

# Enzyme Kinetics, Substitutable Resources and Competition: From Biochemistry to Frequency-Dependent Selection in *lac*

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## ABSTRACT

Trade-offs in catalytic efficiency at the *lac* permease of *Escherichia coli* produce alleles with different substrate specializations that are selectively favored on different galactosides. We show that differential resource utilization during competition for mixtures of galactosides produces frequency-dependent selection at *lac*. However, the polymorphism is protected only in a narrow range of galactoside ratios despite intense selection on the pure galactosides. Hence, stabilizing frequency-dependent selection protecting natural allozyme polymorphisms through differential resource utilization will be sporadic and ephemeral in randomly changing environments. A comparison of predictions, based on first principles, with experimental outcomes reveals an additional, unanticipated source of weak selection.

THERE are many instances in which frequency-dependent selection is expected to act (LEVIN 1988; GAVRILETS and HASTINGS 1995). In allelopathic systems (LEVIN 1988) disruptive frequency-dependent selection favors common genotypes, driving them to fixation. Selection for novelty in influenza hemagglutinin genes (BUSH *et al.* 1999) produces successive waves of new alleles that sweep through the viral population, displacing their older predecessors. In contrast to both these mechanisms, selection for rare alleles stabilizes polymorphisms. *Trans*-species polymorphisms, at the self-incompatibility loci of plants and fungi and (possibly) at the MHC loci of vertebrates (KLEIN *et al.* 1998; but see TAKAHATA and NEI 1990), are testimony to the power of stabilizing frequency-dependent selection in maintaining large quantities of genetic variation over long periods of evolutionary time.

Not surprisingly, stabilizing frequency-dependent selection has sometimes been invoked to protect enzyme polymorphisms (ANTONOVICS and KAREIVA 1988). But here, its very power is its Achilles' heel. Most enzymes show no evidence of *trans*-species polymorphism unless speciation is very recent. Like overdominance, stabilizing frequency-dependent selection on enzymes is, at best, an ephemeral phenomenon confined to within-species polymorphisms.

Genealogical studies provide evidence for balanced polymorphisms within species but have difficulty in distinguishing between stabilizing frequency-dependent selection and overdominance in diploids (TAKAHATA and

NEI 1990; APANIUS *et al.* 1997). Sadly, empirical evidence demonstrating the existence of frequency-dependent selection at enzyme loci is lacking. To some extent this can be attributed to technical difficulties in the design and execution of experiments with higher eukaryotes (ANTONOVICS and KAREIVA 1988), difficulties that render data unreliable. Even the appearance of frequency dependence may prove illusory, as when linkage disequilibrium with a selected locus dissipates over the course of an experiment (NEI 1988).

Yet another problem is that diploid population genetic models lack plausible mechanisms giving rise to frequency dependence at enzyme loci. Indeed, LEVIN (1988) suggests that this absence is an artifact of the way in which the population genetics of sexual eukaryotes are modeled. He points out that frequency dependence is ubiquitous in bacterial population dynamics where both the genetics and the underlying ecological interactions are modeled.

The theory underlying frequency-dependent selection through differential resource depletion has long been known to microbial ecologists and evolutionary biologists (STEWART and LEVIN 1973; TILMAN 1980, 1982; GROVER 1997). If there is a difference between the two disciplines it is that ecologists emphasize competition for essential resources (*e.g.*, silicon, phosphorus, nitrogen), whereas evolutionary biologists should emphasize selection on substitutable resources. The reason for the difference is that competition for essential resources has been implicated in maintaining diversity in many communities, whereas alternative enzymic substrates are invariably very similar (*e.g.*, lactose and galactosyl-glycerol) and hence substitutable. An added advantage of studying bacterial populations is that the problem of distinguishing between stabilizing frequency-dependent

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selection and overdominance is eliminated—bacteria are haploid.

The lactose operon of *Escherichia coli* has long served as a model system for studying the relations between enzyme kinetics, metabolic flux, and fitness (DEAN *et al.* 1986; DYKHUIZEN and DEAN 1990; ZHANG and FERENCI 1999; WATT and DEAN 2000). The permease and the  $\beta$ -galactosidase are highly polymorphic, with permease alleles subject to strong selection in laboratory competition experiments (SILVA and DYKHUIZEN 1993; DEAN 1995) and also in nature to judge by DNA sequence analyses (WAGNER and RILEY 1996).

The lactose pathway is capable of handling a broad array of galactosides that differ in the aglycone moieties attached through a  $\beta$ -bond to the 1C of galactose. This suggests an obvious mechanism for maintaining the polymorphism since environmental variability, in the form of alternative substrates, is delivered directly to the active sites of the enzymes. However, the necessary trade-offs in enzyme activity are rare, and for most alleles the rank order of fitnesses is maintained across all galactosides (DEAN 1995). If selection maintains these polymorphisms it must do so by means other than, or at least in addition to, any selection attributable to the alternative substrates investigated.

One exception to the above generalization is strain TD10C, which carries a deregulated *lac* operon derived from ECOR16, an *E. coli* strain isolated from a leopard (OCHMAN and SELANDER 1984). Strongly favored on lactose, galactosyl-glycerol, and methyl-galactose, strain TD10C is at a strong disadvantage against TD2 (which carries a deregulated K12 operon) on lactulose and galactosyl-arabinose (SILVA and DYKHUIZEN 1993; DEAN 1995). Trivially, both operons could be maintained within the species if, in the face of limited migration, selection favors each in different subpopulations. Yet selection might also protect both operons from loss when they co-occur within a single subpopulation through frequency-dependent selection (overdominance is not possible in a haploid species) generated by differential resource depletion.

Here, we investigate the standard chemostat model of competition for two substitutable resources, borrowing the concept of a quasi-steady state from enzyme kinetics (BRIGGS and HALDANE 1925). This enables us to recast the model (see APPENDIX), producing two simple equations that determine the invasibility of the system and define the boundaries where coexistence is possible. We use competition between TD10C and TD2 on mixtures of methylgalactoside and lactulose as a model system to test the theory and to determine the likelihood that such a mechanism might protect polymorphisms of enzymes with broad substrate specificities. We also describe a new method for following the progress of competition using flow cytometry.

## MATERIALS AND METHODS

**Bacterial strains:** The genetic background used in these experiments is the K12 strain DD320, which has served in all previous experiments (DYKHUIZEN and DAVIES 1980; DEAN *et al.* 1986, 1988; DYKHUIZEN *et al.* 1987; DEAN 1989; SILVA and DYKHUIZEN 1993; DYKHUIZEN and DEAN 1994; DEAN 1995) and which is wild type except for a deletion spanning the *lac* operon. Strain TD2 carries a constitutive (*lacI*<sup>-</sup>) but otherwise wild-type K12 operon. Strain TD10C carries a constitutive (*lacI*<sup>-</sup>) derivative (DEAN 1989) of the operon from strain ECOR 16, isolated from the scat of a leopard (OCHMAN and SELANDER 1984). To ensure that the genetic backgrounds of the competitors were as similar as possible both TD2 and TD10C were reconstructed using P1 (*cml chr100*) generalized transduction (MILLER 1972) to move their *lac* operons into a fresh isolate of DD320.

**Media:** Reconstructed strains were isolated on petri plates composed of minimal Davis salts [7 g K<sub>2</sub>HPO<sub>4</sub>, 2 g KH<sub>2</sub>PO<sub>4</sub>, 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g trisodium citrate in 1 liter of distilled deionized water with 1 ml of 1 M MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.5 ml of 1% thiamine added after autoclaving] supplemented with 15 g/liter Bacto agar and 2 g/liter lactose and purified on Bacto MacConkey-lactose agar plates. Isolates of TD2 and TD10C resistant to the bacteriophage T5 were isolated on Luria broth (LB) rich medium (5 g yeast extract, 10 g tryptone, 10 g NaCl in 1 liter of distilled deionized water with 1 g glucose and 2.5 mM CaCl<sub>2</sub> added after autoclaving).

Competition experiments were conducted in minimal Davis salts supplemented with 5  $\mu$ M FeSO<sub>4</sub> (from a stock solution of 5 mM FeSO<sub>4</sub>, 7.5 mM Na<sub>2</sub>EDTA). The sugars, methylgalactoside, and lactulose, which are the sole sources of carbon and energy, are added to a final summed concentration of 0.1 g/liter, which is sufficiently low as to ensure that growth at steady state in the chemostat growth chamber is limited by their availability (DYKHUIZEN and DEAN 1994). The gratuitous inducer isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added to a final concentration of 10<sup>-5</sup> M to ensure full constitutive expression of the operons. All sugars were sterilized by passage through 0.2- $\mu$ m filters.

**Inoculating chemostats:** Competition experiments are conducted between pairs of strains, one sensitive to the phage T5 and the other resistant, a phenotype conferred by mutations in *fhuA* (which encodes an Fe<sup>2+</sup> ferrichrome receptor) that prevent phage attachment. Each is separately inoculated into a side arm flask, containing minimal Davis salts supplemented with 0.1 g/liter lactose, 5  $\mu$ M FeSO<sub>4</sub>, and 10<sup>-5</sup> M IPTG, and grown to a Klett density of  $\sim$ 150. Inocula for the chemostats are prepared by mixing pure cultures (density  $\sim$ 5  $\times$  10<sup>8</sup> cells/ml) in the appropriate proportions as determined by Klett readings.

**Flow cytometry:** The progress of competition is monitored by periodically sampling cultures and determining the proportion of the culture that is T5R. Typically, each competition experiment is sampled five times per day over a period of 3–5 days. Samples are stored overnight at 4<sup>o</sup> prior to staining and enumeration by flow cytometry.

Staining proceeds as follows. To each 200- $\mu$ l sample of stored cells is added 2  $\mu$ l of a 2% (w/v) chloramphenicol (dissolved in ethanol) stock solution and 20  $\mu$ l of a 5  $\times$  10<sup>11</sup>/ml stock of bacteriophage T5 in LB medium. Following incubation at 24<sup>o</sup> for 1 hr, 20  $\mu$ l of the mixture is added to 1 ml of phosphate buffer [7 g/liter K<sub>2</sub>HPO<sub>4</sub>, 2 g/liter KH<sub>2</sub>PO<sub>4</sub>, 500  $\mu$ M Na<sub>2</sub>EDTA (pH 8.0) passed through a 0.22- $\mu$ m nitrocellulose filter to remove particulate matter] containing 10  $\mu$ l 2% (w/v) chloramphenicol stock solution and 100  $\mu$ M cyanine dye, YoPro-1-iodide (Molecular Probes, Eugene, OR). Samples

are incubated in the dark and enumerated at 30, 45, and 60 min by flow cytometry.

Cells are counted with an Epics XL-MCL flow cytometer (Coulter Corporation, Hialeah, FL) equipped with a 15-mW air-cooled 488-nm argon laser (Coherent). Data acquisition is triggered using sideways light scattering and data are collected for sideways (SS) light scattering, forward (FS) light scattering, and fluorescence between 505 and 545 nm (FL). The discriminator is set at a value of 2, as chemostat-grown *E. coli* cells are small. The  $\log_{10}SS$  vs.  $\log_{10}FS$  plots are gated to remove points, such as the bacteriophage T5, that are too small to be *E. coli* cells. Cell counts are determined from the bimodal  $\log_{10}SS$  vs.  $\log_{10}FL$  plots that show the fluorescent T5-sensitive population well separated from the nonfluorescent T5-resistant population. Cells do not completely stain until the 45- and 60-min samples, which typically show little difference and are considered replicate samples.

**Estimating fitness:** Growth rates are approximately constant in a chemostat at quasi-steady state and the densities of the competitors are given by

$$N_1 \approx N_{10}e^{(\mu_1 - D)t}, \quad (1)$$

$$N_2 \approx N_{20}e^{(\mu_2 - D)t}, \quad (2)$$

where  $N_{10}$  and  $N_{20}$  are the initial values. Taking the  $\log_e$  ratio yields

$$\log_e(N_1/N_2) \approx \log_e(N_{10}/N_{20}) + (\mu_1 - \mu_2)t. \quad (3)$$

Hence, linear regression can be used to estimate the selection coefficient per hour as the slope  $s = (\mu_1 - \mu_2)$  of a plot of the  $\log_e$  ratio of the densities against time (DYKHUIZEN and HARTL 1980).

The magnitude of  $s$  depends on the generation time, which varies with dilution rate  $D$  and with the frequency of the strains. In a quasi-steady state the population density is almost constant,  $d(N_1 + N_2)/dt \approx 0$  and  $D \approx \mu_1 p + \mu_2 q$ . Hence

$$\frac{s}{D} \approx \frac{\mu_1 - \mu_2}{\mu_1 p + \mu_2 q}, \quad (4)$$

which can be rearranged to give

$$w_2^1 = \frac{\mu_1}{\mu_2} \approx \frac{1 + qs/D}{1 - ps/D} \quad (5)$$

as a convenient means to estimate relative fitness from experimental data when  $s/D$  is large. Although Equation 5 is dependent on strain frequencies, this has nothing to do with the frequency-dependent selection discussed earlier. It is simply a correction for the fact that a fitter strain grows faster than the chemostat dilution rate,  $\mu_1 > D$ , and that, as its frequency increases, so its growth must slow down—for at equilibrium, when the competitor is completely displaced,  $\mu_1 = D$ . The values of  $p$  and  $q$  are taken as the mean frequencies of the strains over the period analyzed. The approximation  $w_2^1 \approx 1 + s/D$  (DYKHUIZEN and DEAN 1994) is accurate when either  $s/D$  or  $p$  is small.

The mean fitness estimates obtained with Equation 5 (see APPENDIX) were fitted to the following model of frequency-dependent selection

$$w_2^1 = aw_{2,A}^1 + bw_{2,B}^1 \quad \text{if } p < 0.2 \quad (6)$$

$$w_2^1 = \frac{1}{a/w_{2,A}^1 + b/w_{2,B}^1} \quad \text{if } p > 0.8, \quad (7)$$

using the nonlinear least-squares algorithm available in JMP (SAS Institute), where  $w_{2,A}^1$  and  $w_{2,B}^1$  are the fitnesses on each

pure resource,  $A$  and  $B$ , respectively, the relative proportions of which are  $a$  and  $b$ .

## RESULTS

**Flow cytometry:** Competition experiments are conducted between paired strains of *E. coli*, with TD2 sensitive to the bacteriophage T5 and TD10 resistant or *vice versa*, with TD10 sensitive to the bacteriophage T5 and TD2 resistant. The progress of competition is simply monitored by periodically sampling the chemostat population and determining the proportion of cells that are T5 resistant. T5 resistance does not itself confer a detectable fitness cost (DYKHUIZEN and HARTL 1980; DEAN 1995) and simply hitchhikes along with selected alleles, there being no detectable rate of recombination between *E. coli* clones in chemostats (LEVIN 1981).

In the old method (DYKHUIZEN and HARTL 1980; DYKHUIZEN and DEAN 1994), the proportion of T5-resistant cells in the population was estimated by serially diluting a sample from  $10^8$  cells/ml to  $10^5$  cells/ml in Davis salts. The total count was determined by adding 100  $\mu$ l to each of four tubes of 4 ml of soft LB agar ( $45^\circ$ ), mixing, and overlaying LB plates. Once solidified, a clear surface agar (8 g/liter agar) was laid on top to prevent any colony from bursting through the surface and spreading. The colonies formed on the four plates were counted using an automatic colony counter (early articles used hand counts). The T5-resistant count was determined in the same manner, but with  $10^7$  phage added to the soft LB agar. One person could complete eight chemostat experiments every 2 weeks, including set up, experiment, analysis, clean up, and recovery (of the researcher). Few are the students willing to work so hard, so we endeavored to find a new, less taxing method to help generation X monitor their competition experiments in relative comfort.

Our new method also relies on T5 bacteriophage. When T5 attaches to its receptor, the outer membrane ferrichrome transporter FhuA, the cell membrane transiently depolarizes (BOULANGER and LETELLIER 1992; LETELLIER *et al.* 1997). Only depolarized cell membranes are permeant to YoPro-1, a cyanine dye that fluoresces bright green when tightly bound to nucleic acids. Thus, when a chemostat sample is treated with T5 in the presence of YoPro-1, sensitive cells fluoresce green whereas resistant cells, to which T5 does not attach, remain nonfluorescent (Figure 1). A flow cytometer (Figure 2) is then used to count individual cells. Briefly, cells are injected into a stream of sheath fluid and, as they pass in front of a 488-nm laser beam, light is scattered. Counts are triggered on the basis of sideways 488-nm light scatter (total count) with the number of sensitive cells determined using a second photomultiplier tube that simultaneously collects light between 505 and 545 nm (green fluorescence count). Typically

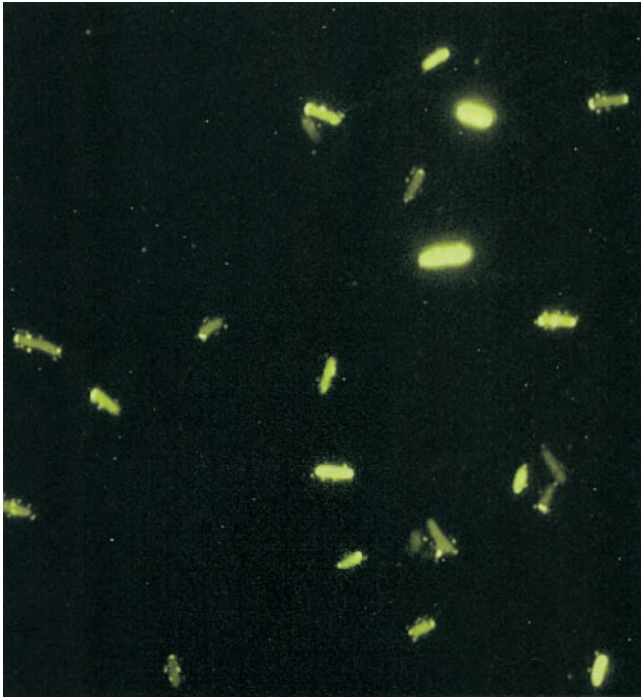


FIGURE 1.—Attachment of bacteriophage T5 (small green dots) to FhuA causes sensitive *E. coli* cells (large green blobs) to fluoresce bright green in the presence of YoPro-1 iodide. Sensitive (fluorescent) and nonsensitive (nonfluorescent) cells are readily distinguished despite the evident variation in the intensity of fluorescence.

25,000 cells can be counted in 30 sec (Figure 3). A single person is capable of completing 12 chemostat competitions per week, with each sampled five times daily.

We find that flow cytometry accurately determines, to within the  $\pm 0.5\%$  accuracy of a pipetman, the proportion of sensitive and resistant cells produced by mixing pure cultures grown in chemostats (Figure 4). The only regions where problems arise are near the extremes of 100%-sensitive and 100%-resistant cells. Samples of 100%-sensitive cells counted at a rate of 1000/sec typically yield 1% nonfluorescent "resistant" counts that can be attributed to the  $\sim 10$  counts/sec nonfluorescent background particles in the cytometer's sheath fluid. Samples of 100% resistant cells produce  $1.08 \pm 0.11\%$  green fluorescent cells. Why a small proportion of resistant cells (or untreated sensitive cells) should have depolarized membranes is not known.

**Control experiments:** Chemostat competition experiments between T5R and T5S isolates of the same strain for limiting glucose reveal evidence of weak selection against the T5-resistant strain (*e.g.*, TD2.T5R *vs.* TD2.T5S:  $s/D = -0.00207 \pm 0.00029$ ). T5 resistance is conferred by mutations (probably internal deletions) in *fhuA*, a gene that encodes the  $\text{Fe}^{2+}$ -ferrichrome transporter that serves as the attachment site for T5 phage. The selection disappears when the chemostat growth medium is sup-

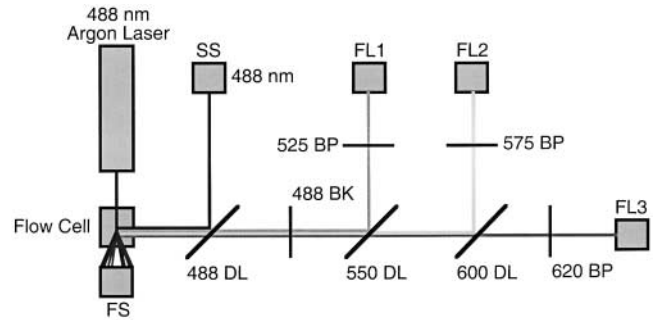


FIGURE 2.—Diagram of the optical bench of the EPICS XL flow cytometer used in these studies. A 488-nm laser is fired at a quartz flow cell. Light scattered forward and sideways by particles passing through the laser beam is detected by two photomultiplier tubes (FS and SS). Light scatter alone can be used to distinguish cells that are sufficiently different in size, shape, or density. More commonly, fluorescence, either natural (*e.g.*, chlorophyll) or synthetic (many dyes are available), allows different cell types to be identified. Light  $>488$  nm passes through a dichroic mirror (488 DL) and a 488 long pass filter (488 BK) into the optical bench. Light  $<550$  nm is reflected by a second dichroic mirror (550 DL) through the  $525 \pm 15$ -nm bandpass filter (525 BP) onto a photomultiplier tube (FL1). Light  $>550$  nm passes through to another dichroic mirror (600DL), which reflects light  $<600$  nm through the  $575 \pm 15$ -nm bandpass filter (575 BP) onto photomultiplier tube FL2. Light  $>600$  nm passes through the  $620 \pm 15$ -nm bandpass filter (620 BP) onto photomultiplier tube FL3.

plemented with  $5 \mu\text{M}$   $\text{FeSO}_4$ . For this reason all chemostat competition experiments are now conducted in media supplemented with  $\text{Fe}^{2+}$ .

Strains TD2 and TD10C are constitutive and express their *lac* operons in the absence of inducers. TD10C is fitter during competition for glucose (DYKHUIZEN and DEAN 1994). Glucose and galactose are structurally similar, being epimers, while the lactose permease transports a wide variety of galactosides. To test if the selection on glucose is attributable to TD10C harboring a lactose permease that transports glucose more efficiently than that of TD2 we allowed these two strains to compete for succinate, a dicarboxylic acid that is unlikely to be transported by the *lac* permease because it has no structural resemblance to a sugar. As expected, no selection is detectable during competition for succinate (Figure 5). These control competition experiments demonstrate that any selection seen during competition for galactosides is attributable to differences between the lactose operons of the competing strains and not to differences accrued during their construction.

**Standard deviations and standard errors:** There are three sources of variability: (1) binomial sampling effects, (2) variable staining, and (3) heterogeneity among replicate chemostat experiments. When sampling is binomial the standard deviation of the frequency of a strain is given by  $\text{SD} = \sqrt{pq/n}$ . With strains equally common ( $p = q = 1/2$ ) and a typical sample size of 50,000,

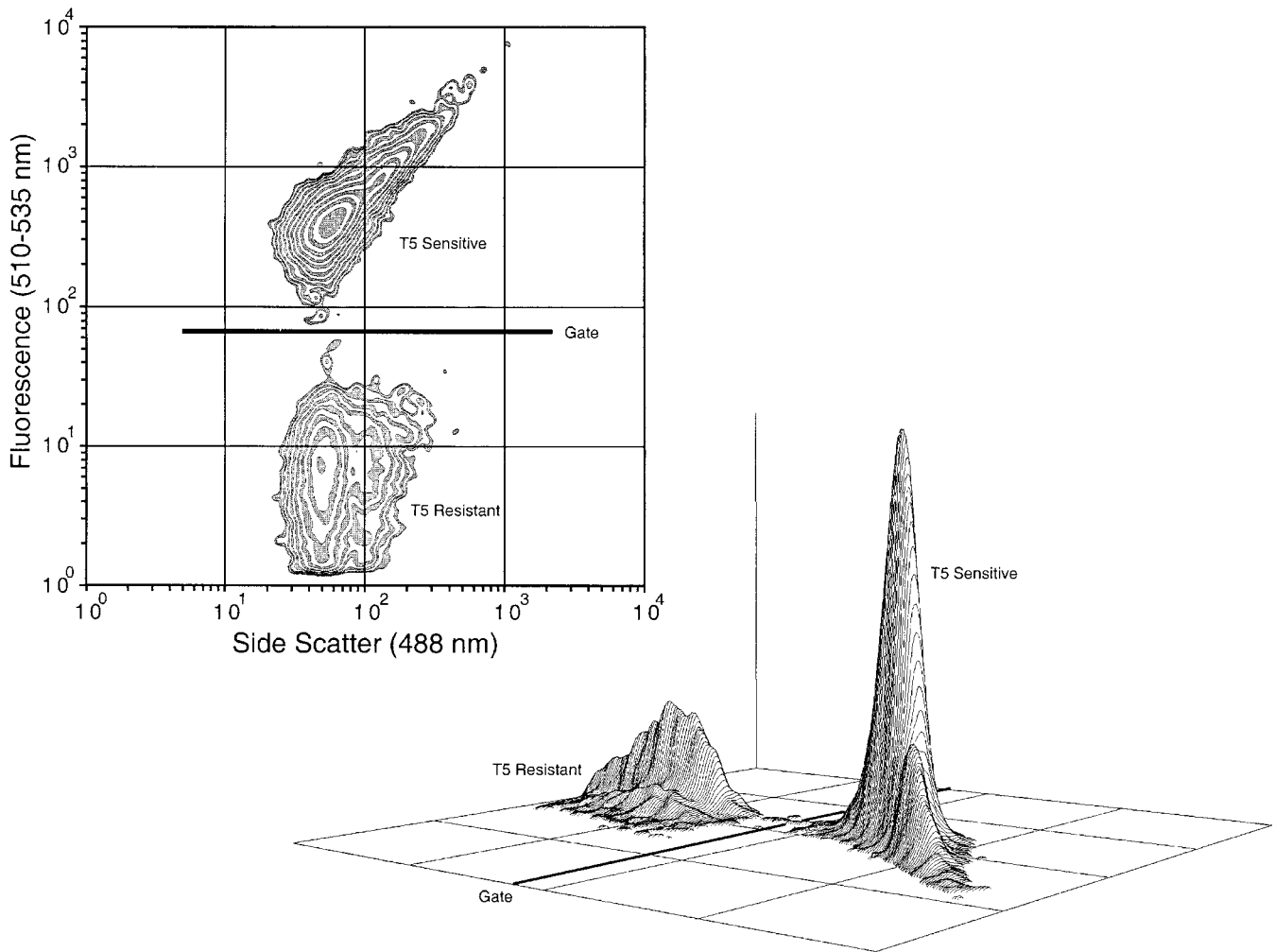


FIGURE 3.—Contour and isometric plots of 26,138 cells from a chemostat sample, stained with YoPro-1 following incubation with T5 and counted in 30 sec using an EPICS XL flow cytometer. The T5-resistant and -sensitive populations are well separated and readily distinguished.

the expected standard deviation among perfect replicate samples is  $SD = \sqrt{(\frac{1}{2})(\frac{1}{2})/50000} = 0.00224$ . Samples stained in YoPro-1-iodide for 45 and 60 min are treated as replicates because the frequencies of stained cells differ randomly. However, the observed SDs among replicates are a little larger than expected ( $SD = 0.0042$  and  $SD = 0.0044$  for TD10C *vs.* TD2 grown on succinate). Hence, variability in staining provides an additional source of variation.

When selection is not too strong (in the range 10–40% methylgalactoside, where  $s/D < \pm 0.075$ ) the bias in using the approximation  $w_2^1 \approx 1 + s/D$  is small ( $< 0.005$ ). Under these circumstances heterogeneity between replicate estimates of  $s/D$  (obtained as the slope of a plot of  $\log_e(\text{TD10C}/\text{TD2})$  *vs.* generations) can be tested using standard pooled regression analyses (SNEDECOR and COCHRAN 1967). These heterogeneity tests are invariably significant ( $P < 0.05$ ). The cause of this second source of experimental variation, attributable to differences between replicate chemostat experiments, has not been identified. Consequently, all standard er-

rors reported in Table 1 are based on direct estimates of fitnesses obtained using Equation 5.

**Competition experiments:** Figure 6 reveals that the fitness of TD10C when rare displays a linear dependency on the abundances of methylgalactoside and lactulose entering the growth chamber. This accords with Equation 6 and earlier results (DYKHUIZEN and DEAN 1994; P. J. N. SILVA and D. E. DYKHUIZEN, unpublished results) showing a similar dependence of fitness of rare strains during competitions for mixtures of lactose and glucose. Figure 6 also reveals that the fitness of TD10C when common displays a curvilinear dependency on the abundances of methylgalactoside and lactulose entering the growth chamber. This accords with the prediction (Equation 7) that the fitness of a strain when common is a weighted harmonic mean of the fitnesses on each resource, with the weights determined by the relative abundances of the resources *entering* the chemostat growth chamber.

The model (Equations 6 and 7) fits the data reason-

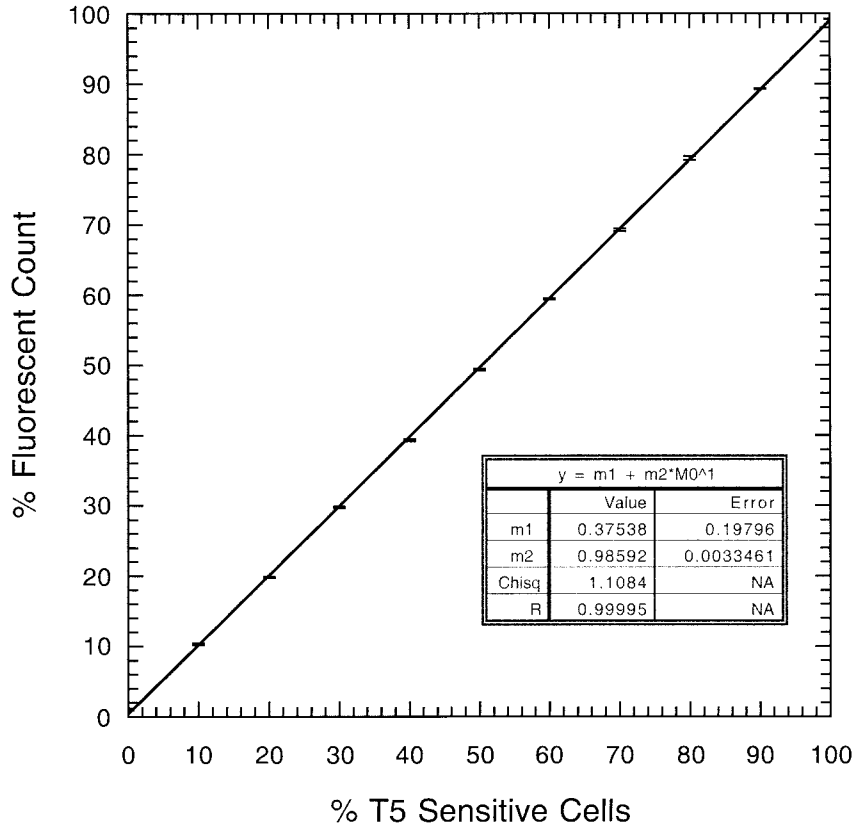


FIGURE 4.—Results from a reconstruction experiment in which pure chemostat-grown cultures of T5-sensitive and T5-resistant cells were mixed in predetermined proportions, stained, and counted by flow cytometry reveal that estimates of the proportion of T5-sensitive cells are unbiased over much of the range, the exceptions being at the extremes of 0 and 100%. The horizontal bars represent the 95% confidence intervals calculated from four replicate counts of each stained sample. The correlation coefficient is 0.99995.

ably well ( $w_{TD2,Lactu}^{TD10C} = 0.9037 \pm 0.0019$  on lactulose and  $w_{TD2,MeGal}^{TD10C} = 1.3166 \pm 0.0030$  on methylgalactoside; Table 2). The former is similar to the previously determined

value ( $w_{TD2,Lactu}^{TD10C} = 0.91 \pm 0.01$ ; SILVA and DYKHUIZEN 1993), while the latter is a little lower than expected ( $w_{TD2,MeGal}^{TD10C} = 1.35 \pm 0.01$ ; SILVA AND DYKHUIZEN 1993),

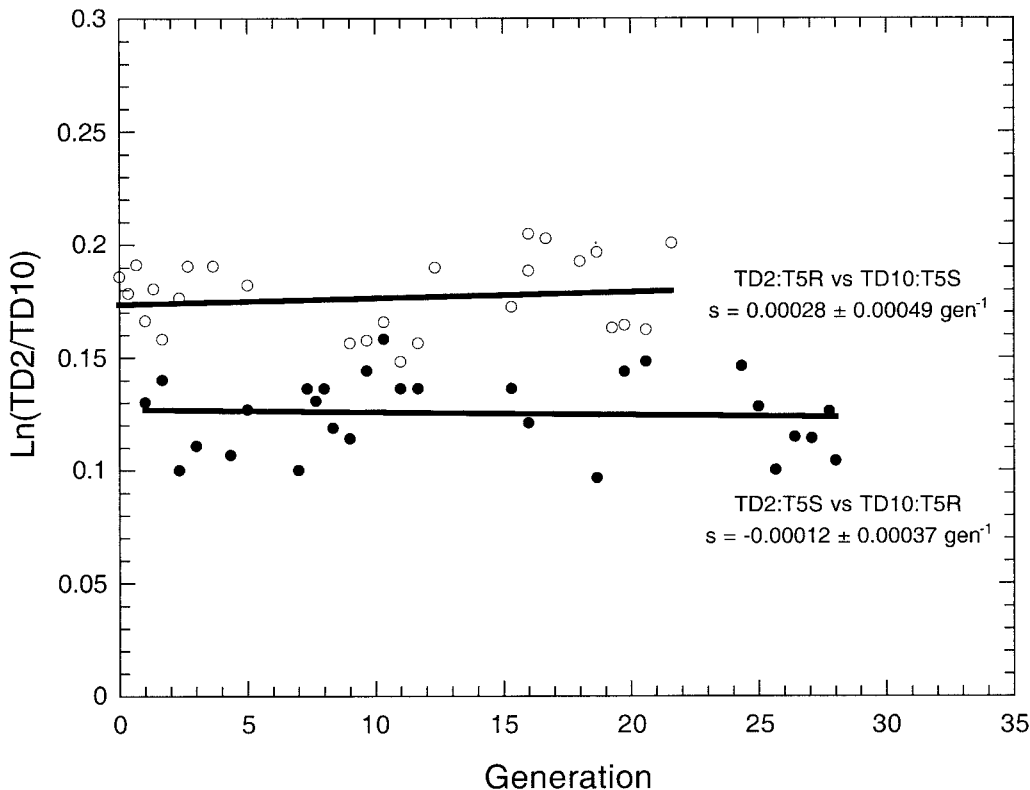


FIGURE 5.—Competition experiments with succinate as the sole resource-limiting growth. The slopes of the regression lines estimate the selection coefficients per generation. The absence of selection during succinate limitation demonstrates that the selection seen on lactulose and methylgalactoside is caused by differences between the *lac* operons of TD2 and TD10C.

TABLE 1

Fitnesses and standard errors of TD10C relative to TD2 as a function of galactoside abundance and frequency

% MeGal <sup>a</sup>	% lact <sup>a</sup>	Frequency of TD10C	No. experiments	Fitness of TD10C ( $w_{TD2}^{TD10C} = \frac{1 + qs/D}{1 - ps/D}$ )
0.00	100.00	<0.2	2	0.9075 ± 0.0037
9.19	90.81	<0.2	4	0.9419 ± 0.0019
18.55	81.45	<0.2	4	0.9768 ± 0.0034
23.29	76.71	<0.2	3	1.0074 ± 0.0042
28.06	71.94	<0.2	4	1.0253 ± 0.0027
32.91	67.09	<0.2	3	1.0473 ± 0.0031
37.78	62.22	<0.2	4	1.0615 ± 0.0064
57.74	42.26	<0.2	3	1.1467 ± 0.0024
78.46	21.54	<0.2	2	1.2370 ± 0.0032
100.00	0.00	<0.2	3	1.3174 ± 0.0043
0.00	100.00	>0.8	3	0.9003 ± 0.0043
9.19	90.81	>0.8		ND <sup>b</sup>
18.55	81.45	>0.8	4	0.9566 ± 0.0025
23.29	76.71	>0.8	3	0.9695 ± 0.0044
28.06	71.94	>0.8	4	0.9847 ± 0.0032
32.91	67.09	>0.8	2	1.0103 ± 0.0036
37.78	62.22	>0.8	3	1.0218 ± 0.0058
57.74	42.26	>0.8	3	1.0994 ± 0.0061
78.46	21.54	>0.8	2	1.1927 ± 0.0071
100.00	0.00	>0.8	3	1.3075 ± 0.0039

<sup>a</sup>Calculated assuming the methanol produced from methylgalactoside hydrolysis is not further metabolized.<sup>b</sup>ND, not determined.

a difference we attribute to the new method for monitoring competitions being far more precise than counting colonies.

There is a notable tendency for the data to fall outside the crescent defined by the arithmetic and harmonic means (Figure 6). This is unexpected. According to the model the perimeter of the crescent defines the maximum possible selection, selection attained only in the limits where one or the other strain is exceedingly rare ( $p \rightarrow 0$  and  $p \rightarrow 1$ ). We necessarily collect data within these limits ( $0 < p < 0.2$  and  $0.8 < p < 1$ ) and had anticipated that data should fall within the crescent. Fitting an arithmetic mean to the TD10C<sub>rare</sub> data yields fitness estimates that are slightly higher than those obtained by fitting a harmonic mean to the TD10C<sub>common</sub> data (Table 2). This suggests that, when common, TD10C is slightly less fit than expected. Competitions for pure resources, where frequency dependence is not expected, also reveal that TD10C is slightly less fit when common than when rare (Table 1). The model was modified by adding an additional parameter ( $\Delta w_{TD10C,common}$ ) to allow for this additional fixed selective difference (Table 2), the cause of which is not known.

Below 23% methylgalactoside TD2 wins the competition while >30.5% methylgalactoside TD10C wins. Between these limits it can be seen that TD10C wins when rare ( $w > 1$ ), but loses when common ( $w < 1$ ). Thus, between 23 and 30.5% methylgalactoside is a region

of coexistence, a region where frequency-dependent selection ensures that neither strain can reach fixation. Although our simple theory predicts a unique equilibrium that depends only on the fitnesses on the single resources and their relative proportions (Equations A20 and A21), it does not accommodate the additional source of selection,  $\Delta w_{TD10C,common}$ . However, we can determine the region where the equilibrium is likely to lie by calculating  $p_{eq}$  both with and without  $\Delta w_{TD10C,common} = 0.0082$ . At 28.06% methylgalactoside the former yields an estimate of  $p_{eq} = 0.5$  and the latter of  $p_{eq} = 0.8$ . This serves to demonstrate that small differences in fitness (~1%) cause dramatic changes in the predicted equilibrium (~30%).

We therefore determined the position of the equilibrium at 28.06% methylgalactoside by experimental means (Figure 7). Long-term chemostat experiments could not be used to follow the approach to equilibrium because periodic selection (the appearance of new advantageous mutants in the chemostat that sweep through the population) would compromise the results. Instead, chemostats were inoculated at different frequencies of TD10C and TD2, and selection was monitored over a period of 25 generations. In no instance did strain frequencies change by >15% (which would correspond to a change of 0.5% in a selection coefficient initially 2.5%) and so the selection appears linear. These experiments reveal an equilibrium frequency for TD10C of ~62%

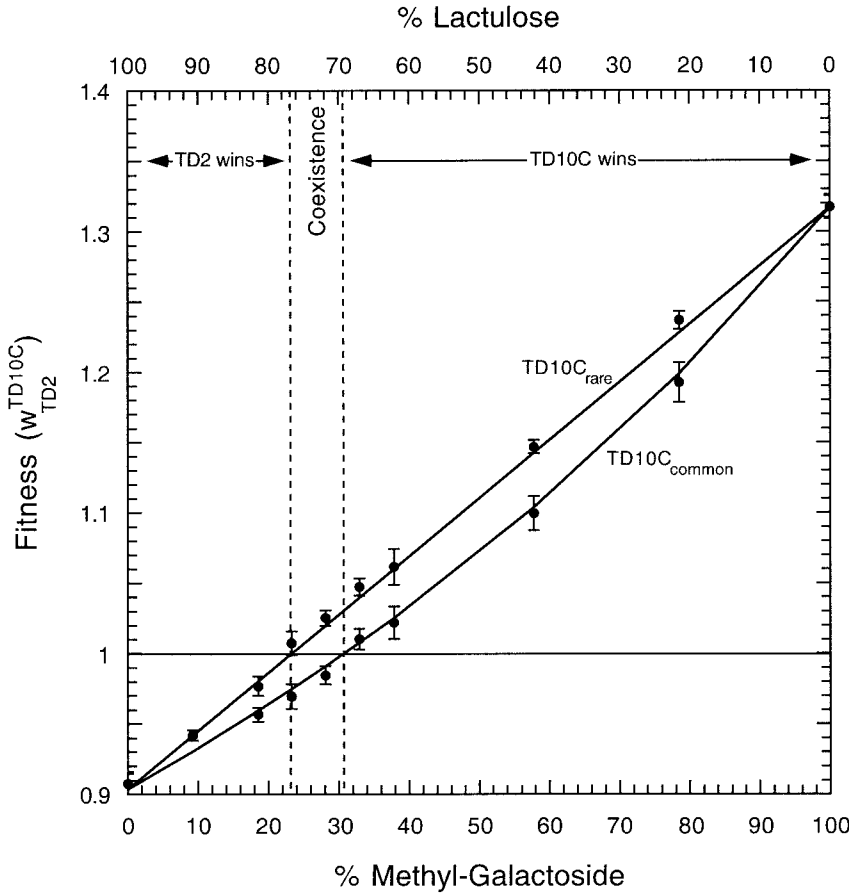


FIGURE 6.—The observed fitness relations between TD10C and TD2 vary across the methylgalactoside/lactulose resource axis and are also dependent on strain frequency. Fitnesses of TD10C were estimated at frequencies between 0 and 20% (rare) and 80 and 100% (common). Between 23 and 30.5% methylgalactoside the fitness of TD10C is >1 when rare and <1 when common. Here, TD10C and TD2 can coexist, maintained by frequency-dependent selection in a balanced polymorphism. The window of coexistence lies a little to the right of that predicted (Figure A3) because of slight differences in the estimated fitnesses on the pure sugars. The error bars designate 95% confidence intervals and unweighted nonlinear regression curves were fitted to Equation 6.

[ $\ln(0.62/0.38) \approx 0.5$ ] with TD10C favored below this frequency and disfavored above. As expected, selection intensifies the farther the initial frequency is from equilibrium.

DISCUSSION

Many years ago GILLESPIE and KOJIMA (1968) observed that enzymes having more than one substrate of extracellular origin (type II loci) tend to be more polymorphic than those with only a single substrate

of intracellular origin (type I loci). Subsequent work supports this pattern (KOJIMA *et al.* 1970; SELANDER 1976; GOJOBORI 1982; GILLESPIE 1991; SINGH 1992) although exceptions are known (SOLE-CAVA and THORPE 1989). HUANG *et al.* (1971) suggested that stabilizing frequency-dependent selection, observed at the esterase-6 locus in *Drosophila melanogaster*, might arise as a consequence of different genotypes utilizing different nutrients. This would provide a powerful mechanism for maintaining genetic variation at type II loci (mostly

TABLE 2

Estimated fitnesses of TD10C when rare and common obtained with alternative models

Model		Estimated fitnesses		
Rare ( $0.0 < p < 0.2$ )	Common ( $0.8 < p < 1.0$ )	$w_{TD2.MeGal}^{TD10C}$	$w_{TD2.Lactu}^{TD10C}$	$\Delta w_{TD10C.common}$
$aw_{TD2.MeGal}^{TD10C} + bw_{TD2.Lactu}^{TD10C}$	$\frac{1}{a/w_{TD2.MeGal}^{TD10C} + b/w_{TD2.Lactu}^{TD10C}}$	$1.3166 \pm 0.0030$	$0.9037 \pm 0.0019$	
$aw_{TD2.MeGal}^{TD10C} + bw_{TD2.Lactu}^{TD10C}$	$\frac{1}{a/w_{TD2.MeGal}^{TD10C} + b/w_{TD2.Lactu}^{TD10C}}$	$1.3218 \pm 0.0030$	$0.9066 \pm 0.0022$	
	$\frac{1}{a/w_{TD2.MeGal}^{TD10C} + b/w_{TD2.Lactu}^{TD10C}}$	$1.3084 \pm 0.0023$	$0.9014 \pm 0.0013$	
$aw_{TD2.MeGal}^{TD10C} + bw_{TD2.Lactu}^{TD10C}$	$\frac{1}{a/w_{TD2.MeGal}^{TD10C} + b/w_{TD2.Lactu}^{TD10C}} - \Delta w_{TD10C.common}$	$1.3194 \pm 0.0021$	$0.9081 \pm 0.0016$	$0.0082 \pm 0.0017$

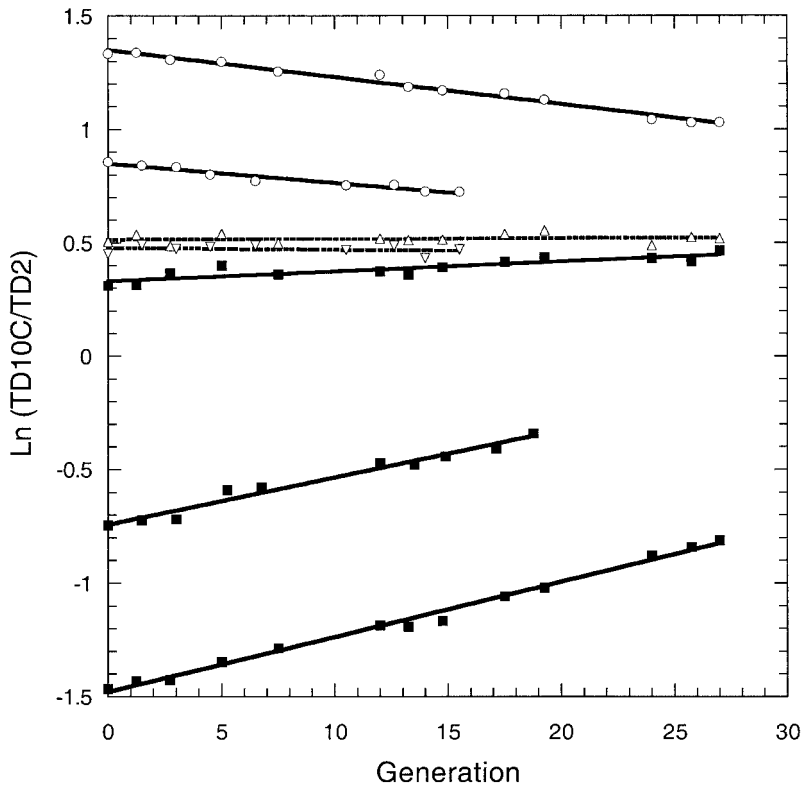


FIGURE 7.—Selection on 28% methylgalactoside reveals an equilibrium near  $\text{Log}_e(\text{TD10C}/\text{TD2}) = 0.5$ , which corresponds to an equilibrium TD10C frequency of 0.62 (dashed lines). Above this frequency TD10 is selected against while below it TD10 is favored. As expected, the farther from equilibrium the more intense the selection.

esterases, peptidases, and other assorted hydrolases with multiple substrates). Subsequent experiments produced conflicting results (compare BIRLEY and BEARDMORE 1977 with DOLAN and ROBERTSON 1975; MORGAN 1976 with YOSHIMARU and MUKAI 1979; and TOSIC and AYALA 1981 with BARNES 1983). Perhaps type II loci are more polymorphic simply because they are subject to less stringent purifying selection (SINGH 1976), or perhaps an appearance of frequency dependence is caused by recombination dissipating linkage disequilibrium with a selected locus (NEI 1988). Many additional examples were proposed, but interest waned as it became increasingly apparent that much of the evidence, although tantalizing, was indirect, subject to artifact, and inconclusive.

We have now demonstrated, unambiguously, that an allozyme polymorphism can be subject to stabilizing frequency-dependent selection. We succeeded because we used an asexual haploid species (to remove any possibility of heterozygote advantage), because we competed genotypes that differed only at one selected locus (to remove any possibility of hitchhiking effects), because our experiments were conducted in chemically defined and highly reproducible environments (chemostats), and because a rigorous mechanistic theory of frequency-dependent selection was thoroughly understood at the outset (differential resource depletion).

We tested the prediction that competition on mixtures of galactosides would generate frequency-dependent selection among *lac* operons through differential

resource depletion. We confirm that, when an operon is favored on one galactoside and disfavored on another, there is a window on the resource axis where selection prevents loss of either allele (Figure 6). The window is predicted with reasonable accuracy from a knowledge of the fitnesses obtained during competition for the pure galactosides: It lies between the intersections of the arithmetic and harmonic mean fitnesses on the neutral line. Since the relationships between enzyme kinetics and fitness are thoroughly understood for the *lac* operon (DEAN 1989, 1995; DYKHUIZEN and DEAN 1994), so the direction and intensity of selection, and the window of coexistence on the resource axis, can be predicted solely from a knowledge of biochemistry.

Our demonstration of frequency-dependent selection at *lac* requires us to reassess differential resource depletion as a mechanism maintaining polymorphisms at type II loci. Chemostat competition experiments reveal that the fitnesses of lactose operons on one galactoside are generally indicative of their fitnesses on other galactosides (SILVA and DYKHUIZEN 1993; DEAN 1995). With trade-offs so infrequent, other factors must be important in maintaining genetic variation at *lac*. Perhaps these act entirely independently, or perhaps they change operon fitnesses in such a way that the frequency-dependent mechanism described becomes operational.

Even given the latter possibility, frequency-dependent selection generated by differential resource depletion remains an unlikely mechanism for protecting allozyme variation at *lac*. Figure 6 reveals that selection coeffi-

cients as large as 10 and 30% on the single resources generate only a narrow 7.5% window on the resource axis where alleles are protected. That an environment should stay so stable for so long over such a narrow range seems implausible. Moreover, the conditions necessary to protect the polymorphism in a changing environment remain essentially unchanged; we simply use the mean fitnesses calculated over all the changes:  $\Sigma(a_i w_{2,A}^1 + b_i w_{2,B}^1) t_i / \Sigma t_i > 1$  and  $\Sigma t_i / \Sigma(a_i / w_{2,A}^1 + b_i / w_{2,B}^1) t_i < 1$ , with time in each environment ( $t_i$ ) measured in generations. Regardless of whether the environment is stable or changeable, frequency dependence engendered by resource depletion is unlikely to protect *lac* polymorphisms for very long.

Nevertheless, the proposed mechanism may act intermittently from time to time. *Lac* polymorphisms might arise, persist for a time, and then dissolve. Permease sequences (WAGNER and RILEY 1996) are in accord with this scenario; the low level of silent polymorphism suggests a selective sweep, while the level of amino acid polymorphism, typical of many *E. coli* genes, suggests rapid diversifying selection. Unfortunately, sequence data alone do not distinguish among the multitude of selection schemes that would generate similar patterns of nucleotide polymorphism. Differential resource depletion generating frequency-dependent selection at *lac* is plausible, but certainly not proven for natural populations.

Competition experiments (EANES 1999; WATT and DEAN 2000) and DNA sequence analyses (SAWYER *et al.* 1987; SAWYER and HARTL 1992; HARTL *et al.* 1994; KENNEDY and NACHMAN 1998) suggest that selection coefficients among allozymes at most loci are far smaller in general than those observed at *lac*. With smaller selection coefficients the window of coexistence is rapidly diminished—to a mere 0.075% of the resource axis with selection coefficients of  $-0.1$  and  $0.3\%$ . In a diploid model, where heterozygotes with intermediate enzyme activities display the dominant fitness phenotypes of the homozygotes and so benefit from overdominance when consuming mixed resources, this window broadens—to 0.15%. The prospect that incoming resource supplies will admit such narrow zones of coexistence in natural habitats is implausible. We conclude that stabilizing frequency-dependent selection through resource depletion, though it may operate sporadically, is an unlikely mechanism for protecting allozyme variation at type II loci.

Crucial to our success in demonstrating the existence of frequency-dependent selection was the development of a highly efficient technique for monitoring chemostat competitions. The new technique depends, as did earlier experiments (DYKHUIZEN and HARTL 1980; DEAN 1995), on competitions between paired strains, one of which carries the selectively neutral marker *fhuA*. This marker prevents the bacteriophage T5 attaching to the cell wall. T5 attachment to unmarked cells leads to depolarization of the cell membrane, which in turn allows YoPro-1 iodide to enter the cell, bind to nucleic acids,

and fluoresce. The progress of the competition is followed simply by determining the number of stained and unstained cells in periodic samples.

Assuming that a flow cytometer is handy, the procedure is less expensive, more rapid, and more accurate than traditional plating techniques. The cost per chemostat sample using the old colony-counting technique is  $\sim \$2.84$  (for materials) compared to  $\sim \$1.64$  using a flow cytometer (calculated assuming a  $\sim \$26.00/\text{hr}$  rental fee for the flow cytometer; cost of materials is  $\sim \$0.20$  per sample). The old upper limit of sampling 8–10 chemostats twice daily is now routinely surpassed by sampling 12–16 chemostats five or six times daily. Finally, flow cytometry is more accurate because the increase in sample size, from 4000 colonies counted to 50,000 cells counted, reduces the SD 3.5-fold. Indeed, we identified an additional source of selection, over and above that predicted from theory. Strain TD10C is less fit than expected at high frequency (or conversely, strain TD2 is more fit when at low frequency) during competition for either galactoside ( $\Delta w_{\text{TD10C.common}} = 0.0082 \pm 0.0017$ ), although not during the control competition experiments for succinate. The cause of this additional selection remains a mystery.

Flow cytometry is readily adaptable. Chlorophyll, green fluorescent protein, and its cogeners, strain-specific fluorescent antibodies, and a wide array of cell-permeant and impermeant fluorescent stains provide ready means to distinguish microbial populations. We anticipate that flow cytometry will increasingly be used to monitor population dynamics in a diverse range of evolutionary and ecological microbial systems.

We thank Robert Jones for his generosity in letting us use his EPICS XL flow cytometer and Ian Molineux for his advice on phage life cycles when developing our new methods. This work was supported by National Science Foundation and National Institutes of Health grants awarded to A.M.D. and D.E.D.

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## APPENDIX

The theory described below is an extension of that derived by DYKHUIZEN and DEAN (1994). The key result, which describes the fitness of a strain as a function of

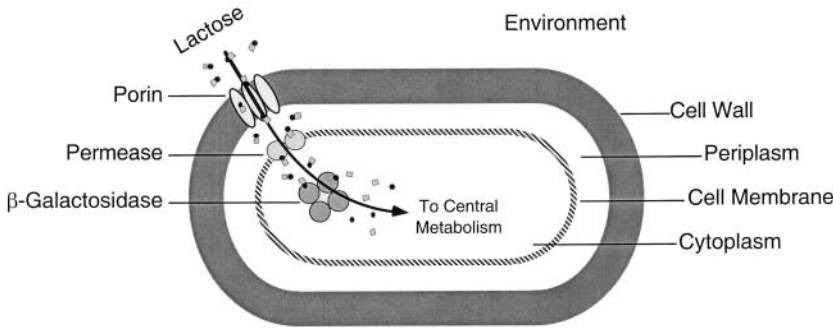


FIGURE A1.—The lactose pathway of *Escherichia coli*. Lactose diffuses passively through the porin pores of the cell wall into the periplasmic space. The *lacY*-encoded permease then actively transports lactose across the cell membrane into the cytoplasm where it is irreversibly hydrolyzed by the *lacZ*-encoded  $\beta$ -galactosidase. The products, glucose and galactose, then enter central metabolism. Lactulose, a dimer of galactose and fructose, uses exactly the same pathway as lactose. Methylgalactoside is also transported across the cell membrane by a second route, the *mgl* transport system, which is fully induced during starvation in chemostats (DEATH and FERENCI 1994).

both frequency and environmental composition (Equation A15), necessitates a somewhat different proof than the earlier results that considered only the fitness of a strain when rare.

**From flux to fitness:** Lactose metabolism by *E. coli* (Figure A1) serves as a paradigm for investigating the relations between Darwinian fitness ( $w$ ), metabolic flux ( $J$ ), resource abundance ( $R$ ), and enzyme activity (WATT and DEAN 2000). When environmental lactose abundance ( $R$ ) is low each step in the pathway is unsaturated and, following KACSER and BURNS (1973, 1981), the flux is given by

$$J = \frac{R}{1/D_{\text{wall}} + K_{\text{m,permease}}/V_{\text{max,permease}} + K_{\text{m,\beta-gal}}/V_{\text{max,\beta-gal}}/K_{\text{eq,permease}}}, \quad (\text{A1})$$

where  $D_{\text{wall}}$  is the diffusion constant of lactose through the porin pores of the outer cell wall,  $K_{\text{m},i}$  and  $V_{\text{max},i}$  are the Michaelis constants and maximum velocities, respectively, of enzyme  $i$ , and  $K_{\text{eq,permease}}$  is the apparent equilibrium constant for lactose uptake across the cell membrane (DEAN 1989). If growth ( $\mu$ ) is proportional to flux then

$$\mu = YJ = \frac{YR}{\Sigma}, \quad (\text{A2})$$

where  $Y$  is the yield coefficient (the number of cells produced per amount of resource consumed) and the denominator of Equation A1 is represented by  $\Sigma$ .

Slow growth in chemostats, a continuous culture device, imposes fierce scramble competition for growth-limiting concentrations of lactose. Starving cells have no appreciable death rate (DYKHUIZEN and DAVIES 1980) and interference competition, mutualisms, and commensalisms are absent (DEAN *et al.* 1988; DYKHUIZEN and DEAN 1994). Consequently, the fitness of strain 1 relative to strain 2 is

$$w_2^1 = \frac{\mu_1}{\mu_2} = \frac{J_1}{J_2} = j_2^1 = \frac{\Sigma_2}{\Sigma_1}. \quad (\text{A3})$$

This equation states that relative fitness is relative growth rate ( $w_2^1 = \mu_1/\mu_2$ ), which equals relative flux ( $J_1/J_2 =$

$j_2^1$ ), and that the latter is given by a ratio of kinetic terms ( $w_2^1 = j_2^1 = \Sigma_2/\Sigma_1$ ). The yield coefficient ( $Y$ ) cancels as we assume that it is identical for all competing strains—they are genetically identical except for the genes controlling lactose metabolism. Hence, by estimating the biochemical kinetic parameters at each step in the lactose pathway, the direction, intensity, and ultimate outcome of competition can be predicted, *ab initio*. That this is so is illustrated in Figure A2.

**$R^*$  and fitness:** The theory outlined is intimately related to the  $R^*$  approach of TILMAN (1980, 1982). Imagine that, as an isolated population approaches its carrying capacity, some abiotic resource becomes sufficiently scarce that growth ( $\mu$ ) is reduced until it precisely matches loss ( $D$ ). Here

$$\mu_i^* = D = YJ_i^* = \frac{YR_i^*}{\Sigma_i}, \quad (\text{A4})$$

where the asterisk denotes an isolated population at equilibrium. Then

$$w_2^1 = \frac{\mu_1}{\mu_2} = \frac{J_1}{J_2} = \frac{\Sigma_2}{\Sigma_1} = \frac{R_2^*}{R_1^*}, \quad (\text{A5})$$

and we see that the concept of Darwinian fitness is intimately related to the ecological concept of  $R^*$ . In other words, fitness can be predicted either by the biochemical approach described or by Tilman's phenomenological approach of  $R^*$ . The advantage of the former is that the mechanistic basis of competitive ability is delineated. The advantage of the latter is that ecological predictions are still possible when mechanistic detail is absent.

**Multiple resources:** Models of scramble competition for two (or more) resources have received many analyses (*e.g.*, LEON and TUMPSON 1975; BALLYK and WOLKOWICZ 1993; SOMMER 1993; VARGAS-MENDOZA and FOWLER 1998; CHESSON 2000). Again, the outcome of scramble competition can be predicted using the  $R^*$  approach (TILMAN 1980, 1982). However small differences in  $R^*$  are notoriously difficult to estimate either directly or from plots of growth rate *vs.* resource concen-

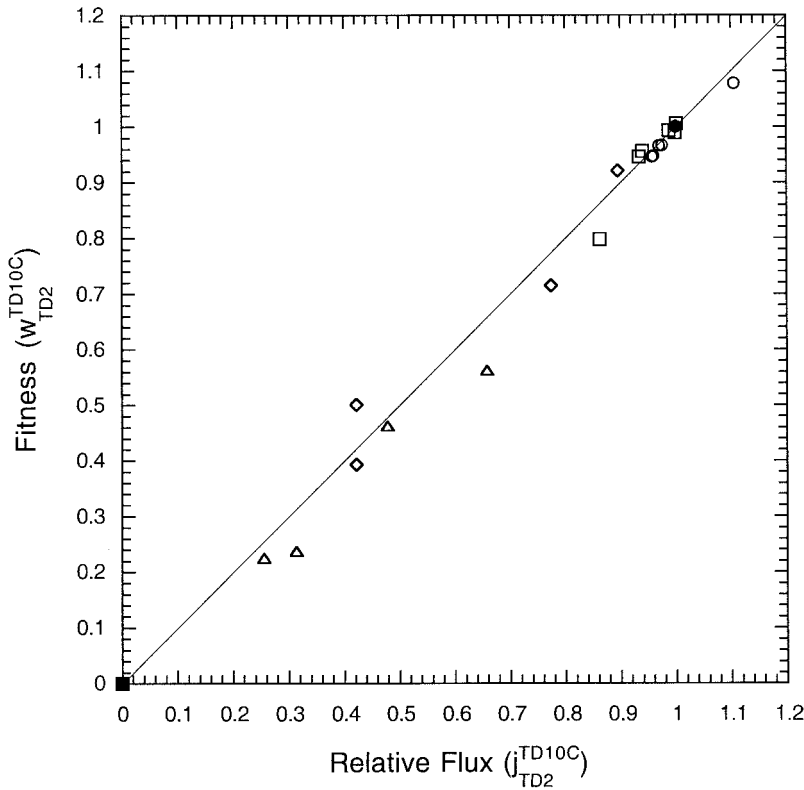


FIGURE A2.—The linear relationship between relative fitness ( $w$ ) and relative flux ( $j$ ) during chemostat competition for lactose. Solid circle, TD2 is taken as a reference; solid square, DD320 (*lac* deletion); open squares, mutant  $\beta$ -galactosidases; open triangles, mutant evolved  $\beta$ -galactosidases; open diamonds, regulated operons induced with IPTG; open circles, natural *lac* operon variants. After DEAN (1989).

tration. Here, we reparameterize a chemostat model to eliminate the need to estimate  $R^*$ 's.

Pure scramble competition between two populations (1 and 2) for two resources (A and B) is modeled using the resource-based approach first introduced by MONOD (1942, 1950) to describe the growth of microbial populations in chemostats. Specifically,

$$\frac{dN_1}{dt} = (\mu_1 - D)N_1, \quad (\text{A6})$$

$$\frac{dN_2}{dt} = (\mu_2 - D)N_2, \quad (\text{A7})$$

where  $N_1$  and  $N_2$  are the densities of the competing populations and  $\mu_1$  and  $\mu_2$  are their respective growth rates.  $D$ , the dilution rate, is the fractional rate of replacement of the mixed culture in the growth chamber by fresh medium. The rates of change in the concentrations of the resources, A and B, are described by

$$\frac{dR_A}{dt} = D(R_{A,0} - R_A) - J_{A,1}N_1 - J_{A,2}N_2, \quad (\text{A8})$$

$$\frac{dR_B}{dt} = D(R_{B,0} - R_B) - J_{B,1}N_1 - J_{B,2}N_2, \quad (\text{A9})$$

where  $R_{A,0}$  and  $R_{B,0}$  are the concentrations of the resources in the fresh medium entering the growth chamber,  $R_A$  and  $R_B$  are the concentrations of resources in the growth chamber, and  $J_{A,i}$  and  $J_{B,i}$  are the rates at which they are consumed by population  $i$ .

Following inoculation the chemostat populations increase in density until both resources are severely depleted, whence  $R_A \ll R_{A,0}$  and  $R_B \ll R_{B,0}$  and fierce competition ensues. Resource concentrations in the chemostat now change so slowly that  $dR_A/dt \approx 0$  and  $dR_B/dt \approx 0$ . In short, the system has entered a quasi-steady state, a concept first introduced to the field of enzyme kinetics by the eminent biochemists G. E. Briggs and J. B. S. Haldane (BRIGGS and HALDANE 1925). In the quasi-steady state

$$DR_{A,0} \approx J_{A,1}N_1 + J_{A,2}N_2 = \frac{R_A}{\Sigma_{A,1}}N_1 + \frac{R_A}{\Sigma_{A,2}}N_2, \quad (\text{A10})$$

$$DR_{B,0} \approx J_{B,1}N_1 + J_{B,2}N_2 = \frac{R_B}{\Sigma_{B,1}}N_1 + \frac{R_B}{\Sigma_{B,2}}N_2, \quad (\text{A11})$$

where the  $\Sigma$ 's again represent the biochemical terms as in Equation A1. Taking the ratio yields

$$\frac{R_{A,0}}{R_{B,0}} \sim \frac{R_A}{R_B} \left[ \frac{N_1/\Sigma_{A,1} + N_2/\Sigma_{A,2}}{N_1/\Sigma_{B,1} + N_2/\Sigma_{B,2}} \right], \quad (\text{A12})$$

and we see that the ratio of resources entering the growth chamber is intimately related to the ratio of resources in the growth chamber at quasi-steady state.

Assume that the growth rates are proportional to the sums of fluxes,

$$\mu_i = Y_{A,i}J_{A,i} + Y_{B,i}J_{B,i} = Y_{A,i} \frac{R_A}{\Sigma_{A,i}} + Y_{B,i} \frac{R_B}{\Sigma_{B,i}}, \quad (\text{A13})$$

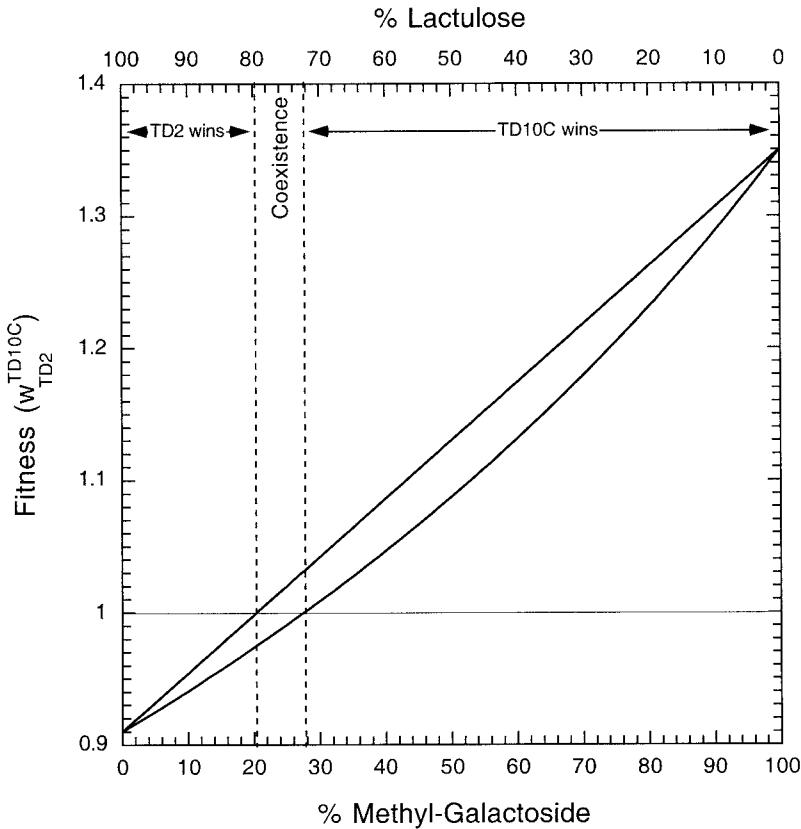


FIGURE A3.—The predicted fitness of TD10C relative to TD2 varies across the methylgalactoside/lactulose resource axis and is also dependent on strain frequency. When TD10C is rare its fitness is expected to be a linear function of resource abundances. When TD10 is common its fitness is expected to be lower than when rare, and the relation between fitness and resource abundances is now expected to be curvilinear. The fitnesses ( $w_{\text{TD2,Lactul}}^{\text{TD10C}} = 0.91 \pm 0.01$ ;  $w_{\text{TD2,MeGal}}^{\text{TD10C}} = 1.35 \pm 0.01$ ) are from SILVA and DYKHUIZEN (1993).

and that the yield coefficients and the biochemical terms are constant regardless of resource abundances. There is no switching between resources, there is no interference between resources, and so the resources are perfectly substitutable. Darwinian fitness is now given by

$$w_2^1 = \frac{\mu_1}{\mu_2} = \frac{Y_{A1}J_{A1} + Y_{B1}J_{B1}}{Y_{A2}J_{A2} + Y_{B2}J_{B2}} = \frac{Y_{A1}(R_A/\Sigma_{A1}) + Y_{B1}(R_B/\Sigma_{B1})}{Y_{A2}(R_A/\Sigma_{A2}) + Y_{B2}(R_B/\Sigma_{B2})}. \quad (\text{A14})$$

This function is dependent on the availabilities of both resources, whereas fitness on a single scarce resource is independent of its availability (Equation A14 reduces to Equation A3 by setting  $R_B = 0$ ). Substituting Equation A12 into Equation A14 and then setting  $Y_{A1} = Y_{A2}$  and  $Y_{B1} = Y_{B2}$  (because competing *E. coli* strains are coisogenic) and cranking the algebra eventually yield

$$w_2^1 \sim \frac{R_{A0}w_{2,A}^1(pw_{2,B}^1 + q) + R_{B0}w_{2,B}^1(pw_{2,A}^1 + q)}{R_{A0}(pw_{2,B}^1 + q) + R_{B0}(pw_{2,A}^1 + q)}, \quad (\text{A15})$$

where  $p = N_1/(N_1 + N_2)$  and  $q = 1 - p$  are the frequencies of strains 1 and 2, and  $w_{2,A}^1 = \mu_{A1}/\mu_{A2} = \Sigma_{A2}/\Sigma_{A1}$  and  $w_{2,B}^1 = \mu_{B1}/\mu_{B2} = \Sigma_{B2}/\Sigma_{B1}$  are the Darwinian fitnesses of strain 1 when competing for single resources.

Equation A15 relates the fitness on the two mixed resources ( $w_2^1$ ) to the fitnesses on the single resources ( $w_{2,A}^1$  and  $w_{2,B}^1$ ) and through these to the underlying biochemistry, the  $\Sigma$ 's. Fitness on the mixed resources is also dependent on the frequencies of the populations

( $p$  and  $q$ ) and to the concentrations of resources entering the growth chamber ( $R_{A,0}$  and  $R_{B,0}$ ). Unlike  $R_A$  and  $R_B$  in the growth chamber,  $R_{A,0}$  and  $R_{B,0}$  are under the direct control of the experimenter and so are known with precision. The outcome of competition can be predicted using  $R_{A,0}$  and  $R_{B,0}$  and estimates of  $w_{2,A}^1$  and  $w_{2,B}^1$ , obtained either from a knowledge of biochemistry or directly from competitions for single resources. The problem of estimating  $R^*$ 's has been eliminated.

**Competitive exclusion and coexistence:** When strain 1 is scarce ( $p \rightarrow 0$ ) Equation A15 simplifies to

$$w_2^1 = aw_{2,A}^1 + bw_{2,B}^1, \quad (\text{A16})$$

where  $a = R_{A,0}/(R_{A,0} + R_{B,0})$  and  $b = R_{B,0}/(R_{A,0} + R_{B,0})$ . We see that the Darwinian fitness of a strain when rare is simply the arithmetic mean fitness weighted by the proportional abundances of the resources entering the growth chamber (DYKHUIZEN and DEAN 1994).

When strain 1 is common ( $p \rightarrow 1$ ) Equation A15 simplifies to

$$w_2^1 = \frac{1}{a/w_{2,A}^1 + b/w_{2,B}^1}. \quad (\text{A17})$$

We see that the Darwinian fitness of a strain when common is simply the harmonic mean fitness weighted by the proportional abundances of the resources entering the growth chamber. The intimate connection between the arithmetic and harmonic mean fitnesses is instantly grasped once

it is realized that the reciprocal of the arithmetic mean fitness of the rare strain is necessarily the harmonic mean fitness of the common strain.

Coexistence is possible when a population is favored on one resource but selected against on the other. This arises because, for any set of positive numbers, the arithmetic mean is necessarily greater than (or equal to) the harmonic mean. For such a population there exists a region along the resource axis where fitness is  $>1$  when rare, yet  $<1$  when common (Figure A3). The zone of coexistence is bounded by

$$w_{2,A}^1 \frac{w_{2,B}^1 - 1}{w_{2,B}^1 - w_{2,A}^1} > a > \frac{w_{2,B}^1 - 1}{w_{2,B}^1 - w_{2,A}^1}, \quad (\text{A18})$$

$$\frac{w_{2,A}^1 - 1}{w_{2,A}^1 - w_{2,B}^1} > b > w_{2,B}^1 \frac{w_{2,A}^1 - 1}{w_{2,A}^1 - w_{2,B}^1}. \quad (\text{A19})$$

Within this region, natural selection shepherds the populations toward the equilibrium

$$p_{\text{eq}} = \frac{a}{1 - w_{2,B}^1} + \frac{b}{1 - w_{2,A}^1}, \quad (\text{A20})$$

$$q_{\text{eq}} = \frac{a}{1 - w_{1,B}^2} + \frac{b}{1 - w_{1,A}^2}. \quad (\text{A21})$$

Thus, fitnesses on single resources can be used to predict the direction and intensity of selection and the existence of polymorphism or allelic fixation across an entire environmental gradient.

**Key assumptions:** The above model applies only when the following assumptions are fulfilled:

1. Starving cells do not die.
2. Resources are substitutable.

3. No interference competition occurs.
4. No mutualisms or commensalisms occur.
5. Fluxes are proportional to resource abundances.
6. Fitness is proportional to the sum of fluxes.

Assumption 1 is the least critical in that the overall architecture of the system remains essentially unaffected by the introduction of death. Moreover, as DYKHUIZEN and DAVIES (1980) have confirmed, *E. coli* cells have no appreciable death rate under the very similar experimental conditions of glucose limitation. Assumption 2 is easily verified. The resources are substitutable because each strain is capable of growth on each individual resource. Assumptions 3 and 4 require that the only ecological interaction be scramble competition. Interference is unlikely given that the competitors are coisogenic. Mutualistic/commensalistic interactions may occur during rapid growth when sugars are in excess, when the acetate produced by fermentation provides an opportunity for cross-feeding. However, in experiments similar to those described, competition is so intense that all sugars are utilized completely and no cross-feeding is detectable (DEAN *et al.* 1988). Assumptions 5 and 6 require that fitness is a linear function of resource abundance. The resources are said to be perfectly substitutable: Consumption of one neither enhances nor interferes with consumption of the other. Implicit in such statements are the assumptions that the rates of enzyme-catalyzed reactions are proportional to the abundance of metabolites in the pathways, that no enzyme becomes saturated with substrate or product, that the presence of one metabolite does not interfere with the metabolism of the other, that the enzymes are constitutively expressed (deregulated), and that allosteric regulation is absent.