fresh medium. The leftmost row of each plate was filled with media but not inoculated to provide a baseline absorbance. The Bioscreen was set at wideband to measure culture turbidity and programmed to shake continuously at 37°C, taking a measurement every 10 min. Runs in DMglucose lasted 24 h, and runs in DMacetate and mixed environments lasted between 40 and 48 h to ensure that cultures reached stationary phase. Isolated colonies from populations 25 to 36 were grown in DMglucose, DM50:50 acetate:glucose, DM90:10 acetate:glucose, and DMacetate. For population 29, four L strains and 10 S strains were analyzed; 12 L strains and 10 S strains of population 31 were analyzed; and 10 L strains and 10 S strains from population 33 were analyzed. The ancestor was run with each population as a consistent comparison.

When grown in DM50:50, containing 50% acetate and 50% glucose, distinct two-phase growth (diauxie) is observed but the second phase spans less of the total growth than the first phase when plotted on a log-scale of optical density. To increase the resolution of the shift between first and second phases of growth, strains were grown in DM90:10, containing 90% acetate and 10% glucose. Because the first phase is shorter and the second phase is longer in DM90:10 than DM50:50, we infer that glucose is used predominantly during the first phase of diauxie, while acetate is used predominantly during the second phase of diauxie.

Data Analysis

To estimate growth curve parameters for each colony, the parameters of growth curves from each replicate well are estimated and the mean of the three or more replicates taken. Each growth curve is first normalized by subtracting the average of the control wells at each time point. The data are ln-transformed, then a line of maximum slope is fit through the first phase of growth. The maximum slope is defined to be the maximum of the slopes of lines fit to a moving window of 10 datapoints. The first lag is the time between the start of the run and the time at which the first line of exponential growth has a value of −5.0. This assumes that all wells are inoculated with the same biomass and is done because the BioScreen measurements are imprecise at low optical densities. This is in accordance with the common definition of lag as the time period until exponential growth is detected (Dalgaard and Koutsoumanis 2001). Differences in initial inoculum size could significantly affect estimates of lag time, hence the lag measurements may be inaccurate and are treated accordingly. The switching point is the point at which the data is more than 0.1 units away from the line of exponential growth. This deviation corresponds to roughly 2.5% of the total growth curve and was chosen because the resulting switching point corresponds visually to where switching occurs from the first phase to the second phase of growth. The switching optical density is the optical density at the switching point. We believe that the switching point corresponds to the point at which glucose is exhausted from the medium, because *E. coli* are known to use glucose preferentially (Saier et al. 1996). The second maximum growth rate is estimated in the same manner as the first, by taking the maximum of the slopes after the switching point. The switching lag is the length of time between the switching point and the point on the second line of exponential growth where the optical density is equal to the switching optical density. In DMacetate, maximum growth rate and lag are estimated. In DMglucose and DM50:50, maximum growth rate, lag, and switching optical density are estimated. Because glucose catabolism produces acetate in *E. coli* (Guardia and Calvo 2001), switching density in DMglucose is the point where cells exhaust the supplied glucose. In DM90:10, first maximum growth rate, first lag, switching optical density, second maximum growth rate, and switching lag are estimated. These parameters are used to generate histograms and perform phenotype ordinations.

Principal components analysis (PCA) can reduce the dimensionality of a dataset by finding statistically uncorrelated (i.e., orthogonal) variables that capture the main features of the data along a small number of axes (McGarigal et al. 2000).

TABLE 1. Correlations between growth parameters and principal components 1, 2, and 3 for populations 29, 31, and 33. For each population, small strains, large strains, and the ancestor were included. Absolute *r*-values with a magnitude greater than 0.5 are in bold type.

	Population 29			Population 31			Population 33		
Parameter	PC1 *r*	PC2 *r*	PC3 *r*	PC1 *r*	PC2 *r*	PC3 *r*	PC1 *r*	PC2 *r*	PC3 *r*
DM90:10 growth rate	0.413	0.004	**−0.855**	**0.679**	0.006	0.175	0.097	**−0.676**	**0.599**
DM90:10 lag	**−0.696**	−0.173	**−0.502**	−0.253	0.117	−0.23	**−0.685**	**0.538**	0.276
DM90:10 switching density	**0.743**	−0.086	0.434	−0.495	0.429	0.089	−0.226	**−0.683**	**−0.596**
DM90:10 second growth rate	**0.595**	**−0.752**	−0.124	0.096	**0.936**	−0.031	**−0.795**	−0.097	0.407
DM90:10 switching lag	**−0.682**	**0.691**	0.03	**−0.503**	**−0.595**	0.317	**0.806**	0.411	−0.21
DM50:50 growth rate	0.286	**0.772**	0.106	0.228	0.08	**−0.704**	**0.844**	−0.273	−0.12
DM50:50 lag	**−0.506**	−0.419	**0.502**	0.131	0.063	**−0.637**	−0.055	**0.798**	−0.324
DM50:50 switching density	**0.831**	0.358	0.034	0.295	0.54	0.218	**−0.536**	**−0.508**	−0.434
DMglucose growth rate	0.053	**0.774**	−0.242	**0.821**	−0.245	0.138	**0.937**	0.019	0.111
DMglucose lag	−0.102	**−0.837**	−0.077	**−0.918**	0.139	0.006	−0.194	**0.818**	−0.097
DMglucose switching density	**0.896**	0.204	−0.018	**0.677**	−0.396	−0.239	−0.414	**−0.502**	−0.214
DMacetate growth rate	**0.784**	−0.497	−0.04	−0.114	0.153	**−0.669**	**−0.685**	0.438	−0.142
DMacetate lag	−0.285	**−0.739**	−0.085	−0.48	**−0.576**	−0.39	**−0.527**	−0.204	−0.057

The 13 growth parameters (see Table 1) estimated from BioScreen data collected from colonies grown in four measurement environments are used as phenotype variables. Each population is analyzed separately, with the ancestor that was assayed in the same BioScreen runs included in the analysis. PCA ordinations are performed with PCORD version 4.0 (McCune and Mefford 1999). The correlation matrix is used because it scales each component of covariance by the variance of the parameters involved, so measurements with different units can be included in the same analysis.

EXPERIMENTAL RESULTS AND DISCUSSION

Increased Diversity

After 1000 generations, all 12 populations evolved on a mixture of glucose and acetate showed diversity in their colony sizes (Fig. 1A, B). Five populations were bimodal. Populations 29, 31, and 33 had one cluster that peaked near zero and a second smaller cluster far from zero. Population 30 had a cluster near zero and a second cluster with a larger variance than the large size cluster of populations 29, 31, and 33. Population 32 had two separated peaks with the majority of colonies falling in the larger of the two size clusters. Four populations were unimodal; two of these had a single large outlier colony. The remaining three populations had a range of colony sizes and may have been composed of more than two types.

Populations 29, 31, and 33 had clear ecological differentiation that was one-to-one with differences in colony size (Fig. 2 shows data for population 29; populations 31 and 33 are similar). We therefore focus our description on the ecological pattern found in these three populations and only briefly mention the ecological characteristics of the other populations.

Heritability

Population 25's colony sizes were not heritable when streaked onto fresh media. This may be due to increased environmental sensitivity or an elevated mutation rate. The colony sizes of populations 26–36 were heritable when streaked. Populations 29, 31, and 33's large and small colonies gave rise to colonies that appeared on plates after 1 day and 2 days, respectively. Moreover, for these three populations offspring colonies had the same growth phenotype in DM50:50 as their parents. Thus, despite some variation in colony size, the ecological attributes of colonies were heritable.

Ecological Differentiation: Principal Components Analysis

PCA was performed on the 13 estimated growth parameters for populations 29, 31, and 33. The populations differed in the growth parameters that made up the resulting principal components and in the distance from the ancestor of the two phenotype clusters. The switching lag in DM90:10 was the only parameter that was a component of PC1 for all three populations. This is in accordance with our visual examination, which suggested that the primary difference between the large and small type growth curves was the time it took to switch from first to second phase of diauxie (e.g., Fig. 2). The three populations all had six parameters that were strongly correlated ($|r| > 0.5$) with either PC1 or PC2: glucose growth rate, glucose lag, glucose switching density, acetate lag, switching lag in DM90:10, and second growth rate in DM90:10. The correlations of the various ecological parameters with the principal components are summarized in Table 1.

All three populations exhibited phenotypic clustering of L and S types (Fig. 3). In population 29, the L types were phenotypically closer to the ancestor than S types along PC1. In population 31, the L types overlapped with the ancestor and the S types clustered near the L types. In population 33, both the L and S types differed from the ancestor symmetrically. These different patterns may indicate a different order of fixation of evolutionary events, for instance, that the L and S types of population 33 diverged after the fixation of more adaptive mutations than in populations 29 and 31.

Ecological Divergence in Growth Parameters

Populations 29, 31, and 33 had a similar pattern of ecological differentiation in terms of growth parameters (Fig. 4). The L types of all three populations had a longer lag when switching to the second phase of growth and a lower growth

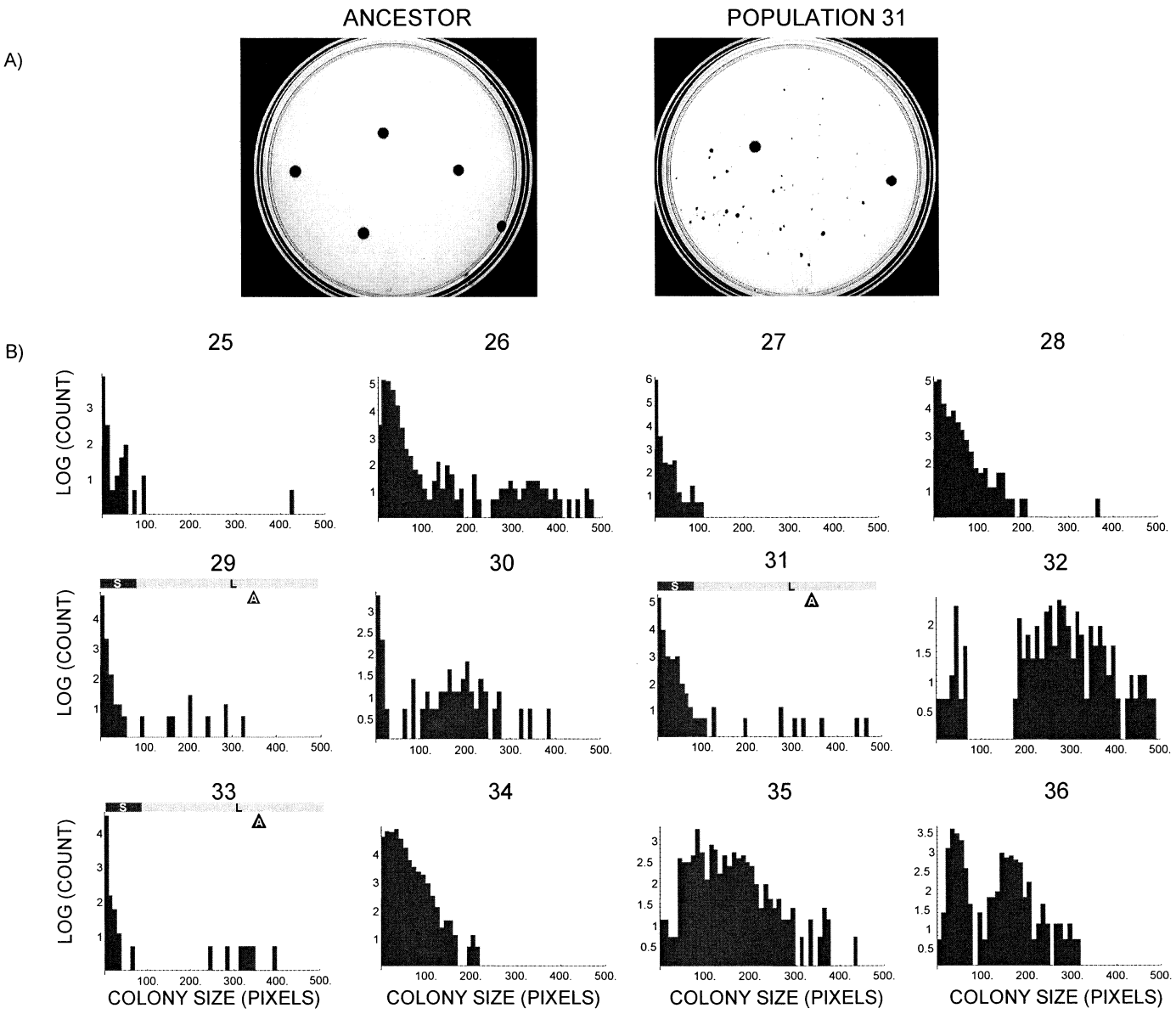

FIG. 1. (A) Plate examples illustrating the difference in the distribution of colony morphologies between the ancestor (left plate) and population 31, which has evolved in a mixed glucose-acetate environment for 1000 generations (right plate). (B) Colony size distributions for all 12 populations evolved in a mixed glucose-acetate environment for 1000 generations. For each population, the distribution is generated with pixel data from at least four plates.

rate during the second phase of growth than the S types (two-tailed *t*-test, $P < 0.05$). Furthermore, the L types had a shorter lag and/or a higher maximum growth rate in glucose, whereas the S types always had both a shorter lag and a higher maximum growth rate in acetate. Maximum growth rate and initial lag are large fitness components for *E. coli* growing in a seasonal environment (Vasi et al. 1994; Lenski et al. 1998), so these differences are not surprising. All three populations exhibited a trade-off between maximum growth rates in glucose and acetate, with the L type of each of these populations exhibiting a higher growth rate in glucose and a lower growth rate in acetate than the S type (two-tailed *t*-test, $P < 0.05$). These results for growth on a single resource contrast somewhat with results for growth on mixed resources: in the first phase of DM50:50, the L types of population 33 had a higher growth rate than the S types, but in both populations 29 and 31 the L and S types had the same growth rate in the first phase of diauxie. We note that all types tended to have a higher growth rate in DM50:50 than in DMglucose, suggesting that acetate was being used simultaneously with glucose during the first phase of growth. Furthermore, both L and S types appeared to use glucose preferentially, so the S type was not using just acetate but rather a combination of glucose and acetate.

There were also differences in the density at which strains switched between first and second phases of diauxie. Populations 31 and 33 had switching density differences in DM50:50 and populations 29 and 31 had switching density differ-

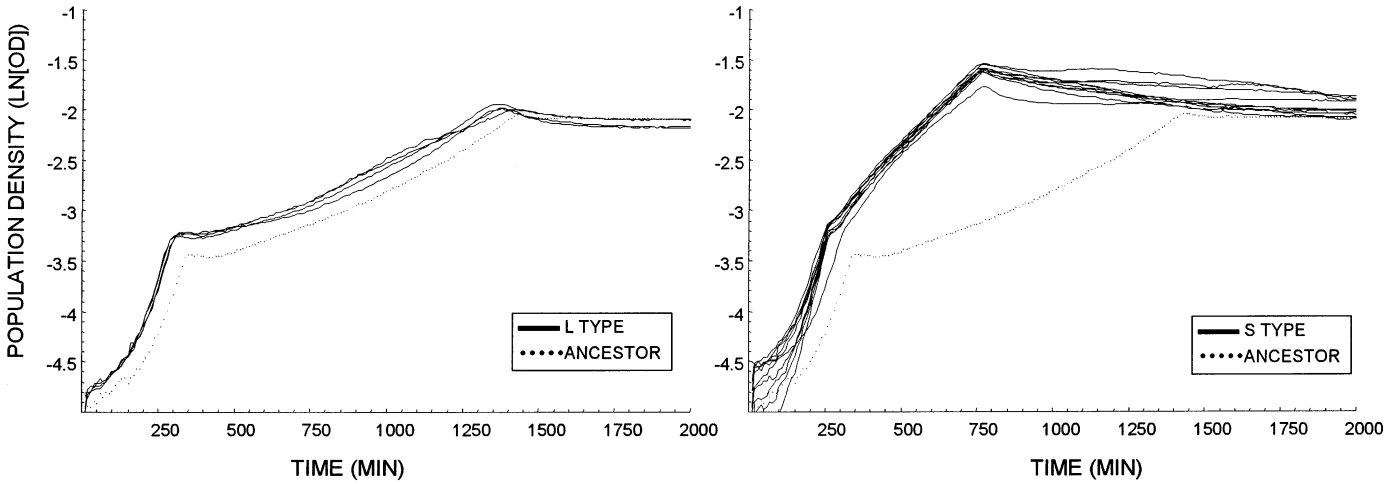

FIG. 2. Growth curves for: (A) L types and (B) S types from population 29 grown in DM90:10. The growth curve of the ancestor is given by the thin dotted line that is set apart from the other curves in the two panels. All strains exhibit diauxic growth patterns, but the pattern differs between L, S, and ancestor.

ences in DM90:10, with the L types switching at a lower density than the S types (Fig. 5). There are two possible interpretations for this result. It may be that L colonies use glucose, the preferred resource, with lower efficiency because of a rate-yield trade-off (Pfeiffer et al. 2001). For instance, fermentative catabolism of glucose can increase a cell's growth rate when the TCA cycle is operating at maximum capacity under oxygen limitation, but at the expense of extracting less energy from the glucose. However, the data are also consistent with the hypothesis that the types differ in the degree to which they use glucose and acetate during the first phase of diauxie. If the S types use some of the acetate during the first phase of growth, more biomass would be produced during this phase in total because all of the glucose would be used up in addition to some of the acetate. Measurements of resource dynamics will be necessary to assess the relative validity of these interpretations.

The pattern of phenotypic change relative to the ancestor differed slightly between the three populations. S strains of all three populations had a significantly shorter switching lag in DM90:10 and a higher maximum growth rate in DMacetate than the ancestor ($P < 5.0 \times 10^{-6}$, Fig. 4). L strains from all three populations had a higher maximum growth rate in DMglucose than the ancestor ($P < 0.01$). In population 29, L strains had a switching lag in DM90:10 that was indistinguishable from the ancestor but had a significantly lower maximum growth rate in DMacetate than the ancestor ($P < 0.001$), whereas S strains had a higher maximum growth rate in DMglucose than the ancestor ($P < 0.001$). In populations 31 and 33, L strains had a significantly lower switching lag in DM90:10 and a higher maximum growth rate in DMacetate relative to the ancestor ($P < 0.05$), and S strains of these two populations had a lower maximum growth rate in DMglucose relative to the ancestor ($P < 0.05$). Thus, relative to the ancestor, the L strains of population 29 apparently had an increased ability to grow quickly on glucose at the expense of a decreased ability to grow quickly on acetate, and S strains of this population had a slightly increased ability to grow quickly on glucose but a greatly improved ability to grow rapidly on acetate. The pattern is inverted in populations 31 and 33, with the L strains of these populations showing improvements in their growth rates on both glucose and acetate relative to the ancestor, whereas the S strains of these populations had large gains in their acetate growth rate with decreased ability to grow quickly on glucose. This suggests that either different physiological constraints were fixed during the initial course of adaptation before diversification occurred or there are multiple ways for evolutionary branching to occur in this system, so that the branching observed in each population reflects the chance order of mutational events.

Overall, it is clear that all three populations were similar in their ecological diversification, but the populations differed in the details and degrees of difference in mean parameter values between L and S types and in the pattern of differentiation relative to the ancestor.

Frequency-Dependent Fitness

To test if the described ecological divergence results in frequency-dependent fitness leading to stable coexistence, we performed invasion experiments between a single S and L type for populations 29, 31, and 33 in DM50:50. In one direction, the L type started at 10% of the population, and in the other direction the S type started at 1% of the population. These proportions were used because initial experiments suggested that the L type invades slowly and the S type can invade rapidly (data not shown). Each type increased in frequency when rare to reach a balanced proportion that was independent of the initial frequencies (Fig. 6A). This frequency fluctuated slightly, but this fluctuation was likely due to measurement error, and both types persisted for the course of the 14-day experiment. Hence, the relative fitness of each type depended on its frequency and each type had a fitness advantage when rare.

To further explore the fitness differences between L and S, we measured colony size frequency over the course of a single 24-h growth cycle in DM50:50 for populations 29, 31,

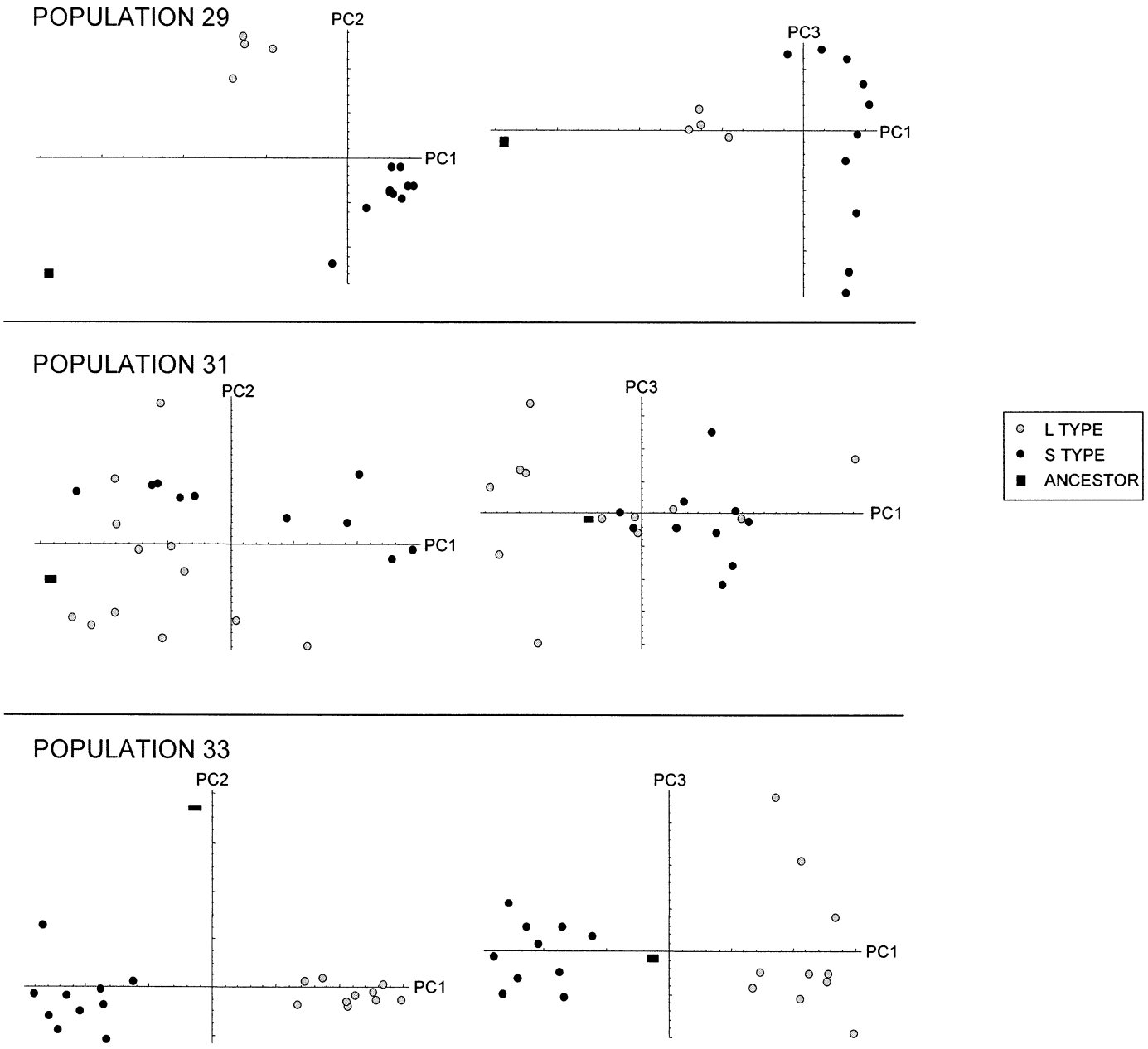

FIG. 3. Principal components analysis of 13 growth parameters for populations 29, 31, and 33. PC1 versus PC2 and PC1 versus PC3 are shown. Each population is analyzed separately, so the principal component axes differ for each population. Each point represents a single colony; the filled square is the ancestor. Clustering of L and S types is apparent in all three populations. For detailed explanations, see text.

and 33. Based on the ecological differences between L and S, we expected that the L type would increase in frequency during the first phase of growth because in population 33 it had a higher growth rate when grown alone in DM50:50 and may also have had a shorter initial lag (data not shown). During the second phase of growth we expected that S would increase in frequency because it had a lower switching lag and higher growth rate in acetate (cf. Fig. 2). This is exactly what was seen, with L rapidly increasing during the first phase of growth and then rapidly decreasing during the second phase of growth (Fig. 6B). These data show that in all three populations there was a competitive interaction between L and S types that reversed in the two growth phases of the adapted environment. During the first phase, the L type was a better competitor, and during the second phase the S type was a better competitor, where ''better'' means that the type can increase in frequency.

Other Populations

Population 34 did not have a distinct visual pattern of dimorphism in colony size, but did exhibit two growth phenotypes. Population 30 had dimorphic colony sizes and the L types had two forms of growth phenotype, while the S type

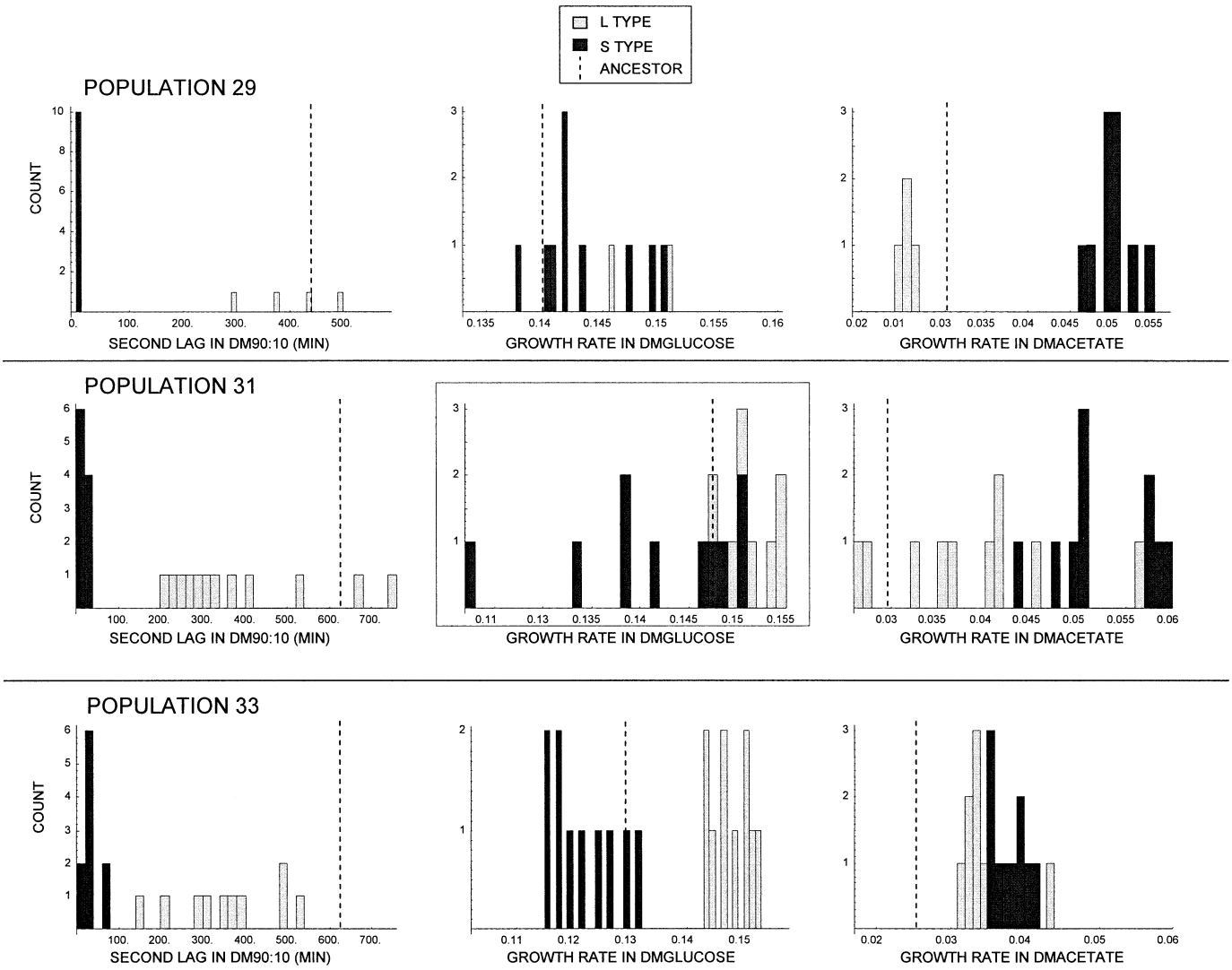

FIG. 4. Histograms of growth parameters for individual colonies isolated from populations 29, 31, and 33. The second lag in 90% acetate plus 10% glucose is significantly longer for L types of all three populations ($P < 0.01$). L types from all three populations have significantly higher maximum growth rate in 100% glucose ($P < 0.05$). Maximum growth rate in 100% acetate is significantly lower for L types of all three populations ($P < 0.05$). The dotted line indicates the mean ancestral phenotype measured during the same experimental run in which data for each set of colonies were gathered. Growth rates in units per 10 min.

had a single form of growth phenotype. Population 30's L type growth parameters were either like those of the S type or like the L types of populations 29, 31, and 33. Populations 25, 26, 27, 28, 32, 35, and 36 did not exhibit two growth phenotypes for the colonies sampled and assayed in the BioScreen, but did possess variation in the growth parameters and were clearly adapted relative to the ancestor. All colonies from populations 25, 26, 27, 28, 32, 35, and 36 had a short switching lag during diauxic growth, similar to the S colonies of populations 29, 31, and 33.

ADAPTIVE DYNAMICS OF DIAUXIC GROWTH

To lend theoretical support to the idea that frequency-dependent selection on diauxie behavior can generate adaptive diversification, we constructed a model for the evolutionary dynamics of diauxie behavior using the framework of adaptive dynamics (Dieckmann and Law 1996; Metz et al. 1996; Geritz et al. 1998). Adaptive dynamics models assume that evolution is mutation limited and that invasion success of rare mutants is determined by the ecological dynamics of the resident population. We therefore begin by describing diauxic growth in serial batch cultures of bacteria that have a certain degree of catabolite repression, which is a form of phenotypic plasticity where metabolism changes in response to nutrient levels (Varma and Palsson 1994; Chang et al. 2002). We then consider the evolution of catabolite repression using pairwise invasion plots and simulations of evolutionary dynamics.

Model Formulation

Our starting point is the Michaelis-Menten model of population growth, which has been widely used to describe the growth of microorganisms (Stewart and Levin 1973; Hansen and Hubbell 1980; Tilman et al. 1981; Edelstein-Keshet 1988). Let $N(t)$ be the density of the bacterial population at

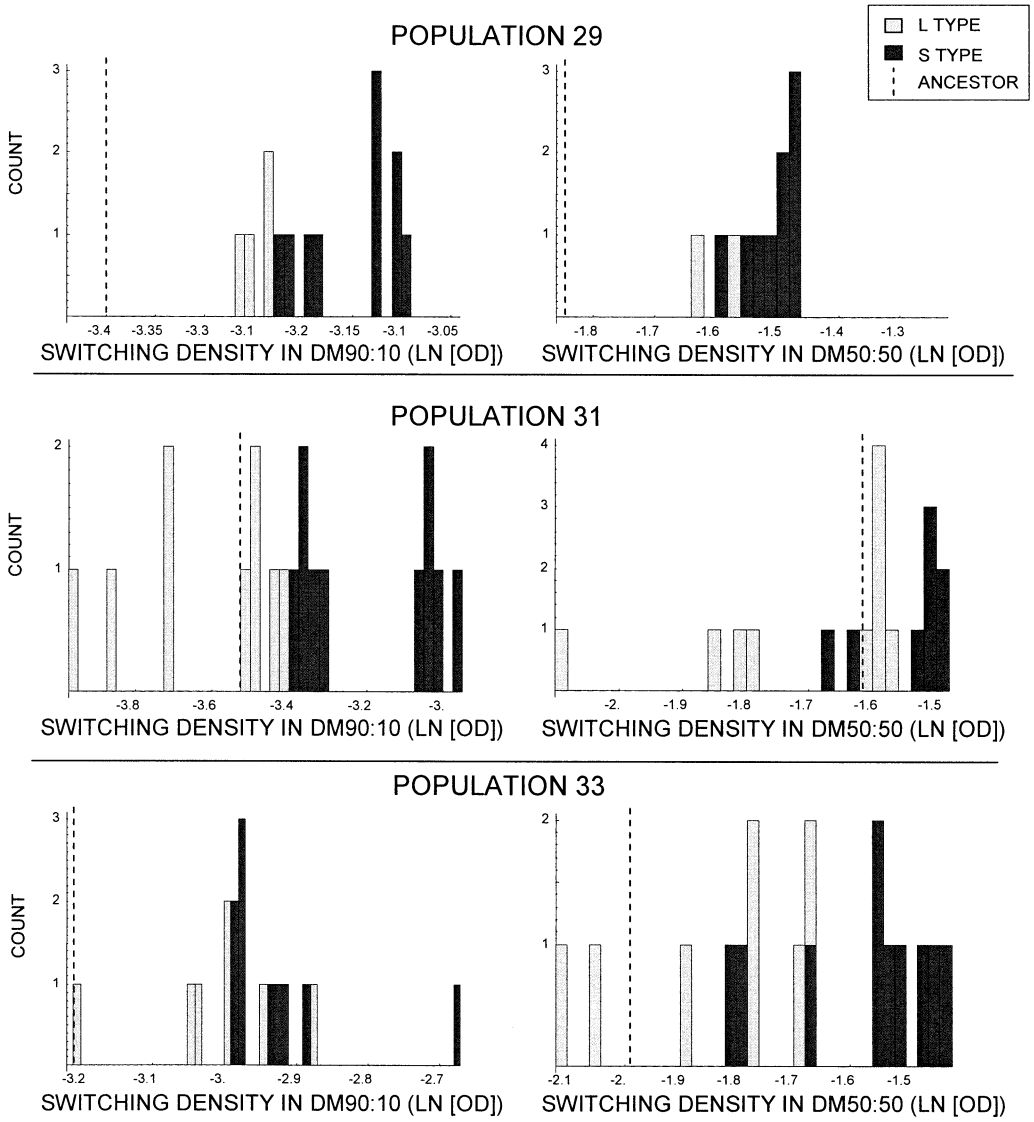

FIG. 5. Switching density of L types in mixed glucose-acetate environments tends to be lower than S types. Population 29: significant in only DM90:10 ($P < 0.0005$); population 31: significant in DM90:10 ($P < 0.01$) and in DM50:50 ($P < 0.05$); population 33: significant only in DM50:50 ($P < 0.01$). The dotted line indicates the mean ancestral phenotype measured during the same experimental run.

time t, and let $X_i(t)$, $i = 1, \ldots, n$ be the concentration of n resources at time t. Then the Michaelis-Menten dynamics for bacterial growth on n resources is given by:

$$N'(t) = \sum_{i=1}^{n} \frac{r_i X_i N}{k_i + X_i} \quad \text{and} \tag{1a}$$

$$X_i'(t) = -\frac{1}{y_i} \frac{r_i X_i N}{k_i + X_i}, \qquad i = 1, \ldots, n. \tag{1b}$$

Here r_i is the maximum growth rate on resource i, k_i is the concentration of resource i at which the growth rate is half the maximal growth rate, and y_i is the yield (for a detailed explanation of these parameters see Edelstein-Keshet 1988). An implicit assumption in the model (eq. 1) is that the resources $X_i(t)$, $i = 1, \ldots, n$ are used additively at all times. However, to model diauxie we have to include preferential substrate use based on catabolite repression, where high-energy carbon sources are used by microorganisms before lower-energy carbon sources. For example, glucose is known to disable *E. coli*'s ability to use other substances such as lactose, melibioise, maltose, and acetate (Saier et al. 1996 and references therein). To describe sequential use of two resources due to catabolite repression, we modify the Michaelis-Menten model for two resources. We take $G(t)$ to be the concentration of the first resource (glucose) at time t, and $A(t)$ to be the concentration of the second resource (acetate) at time t. The corresponding subscripts for the various parameters are, accordingly, g and a.

We assume that a cell has a finite amount of cellular machinery that function in glucose and/or acetate metabolism, and that at the beginning of a batch culture, this cellular machinery is partitioned between growth on glucose and growth on acetate. This constraint forces the cell to choose between catabolizing glucose and acetate: simultaneous use

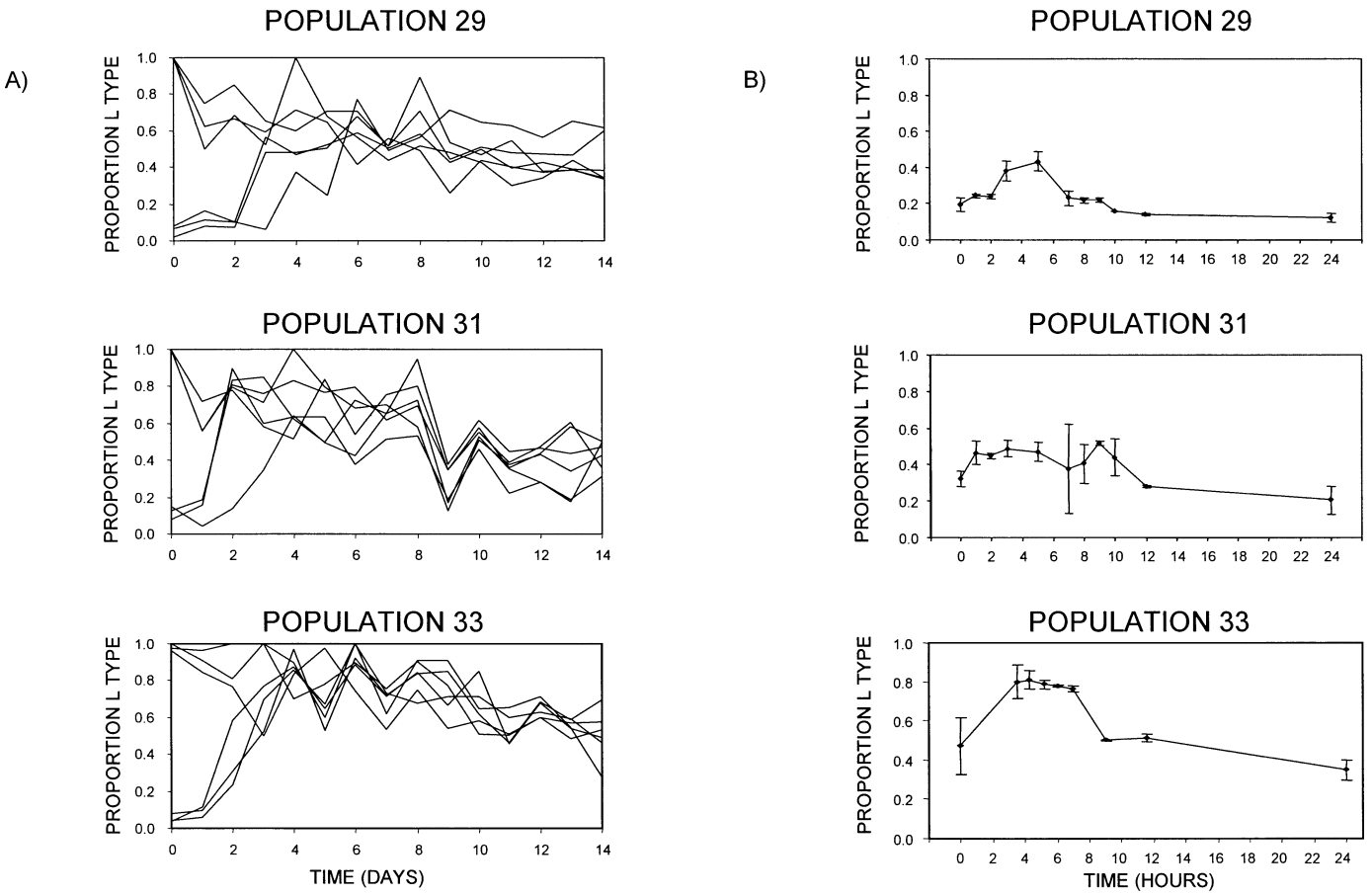

FIG. 6. (A) Results from mutual invasion experiments for populations 29, 31, and 33. The frequency of L types is measured daily for 14 days of serial transfer in 50% acetate plus 50% glucose starting with about 99% L and with 10% L. Each line is a replicate assay. In all cases, the frequency of L types equilibrates at an intermediate level, showing that both L and S types can increase from frequencies. (B) The frequency of a single L strain growing with a single S strain over 24 h for populations 29, 31, and 33. The two strains were grown together for at least three days to reach a stable frequency. Proportions are estimated from plate counts at each time point; error bars represent 95% confidence intervals assuming an underlying normal distribution.

of both substrates is possible but involves a trade-off, so that having a higher growth rate on glucose leads to catabolite repression of acetate consumption. Specifically, we assume that the degree of catabolite repression is determined by the phenotype *cr* of the cell: *cr* is defined to be the proportion of metabolism that is used for glucose catabolism at the beginning of a growth cycle. Similarly, $(1 - cr)$ is the proportion of metabolism used for acetate degradation at the beginning of the growth cycle. It is known that in standard laboratory *E. coli*, growth on glucose leads to the down-regulation of the enzymes in the glyoxylate shunt (Oh and Liao 2000; Oh et al. 2002) and acetyl CoA synthetase (ACS), that brings acetate into central metabolic pathways (Brown et al. 1977). In the experiments of Treves et al. (1998), two cross-feeding types had insertions at different locations in the promoter region of *acs* that led to up-regulation, while four other cross-feeding types had the same single nucleotide substitution T → A that increased the homology to CRP, a transcription factor that is activated by glucose starvation (Saier et al. 1996; Treves et al. 1998). It is interesting to note that the increase in ACS activity differed depending on the insertion sequence, and was highest for the T → A transversion. Therefore, ACS activity appears to be quantitative rather than simply on or off, and it is likely that regulatory changes that affect the glyoxylate shunt are also quantitative. Hence, it is biologically reasonable to assume that catabolite repression is an evolving quantitative character.

Moreover, we assume that the temporal dynamics of resource use is determined by a switching function, which is also determined by the bacterial phenotype. The switching function can be thought of as a decision-making function that determines which resource to use when. We assume that switching depends on the glucose concentration in the medium: once glucose concentration falls below a threshold level, repression of acetate metabolism is relieved and acetate starts being converted into biomass. However, there is a lag period during which intracellular signaling pathways, such as CRP and CRA (Saier et al. 1996), translate this lack of glucose into the activation of metabolic machinery for the catabolism of acetate. We assume that the rapidity of this derepression, the switching lag, is proportional to the phenotype *cr* squared and that after the switching lag has elapsed growth on acetate proceeds. We note that with a linear rather than a quadratic trade-off, evolutionary branching (as explained

below) also occurs for many parameter combinations, but the two emerging phenotypes diverge to the boundaries of the phenotype range, $cr = 0$ and $cr = 1$. Although $cr = 1$ makes sense as a type that uses only glucose during the first phase, a type with $cr = 0$ never uses glucose and hence does not accord with our experimental results, which show that both types use glucose during the first phase of diauxie.

Equations for Diauxic Growth

To implement these assumptions, we multiply the glucose growth term of $N'(t)$ by cr and we multiply the acetate growth term of $N'(t)$ by the switching function $h_a(cr)$. This gives the following equations:

$$N'(t) = cr\frac{r_g GN}{k_g + G} + h_a(cr)\frac{r_a AN}{k_a + A}, \tag{2a}$$

$$G'(t) = -cr\frac{1}{y_g}\frac{r_g GN}{k_g + G}, \quad \text{and} \tag{2b}$$

$$A'(t) = -h_a(cr)\frac{1}{y_a}\frac{r_a AN}{k_a + A}. \tag{2c}$$

The switching function is given by

$$h_a(cr) = \begin{cases} (1 - cr) & \text{when } t < t^*, \\ 0 & \text{when } t^* \le t \le t^* + q \cdot cr^2 \\ 1 & \text{when } t^* + q \cdot cr^2 < t, \end{cases} \tag{3}$$

where t^* is the time at which the glucose concentration $G(t)$ reaches the starvation threshold p, and q is the maximum switching lag. Note that t^* is determined by the ecological dynamics of resource use, whereas p and q are fixed parameters.

The ecological workings of equations (2) and (3) are best explained by considering what happens during a growth cycle. At the beginning of the cycle, glucose concentration is high, so that $h_a(cr) = 1 - cr$, hence the bacteria grow according to additive Michaelis-Menten kinetics and maximal growth rates $cr \cdot r_g$ and $(1 - cr) \cdot r_a$ on glucose and acetate, respectively. As glucose is used and its concentration falls below the starvation threshold p, both glucose and acetate metabolism are shut down due to general inhibition of cellular processes. (Note that the switching function h_a only describes changes in acetate metabolism; we do not explicitly incorporate the shutdown of glucose metabolism, because this shutdown is automatic once the glucose concentration falls below the starvation threshold p; this threshold is assumed to be very small, so that effectively no growth on glucose occurs below this threshold.) During the ensuing switching lag, all metabolism is turned off, which reflects the experimental data presented above (e.g., Fig. 2). However, acetate metabolism starts again after the switching lag given by $q \cdot cr^2$. The trade-off between using metabolic machinery for growth on glucose during the first phase (high $cr \cdot r_g$) and having a long switching lag (high $q \cdot cr^2$) is the basic mechanism driving the evolutionary dynamics in our model. A fundamental assumption of our model is that this trade-off is fixed over evolutionary time.

Adaptive Dynamics of Catabolite Repression

To derive the adaptive dynamics from this ecological background, we assume that the resident population consists of a single strain with phenotype cr, and then consider what happens when a mutant cr' arises in this resident. The invasion fitness of the mutant is simply given by its ecological growth rate: if the mutant's density increases over one growth cycle of the batch culture, its invasion fitness is positive, otherwise it is negative. The dynamics of the mutant, and hence its growth rate over a single growth cycle, is determined by its phenotype cr' and its switching function $h_a(cr')$ according to eq. (3), in which N now denotes the density of the mutant. If the mutant is rare, its effect on the temporal dynamics of the concentrations $G(t)$ and $A(t)$ is negligible, and hence the dynamics of $G(t)$ and $A(t)$ are solely determined by the resident cr. In this way, the invasion success of the mutant phenotype cr' is determined by the resident phenotype cr.

To evaluate the adaptive dynamics from invasion analysis, we use the method of pairwise invasion plots (Geritz et al. 1998; Doebeli 2002). A pairwise invasion plot has the resident phenotype cr along the x-axis and the mutant phenotype cr' along the y-axis; a point (cr, cr') in this plot is filled in if the mutant cr' can invade the resident cr, otherwise the point is left blank. The evolutionary dynamics of the phenotype cr can be derived from these invasion plots (Geritz et al. 1998). In our model, two basic types of evolutionary scenarios occur, and these are illustrated in Figure 7. First, with a low switching cost, the model has an evolutionarily stable strategy (ESS) at a high value of cr. Figure 7A illustrates the case of an ESS at $cr = 1$, where the whole region above the diagonal is filled in. This means that for all resident phenotypes, mutants cr' with $cr' > cr$ can invade, whereas mutants with $cr' < cr$ cannot. In other words, there is directional selection for higher cr irrespective of the resident phenotype. Therefore, the outcome of the evolutionary process is a population that is monomorphic for $cr = 1$, corresponding to full catabolite repression of acetate metabolism during the first phase of diauxie. This evolutionary dynamic is illustrated in Figure 7B, which was produced by starting out with a resident population that is monomorphic for a small value of cr and testing resident populations continually with newly arising mutants $cr' = cr \pm \varepsilon$, where the mutation size ε is chosen uniformly randomly in the interval [0, 0.01]. If the randomly chosen mutant has a positive growth rate, it becomes the new resident, which results in the evolutionary dynamics tending toward the highest allowable value of cr.

The second basic scenario revealed by our model occurs for high switching costs and is very different from the first because it leads to evolutionary branching (i.e., adaptive diversification). In this case, the dark regions in the pairwise invasion plot meet at a point on the diagonal. The corresponding resident phenotype cr^* has the following properties. For resident phenotypes $cr > cr^*$, only mutants with $cr' < cr$ can invade (dark region below the diagonal for $cr > cr^*$ in Fig. 7C); that is, for $cr > cr^*$ selection is directional toward lower cr-values, toward cr^*. For $cr < cr^*$, only mutants with $cr' > cr$ can invade (dark region above the diagonal for $cr < cr^*$ in Fig. 7C), and selection is directional toward higher

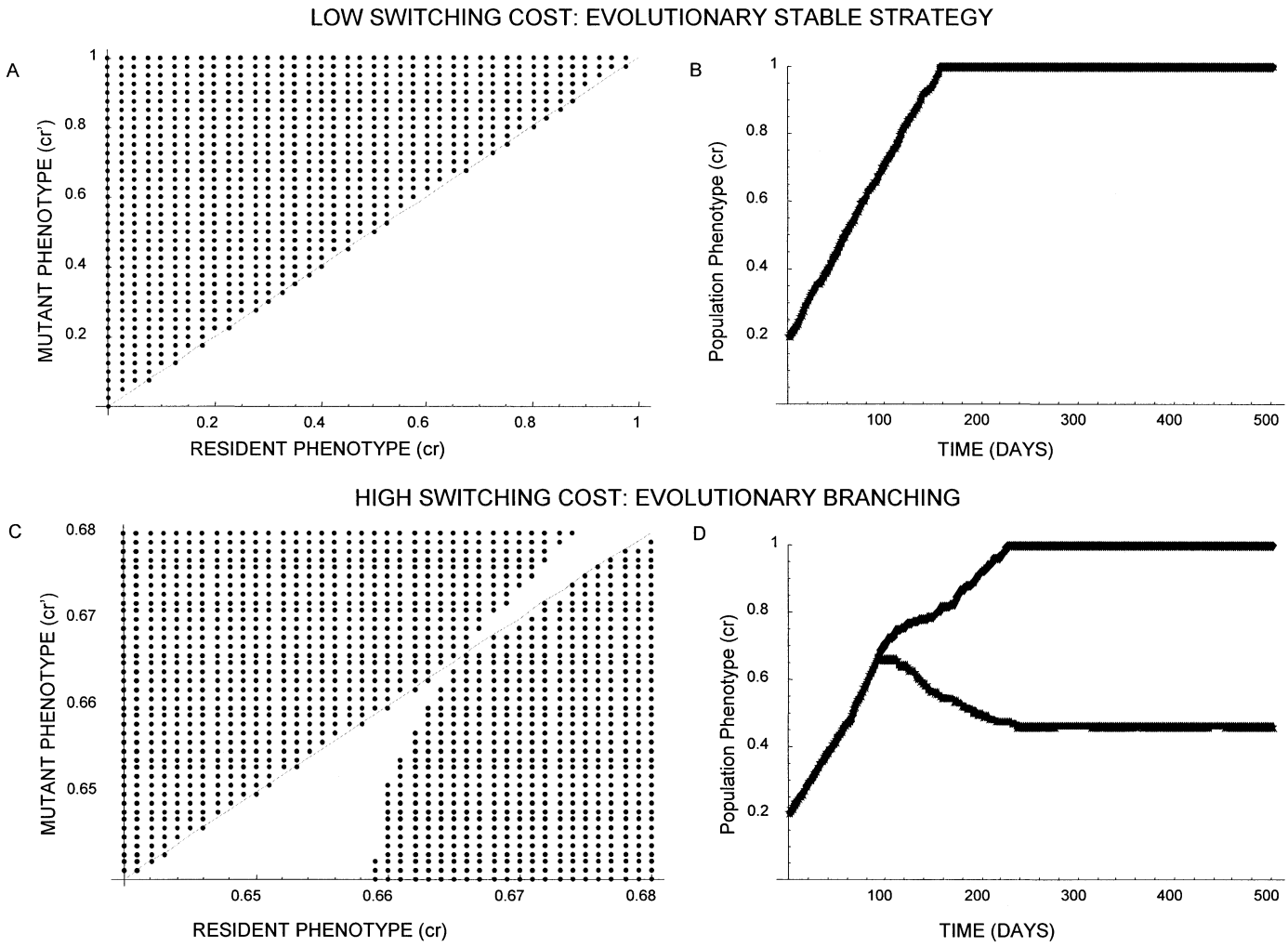

FIG. 7. Low switching cost gives an evolutionary attractor at full catabolite repression, whereas high switching cost leads to evolutionary branching. (A) In the pairwise invasion plot, each filled point represents a mutant-resident combination in which the mutant can invade. Because all points above the diagonal are filled, any resident value of *cr* can be invaded by mutants *cr*′ with *cr*′ > *cr*, that is, there is directional selection for higher values of the phenotype *cr*. (B) The evolutionary dynamics resulting from the regime of directional selection shown in (A): the phenotype *cr* converges evolutionarily to *cr* = 1. Parameters: rg = 1, kg = 0.1, yg = 1, ra = 0.2, ka = 0.1, ya = 0.2, **q** = 5, p = 0.001, c0 = 5, a0 = 5. (C) With a high switching cost, the pairwise invasion plot reveals existence of an evolutionary branching point at *cr* = 0.665. The plot shows that at resident phenotypes above the branching point, there is directional selection for lower values of the phenotype *cr*, and at resident phenotypes below the branching point there is directional selection for higher values of *cr*. Once the resident is at the branching point, all phenotypes can invade. (D) The evolutionary dynamics corresponding to the pairwise invasion plot in panel (C) show convergence to the branching point and subsequent evolutionary branching, that is, adaptive splitting into diverging phenotypic clusters. Parameters: rg = 1, kg = 0.1, yg = 1, ra = 0.2, ka = 0.1, ya = 0.2, **q** = 20, p = 0.001, c0 = 5, a0 = 5.

cr-values, that is, again towards *cr**. Thus, independent of the starting point, the population evolves toward *cr**, which is therefore an evolutionary attractor. However, when the resident is at *cr**, all mutants can invade, because in the pairwise invasion plot, the vertical line through *cr** is wholly contained in the dark region.

It follows from these considerations that after the population has evolved to the attractor *cr**, it will give rise to two diverging phenotypic clusters (Geritz et al. 1998). This is illustrated in Figure 7D, which was produced similarly to Figure 7B, except that after branching occurs we now assume two starting resident populations, that is, two strains or branches, one that was monomorphic for a *cr*-value that was slightly below *cr** and one that was monomorphic for a *cr*-value that was slightly above *cr**. At any given point on the evolutionary trajectory, that is, for any given pair of resident values of *cr*, the two resident strains jointly determine the resource dynamics, from which the invasion fitness of newly arising mutants can be calculated. If a mutant arising in one of the resident strains has a positive growth rate, it replaces that resident strain and becomes the new resident in that branch. This results in two diverging branches, and at the evolutionary endstate one branch has a *cr*-value of one, whereas the other branch has an intermediate *cr*-value (Fig. 7D). Bacterial growth dynamics for these two coexisting strains are shown in Figure 8, which illustrates that the patterns of diauxie occurring in these strains are in qualitative agreement with the patterns found in our di-

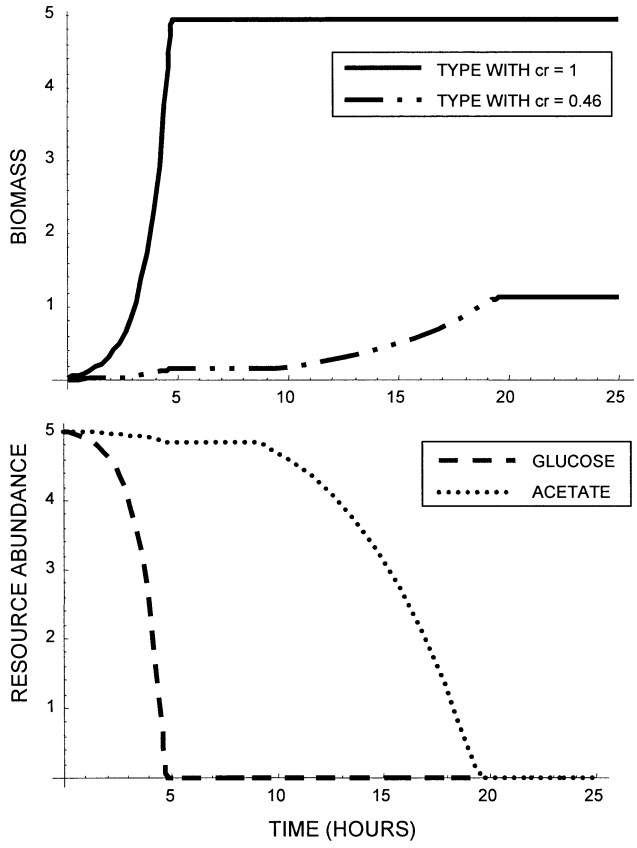

FIG. 8. Ecological dynamics of the two coexisting phenotypes that emerge as a result of the evolutionary branching shown in Figures 7C, D ($cr = 1.0$ and $cr = 0.46$). The top panel shows the growth curves of the two types during a single growth cycle in a simulated batch culture. The bottom panel shows the corresponding dynamics of the two resources. The parameters are as in Figures 7C, D.

morphic experimental populations (cf. Fig. 2). Initially, the type with $cr = 1$ (L) has a higher growth rate and increases in frequency. The type with $cr = 0.46$ (S) depletes some of the acetate during this phase, and if grown by itself it would reach a higher switching density. When glucose goes below the threshold concentration at $t = 5$, both types cease growth. After 0.46^2q has elapsed from the switching point, S resumes growth and depletes the remaining acetate before L's switching lag of $1.0q$ is over.

Summary of Model Results

Figure 7 illustrates that increasing the maximal switching cost q induces a transition from regimes of directional selection leading to ever higher values of cr to regimes leading to evolutionary branching. The transition point varies depending on the other parameters in the models, but the general conclusion from our numerical investigations of the adaptive dynamics of diauxie is that the system exhibits evolutionary branching when the maximal switching cost q is sufficiently high. High values of q induce steep trade-offs, which in turn can induce strong frequency-dependence when resource availability is fluctuating: invasion success of mutants depends on the resource dynamics generated by the resident. Thus, evolutionary branching is due to frequency-dependent competition in a fluctuating environment. Overall, our theoretical results strongly support the notion that adaptive diversification occurred in the experimental populations described in the previous section due to frequency-dependent selection on phenotypic plasticity in the form of diauxie.

GENERAL DISCUSSION

In this paper we have argued that frequency-dependent selection on ecological phenotypes of experimental *E. coli* populations grown on a mixture of resources has led to adaptive diversification. Thus, our experimental system is a model for investigating how ecological interactions can generate adaptive splitting under fully sympatric conditions. Consumption of resources in our experimental batch cultures creates an environment in which the availability of resources fluctuates due to consumption. In such fluctuating environments, it is well known that *E. coli* exhibit diauxic growth: they use the preferred resource glucose when it is abundant, but then switch to acetate (or the other less-preferred resource) once glucose concentration has dropped (reviewed in Saier et al. 1996). Diauxic growth has emerged as an important example of metabolic reprogramming (Chang et al. 2002; Mahadevan et al. 2002). This phenomenon is clearly related to the concept of phenotypic plasticity, with organisms altering their metabolism based on resource availability. Our work shows that in many cases, rather than evolving to a single optimal level of plasticity (Agrawal 2001), different types of plasticity, that is, different strategies of temporal resource use in a fluctuating environment, can arise adaptively from a common ancestor and then coexist. This phenotypic plasticity is heritable, and our invasion analysis (Fig. 6) shows that different diauxie types can mutually invade each other. Therefore, selection on diauxie is frequency dependent: the type of plasticity, or diauxie behavior, that is advantageous for a mutant strain depends on the diauxie type of the current resident strain. It is the ecological dynamics of resource use that create the frequency dependence in the system, and hence a detailed understanding of these dynamics can lead to an understanding of the evolutionary process that in turn shapes the ecology.

We investigated the evolutionary dynamics resulting from this eco-evolutionary feedback using a mathematical model based on the framework of adaptive dynamics (Metz et al. 1996; Geritz et al. 1998). The model assumes that there is a trade-off between growth rate during the first phase of diauxie and the time lag during the switch to acetate. If this trade-off is strong enough, the model exhibits evolutionary branching of diauxie types, a process during which a genetically uniform lineage splits into two diverging phenotypic clusters due to frequency-dependent ecological interactions. Evolutionary branching has been developed as an important theoretical metaphor for adaptive splitting of lineages under fully sympatric conditions (e.g., Doebeli and Ruxton 1997; Geritz et al. 1998; Dieckmann and Doebeli 1999; Doebeli and Dieckmann 2000, 2003). The ecological diversification occurring in our experimental *E. coli* populations is perhaps the first direct empirical confirmation of evolutionary branching that ties ecological data to a firm theoretical framework. Taken together, our empirical and theoretical results thus con-

tribute to elucidating ecological selection pressures that can lead to adaptive splitting in sympatry.

To further analyze the correspondence between theory and experiments, it will be very interesting to describe the ecological dynamics of the strains present in the experimental lines at different evolutionary time-points. Such a dynamical perspective is crucial for understanding the evolution of diversity, yet it seems to have been somewhat neglected in previous experimental work on diversity in microorganisms. Although very valuable in its own right, this work was mainly concerned with the maintenance of diversity (Rainey et al. 2000; Kassen et al. 2000; Buckling et al. 2000; Kassen 2002), rather than with its evolutionary origin through adaptive splitting of genetically uniform ancestral populations.

A notable exception is the study of the evolution of cross-feeding polymorphisms in populations cultured on a single resource (Helling et al. 1987; Rosenzweig et al. 1994; Treves et al. 1998; Rozen and Lenski 2000). Because cross-feeding involves consumption of by-products that arise during metabolism of the primary resource, these polymorphisms effectively also evolve on a mixture of resources. Therefore, our results are likely to be relevant for understanding the evolution of cross-feeding in batch cultures. In fact, preliminary analysis of experimental populations that evolved on batch cultures with glucose as the sole resource suggests that a similar pattern of adaptive diversification occurred (data not shown). In particular, such populations also became polymorphic for diauxie behavior, and it will be very interesting to investigate the differences in the diversity of diauxie behavior between the populations that evolved on glucose only and those that evolved on a mixture of resources. Such a comparative analysis should yield valuable insights for understanding the phenomenon of cross-feeding.

Key to the process of adaptive diversification is the set of ecological constraints dictated by the physiology of specific organisms. The existence of the trade-off between growth in the first and second phases of diauxie assumed in our adaptive dynamics model is supported by our experimental data, which shows that the proportion of L cells increases during the first growth phase but subsequently decreases during the second phase because S cells have a much shorter switching lag as well as a higher growth rate on acetate. Three enzymes are required for *E. coli* to catabolize acetate: ACS brings acetate into the central carbon pathway, and isocitrate lyase in conjunction with malate synthase form the glyoxylate shunt that bypasses the loss of carbon as CO_2 during the full tricarboxylic acid (TCA) cycle but does not provide as much energy as the entire TCA cycle (for a review see Cozzone 1998). Because the TCA cycle is the main pathway by which glucose is catabolized, changes in the regulation of these three enzymes would likely influence maximum growth rate on glucose. In addition, patterns of gene expression during diauxie show that total mitochondrial RNA levels decrease during the switching phase, because the transcription apparatus is inactivated (Chang et al. 2002). These considerations suggest a cost to switching between growth on different substrates (at least in the standard *E. coli* strains used in laboratory experiments), which could lead to a trade-off between the degree of preference for glucose, and hence total growth rate, during the first phase of diauxie, and the amount of metabolic reprogramming required (i.e., the cost) for switching to acetate. Our model shows that such a trade-off is sufficient for the evolution of a dimorphism in diauxie behavior. Thus, the model provides a tentative explanation not only for the coexistence of different diauxie types, but also for the dynamics of their evolutionary origin.

Our model is a simplification of the experimental system, because we do not allow maximum growth rates, lags, or yields in glucose and acetate to evolve independently of the degree of catabolite repression. With a higher value of *cr*, a strain would exhibit a higher maximum growth rate in glucose than a strain with a lower value of *cr* because the glucose growth term is always multiplied by *cr*, but all strains are assumed to have the same maximum growth rate in acetate. Our empirical data shows that this is not the case, and earlier theoretical work has shown that a trade-off function with positive curvature between maximum growth rate on glucose and maximum growth rate on acetate is sufficient for evolutionary branching to occur in both constant and seasonal environments when resources are used nonpreferentially (Doebeli 2002). Future work should assess the relative importance of these two types of trade-offs. Furthermore, we assume that the constraint of the system (i.e., the switching cost) is fixed through evolutionary time. A more detailed model that incorporates the physiological basis of constraints in the system will be required to explore the evolutionary trajectories of phenotypes with changing constraints. Such a model will require a more thorough understanding of the genetic differences that give rise to different ecological phenotypes in our system.

Perhaps the most promising way in which the present work can be extended is the investigation of the genetic mechanisms underlying the observed evolutionary diversification. It is likely that the genes responsible for this diversity regulate the activation and inhibition of metabolic pathways. For example, Treves et al. (1998) found that the cross-feeding strains that evolved in their chemostat cultures had mutations in the promoter region of the *acs* locus and were associated with increased activity of ACS, an enzyme used in acetate uptake (Brown et al. 1977). All strains occurring in our experiments, including the ancestral strain, appear to posses the T $\rightarrow$ A transversion that was found in four of Treves et al.'s (1998) populations (M. Friesen, M. Bertrand, M. Doebeli, unpubl. data), so this particular mutation does not seem to be responsible for the diversity observed in our populations. We are currently investigating changes in activity levels of enzymes and regulatory proteins involved in the glyoxylate bypass, which are known to be differentially regulated during growth on acetate and glucose (Oh and Liao 2000; Oh et al. 2002). Understanding the genetic changes underlying the evolution of diversity in our populations would give general insights into how genetic regulation of phenotypic plasticity can foster evolutionary diversification. Phenotypic plasticity is implicated in many putative processes of adaptive speciation (Dieckmann et al. 2003), and it would clearly be of great value to have a model system in which the significance of phenotypic plasticity for speciation processes can be investigated in genetic and ecological detail.

We realize that there is a basic limitation to the relevance of our results for the problem of speciation: our *E. coli* strains

are asexual, and hence problems regarding the evolution of reproductive isolation do not arise. However, we think it is important to realize that there is actually a great advantage in using asexual microorganisms for studying the evolution of diversity: by avoiding the problem of gene flow between emerging lineages, it is much easier to elucidate basic ecological mechanism, such as frequency-dependent selection on phenotypic plasticity, that can generate adaptive splitting. Therefore, we think that experimental model systems such as ours are very promising tools for studying fundamental questions about speciation. For the die-hards, we close by noting that because *E. coli*'s ability to sexually recombine is conferred by a plasmid, it is possible that this model system could even be used to study the evolution of reproductive isolation in populations that are under disruptive selection due to frequency-dependent competition.

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