MICROBIOLOGY REVIEWS, June 1983, p. 150–168
0146-0749/83/020150-19$02.00/0

Vol. 47, No. 2

Selection in Chemostats

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INTRODUCTION

In this paper we discuss some new uses for an old technique. The technique is the continuous culture of microorganisms, which in one form or another is familiar to every microbiologist. The particular continuous-culture device we will consider is the chemostat. A chemostat is basically a culture vessel having an input aperture for the influx of sterile nutrient medium from a reservoir and an overflow aperture for the efflux of exhausted medium, living cells, and cellular debris. The device (and the term "chemostat") was invented by Novick and Szilard (103); the "bactogène" is a virtually identical device developed independently and simultaneously by Monod (95). In practice, a chemostat apparatus is complicated by various attachments for aeration of the medium and for the prevention of contamination, and the overflow is often controlled by means of a siphon. The rate of cell division within the chemostat can be varied by a factor of 10 by appropriate adjustment of the rate of inflow, but an equilibrium will eventually be reached in which the number of new cells created by division will be exactly balanced by the number of cells siphoned off in the overflow. This equilibrium cell density is determined almost exclusively by the concentration of limiting nutrient in the reservoir. Thus, chemostats provide an environment in which cell division is continuous but population size is held constant, and the two parameters can be independently manipulated.

Although chemostats maintain a continuous culture by means of a constant influx of nutrients, other continuous-culture apparatus maintain a constant value of different parameters. The best known of these devices is a turbidostat, which maintains a constant turbidity of an exponentially growing culture by means of a photocell that regulates nutrient inflow by negative feedback (13). Less well-known devices include the pH auxostat, which maintains a constant pH (82), as well as other apparatus based on specific electrodes that maintain constant oxygen, nitrate, or ammonium (14). Brown and Oliver (12) have developed an ingenious feedback system in which the addition of ethanol to a continuous culture of *Saccharomyces uvarum* is used to control growth rate. The growth rate is monitored by the output of CO_2 which controls the rate of inflow of ethanol, maintaining a constant selection pressure for ethanol tolerance; they obtained mutants with an ethanol tolerance up to a level of 12% (wt/vol). These devices, and chemostats as well, are all different from one another and different from the classical fermentor, which although it can be used as either a chemostat or turbidostat, in most applications is basically a vessel for growing large, dense batch cultures.

Here we focus on experimental work involving chemostats because the apparatus is by far the simplest and the literature is the most extensive. We will restrict ourselves to the use of chemostats to study differential growth rates associated with induced, naturally occurring, or genetically engineered mutations, as well as with the growth rate effects of plasmids, temperate phage, and transposons. Other areas of the literature include rich and diverse studies of the dynamics of growth in chemostats (e.g., 22); inquiries into the nature of single-nutrient and dual-nutrient limitation (e.g., 25, 30); experiments on the physiology and metabolism of cells growing under specified chemostat conditions (e.g., 115, 116, 126); and studies of microbial

ecology involving single or mixed species in chemostats with single or mixed resources (e.g., 36, 45, 54, 68, 114, 117). An excellent summary of various aspects of chemostats, including their use in the study of mutation rates, has been published by Kubitschek (71). More recent reviews focusing on selection are by Harder et al. (57) and Calcott (16).

Chemostat Theory

A theoretical basis for understanding the dynamics of competition for a limiting resource in a chemostat has been developed by Monod (94, 95) and studied by Herbert et al. (64), Powell (108), Moser (96, 97), Tempest (113), and Hsu et al. (66). The equations are based on two fundamental assumptions, the first being that the specific growth rate (μ) of a strain relative to its theoretical maximum (μ_{max}) is described by a conventional Michaelis saturation equation involving the external substrate concentration of the form

$$\mu/\mu_{max} = S(t)/[K_S + S(t)]$$

where $S(t)$ is external substrate concentration within the chemostat culture at time t and K_S is the saturation constant or "assimilation parameter" corresponding to the substrate concentration giving a half-maximal specific growth rate. The second fundamental assumption is that substrate is converted into cell density (x) according to a constant yield coefficient (y) so that

$$dx(t)/dt = -y\, dS(t)/dt$$

For a monoculture chemostat the Monod equations are, therefore,

$$\frac{dS(t)}{dt} = [S_R - S(t)]D - \frac{\mu_{max}x(t)S(t)}{y[K_S + S(t)]} \quad (1)$$

$$\frac{dx(t)}{dt} = \frac{\mu_{max}x(t)S(t)}{K_S + S(t)} - Dx(t) \quad (2)$$

where D is the dilution rate of the chemostat. S_R represents the substrate concentration in the chemostat reservoir.

At equilibrium, $dx(t)/dt = 0$, so, from equation 2,

$$S(t) \rightarrow \hat{S} = K_S D/(\mu_{max} - D) \quad (3)$$

and this equilibrium ($\hat{S}$) value of $S(t)$ is attained at an exponential rate (39, 66).

With the value of $\hat{S}$ given in equation 3, the specific growth rate of cells in the chemostat is simply D, so that the doubling time in the chemostat is given by (ln 2)/D. The quantity (ln 2)/D is usually referred to as the generation time in the chemostat. At equilibrium, $dS(t)/dt = 0$ also, and the density of cells is, from equation 1,

$$\hat{x} = y(S_R - \hat{S})$$

However, $\hat{S}$ is typically very much less than S_R. In one experiment involving glucose limitation, for example, S_R was set at 500 μg/ml and μ_{max} and K_S were found to be 0.83/h and 7 μg/ml, respectively (33). With a doubling time of 2 h, D = (ln 2)/2 = 0.35, and therefore $\hat{S}$ = 5.1 μg/ml, which is smaller than S_R by a factor of 100. Consequently, the equilibrium cell density is little affected by $\hat{S}$ and therefore little affected by D, which is the basis of the assertion made earlier that chemostats permit virtually independent control of generation time and cell density.

For two competing strains the Monod equations are

$$\frac{dS(t)}{dt} = [S_R - S(t)]D - \frac{\mu_{max}(1)x_1(t)S(t)}{y_1[K_S(1) + S(t)]} - \frac{\mu_{max}(2)x_2(t)S(t)}{y_2[K_S(2) + S(t)]} \quad (4)$$

$$\frac{dx_1(t)}{dt} = \frac{\mu_{max}(1)x_1(t)S(t)}{K_S(1) + S(t)} - Dx_1(t) \quad (5)$$

$$\frac{dx_2(t)}{dt} = \frac{\mu_{max}(2)x_2(t)S(t)}{K_S(2) + S(t)} - Dx_2(t) \quad (6)$$

where the symbols and convenient units for limiting organic carbon are as follows:

t = time in hours

$S(t)$, S_R = concentration of the limiting substrate in the chemostat at time t and in the chemostat reservoir, respectively, in units of micrograms per milliliter

$\mu_{max}(i)$, $K_S(i)$, y_i = maximum specific growth rate, saturation constant, and yield constant of strain i (i = 1, 2). Units of μ_{max} are hour^{-1}; K_S, micrograms per milliliter; and y, cells per microgram of substrate

$x_i(t)$ = density of strain i (in cells per milliliter) at time t (i = 1, 2)

D = fraction of the chemostat volume replaced per hour by inflow from the reservoir.

As is the case with monoculture chemostats, $S(t)$ decreases from S_R at an exponential rate. After this initial rapid decrease, $S(t)$ changes slowly and has a value approximately what it

would be were only the better of the two strains present in the chemostat. The value that $S(t)$ approximates is given by equation 3 with the appropriate values of μ_{max} and K_S.

At this quasi-steady state in which $S(t) \cong \hat{S}$, the most important implication of the Monod equations relative to competition is that

$$\frac{d \ln[x_1(t)/x_2(t)]}{dt} = \frac{\mu_{max}(1)\hat{S}}{K_S(1) + \hat{S}} - \frac{\mu_{max}(2)\hat{S}}{K_S(2) + \hat{S}} \quad (7)$$

which follows from equations 5 and 6. All quantities on the right-hand side of equation 7 are constants; thus a plot of $\ln(x_1/x_2)$ against time is expected to yield a straight line with a slope equal to the right-hand side of equation 7. This slope provides a convenient measure of the relative competitive ability of the two strains, and methods of estimation of the slope and its various components are discussed by Dykhuizen and Hartl (38).

A second principal model for the dynamics of chemostat growth has been developed by Droop (29) and studied further by Droop (30, 31) and Goldman and McCarthy (53). As distinguished from the Monod model, which focuses on external substrate concentration $S(t)$, the Droop model focuses on internal substrate concentration, symbolized as $Q(t)$ and measured in units of mass per cell. In the fundamental Droop equation the specific growth rate of a strain is given by

$$\mu = \mu_{max}\left[\frac{Q(t) - K}{Q(t)}\right]$$

where

μ_{max} = maximal specific growth rate corresponding to $Q(t) \rightarrow \infty$

K = minimum concentration of limiting nutrient per cell required before growth can proceed (note that this K has a very different meaning than the saturation constant K_S in the Monod model.)

$Q(t)$ = concentration of limiting nutrient per cell ("cell quota"), which equals the reciprocal of the yield when excretory losses can be neglected

For two strains competing for a limiting resource the Droop analogs of equations 5 and 6 become

$$\frac{dx_1(t)}{dt} = \frac{\mu_{max}(1)[Q_1(t) - K_1]x_1(t)}{Q_1(t)} - Dx_1(t) \quad (8)$$

$$\frac{dx_2(t)}{dt} = \frac{\mu_{max}(2)[Q_2(t) - K_2]x_2(t)}{Q_2(t)} - Dx_2(t) \quad (9)$$

where the subscripts 1 and 2 denote strains 1 and 2. As a chemostat is progressing toward a steady state, $Q_1(t) \rightarrow \hat{Q}_1$ and $Q_2(t) \rightarrow \hat{Q}_2$, where the circumflexes denote equilibrium values for the two strains. Thus, at the steady state

$$\frac{d \ln[x_1(t)/x_2(t)]}{dt} = \frac{\mu_{max}(1)[\hat{Q}_1 - K_1]}{\hat{Q}_1} - \frac{\mu_{max}(2)[\hat{Q}_2 - K_2]}{\hat{Q}_2} \quad (10)$$

which again indicates expected linearity in a plot of $\ln(x_1/x_2)$ against time. It is to be noted that the Monod model (equation 7) contains four strain-specific parameters, whereas the Droop model (equation 10) contains six. For modeling the growth dynamics of single strains in chemostats, the model seems to make a difference. The Monod model seems to be most valid when the limiting nutrient is a source of carbon or energy, whereas the Droop model seems best suited for limiting nitrogen, phosphorus, or vitamins (31, 53). However, when interest is focused on the change in relative proportion of two competing strains, as it is in studies of differential growth rates, then choice of model is no longer critical because both models imply that

$$\ln[x_1(t)/x_2(t)] = \ln[x_1(0)/x_2(0)] + st \quad (11)$$

where $x_1(t)$ and $x_2(t)$ represent the relative proportion (or number) of the two competing strains at time t (conveniently measured in hours) and s is a measure of differential growth rate per unit time. The strain designated by x_1 is favored, neutral, or disfavored relative to the strain designated by x_2 as $s > 0$, $s = 0$, or $s < 0$. An appropriate statistical method for analyzing data relative to chemostat competition is to estimate $x_1(t)$ and $x_2(t)$ for various t and then estimate the slope (s) by linear regression of $\ln[x_1(t)/x_2(t)]$ against t, significance tests being carried out by means of analysis of variance. If one of the strains is resistant to a phage or antibiotic, estimates can conveniently be carried out by plating appropriately diluted chemostat samples on nonselective and selective plates; the latter can be used to estimate the density of the resistant strain directly, and the density of the other strain can then be estimated by the difference between the total (nonselective) and selective plates.

Of course, the simple dynamics of selection outlined above require that the competing organisms be competing for the same limiting substrate. If other phenomena are occurring, such as one strain tending to clump or to stick to the chemostat walls and so avoid washing out of the chemostat at the theoretical rate, then the

dynamics of the interactions become more complex. The theory also breaks down when the competing strains interact in any way other than by competition for substrate, such as when one of them produces substances that promote or inhibit growth of the other.

The selective difference between two competing strains is usually reported in one of two ways, either as the selection per hour (s from the linear regression in equation 11) or as the selection per generation [calculated as (ln 2)s/D]. Preference for selection per generation is sometimes based on the view that this quantity will be relatively independent of generation time (i.e., dilution rate), but this is by no means the case (33). There is also the view that selection per generation is the more natural way to measure selection, as this is the common manner of quantifying selection in discrete-generation models in population genetics (58). However, in chemostat populations, time is continuous and the generations are overlapping, and in this case the natural measure of selection is selection per unit time (27). In population genetics, such continuous-time measures of selection are known as Malthusian parameters (27), and the relationship between these measures and more conventional measures of selection are nontrivial unless the amount of selection is small. Consequently, in this review we report selection coefficients per unit time wherever possible, but in each case will also note the relevant dilution rate.

Although the Monod model and the Droop model of chemostat growth have been the most widely used and investigated, other theoretical approaches are possible. Among these are models discussed by Williams (119, 120), Dabes et al. (28), Button (15), Hellingwerf et al. (63), and a variety of models discussed by Fredrickson et al. (46) and Chiu et al. (22).

Periodic Selection

One important aspect of a chemostat is that the organisms within it represent an evolving or at least potentially evolving system. This point is illustrated in Fig. 1, which is taken from Kubitschek (72, after 101). Theoretically, a mutation at low frequency should increase linearly in frequency because of recurrent mutation, provided that neither the nonmutant nor the mutant organism has a selective advantage (104). Figure 1 shows this initial linear increase in the number of bacteriophage T5-resistant (*tonA*) cells in tryptophan-limited chemostats in *Escherichia coli* strain B/r/1 *trp* at a generation time of 2.8 h. (*tonA*, at 3 min on the standard *E. coli* genetic map [8], is involved in iron uptake.) Although T5 resistance has no detectable effect on growth rate in chemostats limited for tryptophan, methionine, succinate, glucose, or phosphate (72),

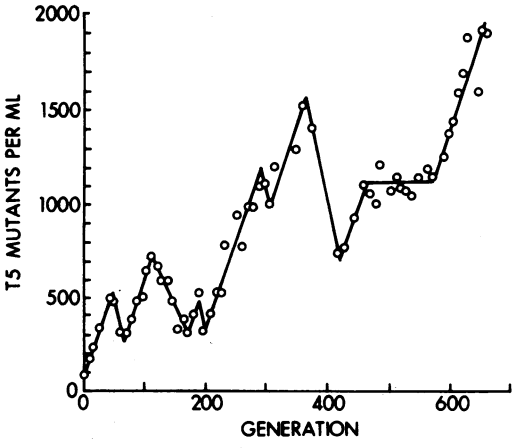

FIG. 1. Change in frequency of phage T5-resistant mutants in a tryptophan-limited chemostat of *E. coli* B/r/1 *trp* at a generation time of 2.8 h. Overall cell density was ca. 2.5×10^8 cells per ml. (From 72, after 101, with permission of Cambridge University Press.)

there are growth rate effects of T5 resistance when chemostats are limited for nitrogen (97). In this latter case, the initial increase in T5 resistance is nonlinear; this sort of selection, which can be attributed to a pleiotropic effect on growth rate of the particular mutation under study, has been termed "specific selection" by Atwood et al. (6, 7) because it is specific to the known mutation.

There is, however, another sort of selection that is of importance in chemostats. The initial linear increase in T5 resistance does not continue indefinitely (Fig. 1). Sooner or later a selectively favored mutation arises at another locus, almost always within the much more abundant T5-sensitive population, and as the clone bearing the favored mutation increases in frequency at the expense of all others, the frequency of the T5 resistance marker drops precipitously. When the favored clone reaches a sufficiently high frequency, mutations to T5 resistance begin to occur in this genotype, and this mutation pressure causes another linear increase in T5 resistance. At some later time a different favorable mutation occurs, again most likely within the more abundant T5-sensitive population, and the whole process regarding T5 resistance repeats itself. Repeated episodes of this sort lead to the jagged, erratic T5 resistance curve shown in Fig. 1.

The sort of selection described above, which is not associated with the mutation under study, has been termed "nonspecific selection" by Atwood et al. (6, 7). Nonspecific selection brings about a hitchhiking effect for all genes initially present in the favored clone, as exempli-

fied for the T5-sensitive gene in Fig. 1. Atwood et al. also refer to nonspecific selection as "periodic selection," and this is the term most widely used for the phenomenon. This term is perhaps unfortunate in two respects. First, periodic selection suggests a special type of selection, although the selection itself is entirely conventional; it is distinguished from other types of selection only in that the genes responsible for it are unknown. Second, periodic selection suggests a mathematical periodicity or regular recurrence at predictable intervals; periodic selection is, in fact, distinguished partly by its unpredictability of time of occurrence.

At least three aspects of periodic selection are to be considered. First, it is a potentially important phenomenon in microbial evolution, as hitchhiking effects can be quite pronounced; this aspect of the situation has been considered theoretically by Kubitschek (72), Koch (70), and Levin (77). Second, periodic selection can be considered as a phenomenon occurring with continuous culture that is worthy of investigation in its own right. And third, periodic selection can be considered as a nuisance factor that can confuse the results and interpretation of other types of chemostat studies. These latter two points of view warrant a brief discussion.

Surprisingly little is known about the genetic or physiological basis of the favorable mutations involved in periodic selection, although they surely differ according to the limiting nutrient. An early study of such a mutation arising in a tryptophan-limited culture was carried out by Novick and Szilard (104), who determined that the favored strain had a decrease in its K_S (recall that K_S is the parameter in the Monod model corresponding to the substrate concentration at which the growth is half its maximum), without a change in maximum specific growth rate. Hartl and Dykhuizen (39, 59) sampled glucose-limited chemostats every 100 h for a total of 500 h and charted the course of increase in competitive ability by competing each sample against a comparable sample isolated 100 h previously. Except for the first 100 h, in which changes in maximum specific growth rate were significant, most of the evolution involved reduction in the value of K_S, which is in agreement with Novick and Szilard's (104) result. However, whereas such experiments reveal the basis of selection in a general sense, they leave its molecular and physiological mechanisms unresolved. Paquin and Adams (106) have studied evolution in haploid and diploid yeast chemostats limited for glucose and observed a significant change in cell shape toward a more elongated longitudinal section; over a period of 250 generations, the geometry of the cells changes so as to give an overall 13% increase in the ratio of surface area to volume. Again, the genetic basis of such changes is unknown.

In a few cases the genetic basis of periodic selection has been resolved. For example, *E. coli* chemostats limited for lactose regularly and predictably undergo a takeover by lactose-constitutive cells within the first 100 h (37, 65, 102, 112). Interestingly, the selective advantage of the constitutive mutation is markedly reduced in the presence of the gratuitous inducer isopropyl-β-D-thiogalactoside, and it is eliminated altogether at isopropyl-β-D-thiogalactoside concentrations above 6 μM (36). Such selection for constitutive mutations is by no means unusual and has, indeed, been used to select D-serine deaminase constitutives in *E. coli* (11) and mandelate constitutives (61) and mandelamidase constitutives (75) in *Pseudomonas putida*.

The reproducibility of selection for lactose constitutives suggests that independent occurrences of the initial periodic selection event may involve the same loci. McDonald (89) studied two *E. coli* strains that had arisen and been selected during the course of serial subculture in glucose medium (not chemostats). The gene involved in the selection was found to be allelic in both strains and to be linked to *rpsL* (streptomycin resistance, ribosomal protein L12; map position, 72 min). Thus, evolution in independent serial cultures had led to the fixation of genotypically and phenotypically similar mutants.

Another example of recurrence of mutations responsible for periodic selection events is found in the work of Collins et al. (23) involving the serine-glycine-alanine uptake system, which is associated with the cotransport of protons described as proton symport. Unselected *E. coli* strains exhibit a slow increase in pH of the extracellular medium associated with alanine transport that is consistent with a proton symport mechanism having a stoichiometry of 1 proton/alanine. A strain evolved in an alanine-limited chemostat for 2 months was found to have a stoichiometry of 4 protons/alanine. In an independent chemostat sampled frequently, the evolving strain was found to change from a stoichiometry of 1 proton/alanine to 2 protons/alanine after about 4 days, and from 2 protons/alanine to 4 protons/alanine after about 7 days. This latter value was maintained thereafter until the end of the experiment (about 1 month). Such changes in proton symport are energetically inefficient but allow the cells to maintain a much greater concentration gradient than otherwise, which is particularly important when the substrate limits growth as it does in chemostats. As compared with unselected cells, the mutant with the stoichiometry of 4 protons/alanine had decreased by 20-fold the concentration of alanine

at which its growth rate was half-maximal. This experiment not only illustrates the repeatability of periodic selection under these conditions, but it also serves to emphasize the importance of transport mechanisms of substrates that are limiting to growth. In addition, the experiment illustrates very well the usefulness of chemostats in selecting mutants for which no obvious traditional approach to mutant selection exists.

Supplementing the direct evidence for the reproducibility of early periodic selection events is a great deal of circumstantial evidence. In the long-term study of Dykhuizen and Hartl (39, 59), for example, little divergence in competitive ability was found among strains that had been evolving for the same length of time in replicate chemostats, even though the overall amount of increase in competitive ability was enormous. Similarly, Chao and Cox (20), studying periodic selection in chemostats containing the mutator allele *mutT* (map position, 3 min) and wild-type *E. coli*, observed that the amount of selection associated with the mutant favored by periodic selection varied little from chemostat to chemostat; they suggest that the higher-fitness mutants that arise in these chemostats are phenotypically similar, if not identical, which leads to the speculation that they may be genetically very similar. Whatever the genetic basis of such adaptive mutations, they seem to be specific to the particular growth conditions, as exemplified by the finding of Helling et al. (62) that favored mutations that arise in glucose-limited chemostats provide no selective advantage in fructose-limited chemostats.

Little research has been carried out on the long-term statistical characteristics of periodic selection. Interesting but unresolved issues pertain to the interval between successive periodic selection events, such as whether the time interval between successive periodic selection events increases, decreases, or remains the same over the course of a long-term chemostat experiment. One experiment of this sort was carried out by Novick and Szilard and analyzed by Kubitschek (72); in this experiment the frequency of T5-resistant mutants in tryptophan-limited chemostats was monitored for 1,500 hours (600 generations under these conditions). During the course of the experiment, six periodic selection events were observed; the first took place after about 170 h, and the others occurred at intervals of about 240 h with a slight tendency for the intervals between successive events to increase. The selective advantage of each new favored mutation was also estimated, and it was substantially smaller in the last three events than in the first three. A similar result was obtained by Hartl and Dykhuizen (59) who, in a shorter experiment involving only 500 h, found much smaller adaptive changes in the last 300 h than in the first 200 h. However, the statistical characteristics of periodic selection are very poorly known and deserve further attention.

A systematic study of the long-term evolution of *Saccharomyces cerevisiae* due to periodic selection in phosphate-limited chemostats has been carried out by Hansche and collaborators (43, 44, 55, 56). Strain S288C was grown for 1,000 generations with limitation by β-glycerophosphate at pH 6.0. The pH optimum of the normal acid phosphatase, coded by the *phoE* locus on the right arm of chromosome II, is 4.2, and a pH of 6.0 reduces its activity by about 70%. At about 180 generations an increase in cell density was noted. This was found to be associated with a single mutation, leading to a 30% increase in the efficiency of utilization of the orthophosphate liberated by the acid phosphatase. By about 400 generations a second perceptible increase in cell density had occurred in the chemostat. The strain associated with this increase was designated M2, and it was found to have a mutation in or near the *phoE* structural gene, leading to a 60% increase in activity at pH 6.0, an increased activity over a wide range of pH, and a change in pH optimum from 4.2 to 4.8. A third major change in the chemostat was detected after about 800 generations. This was due to a mutation that led to cell clumping, which reduced washout of cells through the overflow and thus led to the equivalent of an increased survival time in the chemostat (43).

The mutational changes in phosphate-limited yeast chemostats are to some extent reproducible. In a separate experiment limiting for β-glycerophosphate, Francis and Hansche (44) observed an increase in cell density after 290 generations. The mutant strain, designated M4, had a fourfold-increased activity at pH 6.0 and a change in pH optimum of from 4.2 to 5.2 with little or no change in K_m. Crosses between M2 and M4 gave asci that segregated 2:2 for pH optimum, suggesting that the mutations involved were allelic. In this experiment a clumping mutation also occurred, this time its effects being noted at generation 650.

Further evolution of chemostat-derived strains leads to other reproducible genetic changes. In one case, Hansche (55) maintained a strain for 1,000 generations on limiting uridine 5′-monophosphate at pH 6.0. The strain had previously been maintained for 1,000 generations on limiting β-glycerophosphate. (Uridine 5′-monophosphate is hydrolyzed at about half the rate of β-glycerophosphate.) At the end of this second 1,000 generations, the evolved strain exhibited an overall fourfold greater activity on uridine 5′-phosphate than its parent. About half of this increase was due to the additive effects of

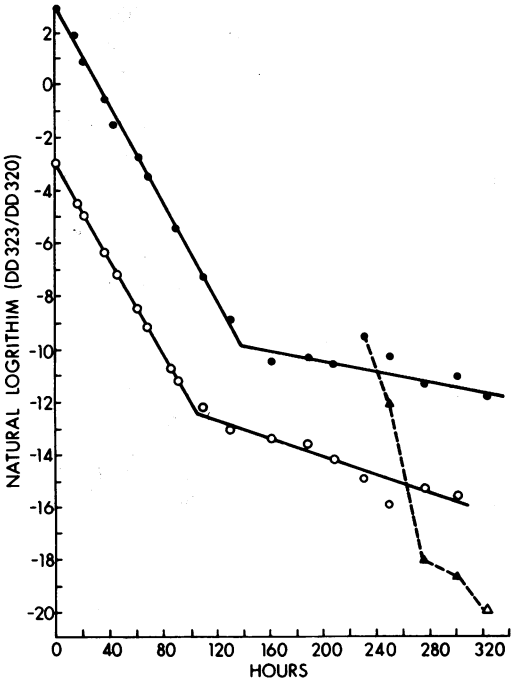

FIG. 2. Selection against strain *E. coli* DD323 in maltose-limited chemostats (solid lines). Note the abrupt change in slope at 100 to 140 h. Transfer of culture to a fresh chemostat markedly increases the selection (dashed line), indicating that the previous decrease in slope is due to wall growth.

two unlinked mutations. One, called *abs*, inhibits abscission of daughter cells from the mother cell and leads to formation of spherical clumps of 40 to 60 cells; Hansche suggests that this mutation increases the capacity of the cell wall to accommodate acid phosphatase molecules. The second mutation, called *wal*, in the absence of the *abs* mutation, leads to cells with little or no wall, which under usual culture conditions results in cells having little geometrical integrity. The other half of the overall activity increase was due to the appearance of a new acid phosphatase gene unlinked to the *phoE* locus. The enzyme produced by the new gene was identical in activity and kinetic properties to that in the parental strain, and Hansche (55) proposed its origin as a duplication.

Hansche et al. (56) studied five acid phosphate duplications that had arisen in chemostat culture. Two of these arose spontaneously and three were induced by UV light. All five duplication events involved the transposition of the *pho* locus and all distal loci on chromosome II to the terminus of a nonhomologous chromosome. No tandem duplications were observed. Among the five duplications only one was mitotically stable; the others were lost at the rate of about 2% per mitotic division. However, selection for stability in one of the unstable clones resulted in a 1,000-fold increase in stability.

In addition to the pH optimum mutants, the clumping mutants, *abs*, *wal*, and the duplications, in at least one case there arose a strain producing acid phosphatase constitutively. This constitutivity was found to be due to a mutation in *pho-80*, which codes for the acid phosphatase repressor that normally represses *phoE* in the presence of inorganic phosphate (73). Clearly, evolution in chemostats has led to significant changes in the cells themselves (*abs*, *wal*) and to extensive evolution of acid phosphatase, its gene number, and its regulation.

As for periodic selection as a nuisance factor, the most important implication is that curves for mutation or selection cannot be expected to remain linear for more than the first 100 to 200 h of chemostat culture. Eventually periodic selection will occur and thereby obscure the phenomenon under study. Moreover, if strains are to be preadapted to chemostat conditions before being placed in competition with one another, it is important that they be preadapted under as nearly identical conditions as achievable and for the same length of time. Even so, the possibility of evolutionary divergence among replicate chemostats requires that the final experiments be accompanied by the appropriate controls.

Wall Growth

There is another mechanism whereby a selection curve may not follow its expected course, and this is wholly distinct from periodic selection. The mechanism is wall growth, the attachment and growth of organisms on the surface of continuous-culture vessels. The various causes of wall growth are rarely distinguished. One type involves "sticky" mutations that sometimes arise during the course of a chemostat experiment; such wall growth usually results in a thick, visible film, and its importance depends on the particular nature of the experiment. The type of wall growth in question here involves the innate tendency of certain organisms to attach to glass surfaces (24). This attachment occurs immediately upon initiation of the culture and results in a virtually invisible film. In tryptophan-limited *E. coli* chemostats maintained at a density of 10^8 cells per ml, this wall population accounts for <1% of the total population (98). In any event, these attached cells form a relatively permanent population that experiences a different selective regime than occurs in the liquid. Indeed, cells attached to the wall tend to preserve their initial frequencies even though the frequencies in the liquid medium may change by many orders of magnitude. As cell division occurs, the wall population serves as a constant source of migra-

tion into the liquid medium. If a strain is disfavored by selection, the initial selection will appear quite conventional because at first the migrants constitute a miniscule proportion of the disfavored type. However, the disfavored strain can never be reduced to a frequency smaller than that provided by the wall population. Indeed, as the disfavored strain decreases in frequency, the migrants constitute an increasingly larger proportion of its total numbers, so that the migration begins to counteract the selection. At some point the selection curve undergoes a perceptible change in slope, which superficially appears as a pronounced decrease in the rate of selection. Examples of these dynamics are illustrated in the solid curves in Fig. 2, which show selection involving DD320 (Str^s Lac^-) and DD323 (Str^r Lac^+) in maltose-limited chemostats at a doubling time of 2.3 h (Dykhuizen, unpublished data).

Normally, wall growth is not a serious problem in short-term chemostats (i.e., up to about 100 h), and it happens rarely and apparently randomly (e.g., 98). Moreover, wall growth is of less concern at intermediate or high population densities or at low dilution rates because a smaller proportion of the cells in the liquid will have been derived from the wall population. Considerable variation in wall attachment characteristics occurs among species. *E. coli* and *Serratia marcescens* are quite high in their wall attachment propensity, *Aerobacter aerogenes* is intermediate, and *Bacillus cereus* and *B. subtilis* are very low (74). Fortunately, when there is doubt about the cause of an apparent change in the rate of selection, the hypothesis of wall growth is very easy to test. One simply initiates a new chemostat from the liquid medium in the old one. If the rate of selection increases dramatically, wall growth is very likely involved. Otherwise genetic or physiological changes in the cells are to be suspected. This test is illustrated by a chemostat in Fig. 2 (dashed curve), which was initiated at 230 h with half of the liquid volume in the chemostat indicated by the solid dots; the dramatic increase in rate of selection in the newly initiated chemostat is evident. A systematic study of various aspects of wall growth in *E. coli* chemostats has recently been carried out by Goguen (Ph.D. thesis, University of Massachusetts, Amherst, 1980).

MUTANTS

Chemostat studies are particularly useful for determining quantitatively the functional effects of known mutations. In such studies the phenomena of periodic selection and wall growth are rarely significant problems because the chemostats are initiated with known frequencies of two appropriately marked competing strains and are maintained for a relatively short period (e.g., 100 h) to estimate the initial change in relative frequency of the strains. The method is sufficiently sensitive to detect very small differences in growth rate. Using the sampling procedure of Dykhuizen and Hartl (38), for example, differences in growth rate as small as 0.005 per h can be detected with relative ease. Because of this sensitivity, the degree to which the competing strains are isogenic becomes a critical issue, and various types of control experiments are necessary. We (38, 60) have distinguished three types of controls. A positive control involves a pair of strains that undergo competition in a chemostat under conditions in which the phenomenon of interest can readily be observed. For example, in studying the functional effects of a leaky mutation, an appropriate positive control would involve a deletion mutation in competition with wild type in chemostats limited for the substrate of the enzyme in question; the role of the positive control is to demonstrate that selection in the anticipated direction can be detected under the chosen conditions. That having been demonstrated. the negative control comes into play. The negative control involves the same strains as the positive control, but now the conditions are so chosen that the selection disappears. In dealing with a specific enzyme, for example, the ideal negative control involves a chemostat limited for the product of the reaction or for a substrate whose metabolism does not involve the enzyme in question. In the negative control the selection observed in the positive control should disappear, which leads to the inference that the gene in question is responsible for the observed selection. The role of the negative control is basically to detect effects that may be due to unrecognized genetic differences beteween the strains. Yet a third type of control is a strain control, which is necessary whenever a genetic marker other than the mutation of interest is used to monitor the frequency of the competing strains, and its purpose is to verify that the marker itself is not selected under the experimental conditions. Useful genetic markers for chemostats in *E. coli* include *tonA* (72) and *rpsL* (Dykhuizen, unpublished data; but see 41), which have negligible effects of their own in glucose-limited chemostats, and those in *S. cerevisiae* include canavanine resistance (1).

Although diploid and haploid strains of *S. cerevisiae* can scarcely be considered "mutants" of one another, it suits our organization to discuss them under this heading. Adams and Hansche (1) have studied virtually isogenic haploid and diploid strains in chemostats limited for glucose, inorganic phosphate, or organic phosphate. (So far as is known the strains were genetically identical except for the mating type

locus and the canavanine resistance locus.) With limitation by glucose or inorganic phosphate, the haploid and diploid strains were found to be competitively equal; with limitation by organic phosphate the haploid strain was found to be competitively superior to the diploid. Adams and Hansche (1) relate these results to the nature of the limitation and to cellular geometry as measured by the ratio of surface area to volume. With glucose limitation the surface area/volume ratio of diploids and haploids is virtually identical, so little selection is expected. The surface area/volume ratio in diploids is reduced with limitation by inorganic or organic phosphate. However, Adams and Hansche (1) argue that growth rate under limitation by inorganic phosphate is determined by efficiency of substrate utilization as opposed to uptake. In this case the surface area/volume difference would have no consequence. With limitation by organic phosphate, they argue, growth rate is limited by uptake, and in this case the haploids displace the diploids by virtue of their greater surface area/volume ratio. (For further discussion see 118).

One of the earliest chemostat studies of the functional effects of laboratory mutants was that of Zamenhof and Eichhorn (125). Prompted by the hypothesis of Lwoff (81) that auxotrophic mutants would have a selective advantage over isogenic prototrophs in nonselective media because of energetic economy due to fewer biosynthetic steps, they examined the effects of His^- and Trp^- auxotrophs in *B. subtilis*. When grown in the presence of histidine, the His^- mutant was favored at the rate of about $s = 0.05$ per h ($D = 0.17$), and in the presence of tryptophan, the Trp^- mutant was favored at about the same rate. This is a remarkably high rate of selection inasmuch as, under these conditions, the tryptophan-synthesizing genes have only the residual activity of a repressed operon. Nevertheless, the strains involved in the competition were isogenic (the prototroph was selected as a spontaneous revertant of the auxotroph in each case), and the basic result for Trp^- has been confirmed by Dykhuizen (33) and extended to tyrosine auxotrophs by Mason and Slater (86). Zamenhof and Eichhorn also competed an anthranilate-requiring strain against a tryptophan-requiring strain in the presence of tryptophan. Selection favoring the anthranilate-requiring strain occurred at the rate of 0.288 per h, favoring their view that earlier metabolic blocks are favored over later ones, presumably because of energetic economy; but in this case the strains were evidently not isogenic so the interpretation of the result is thrown in doubt.

Whereas the results of competition between auxotrophs and prototrophs in nonselective media are clear and reproducible, the interpretation based on energetic economy has not been confirmed. The experiments lack a negative control, for example. Presumably, if energetic economy is involved, the selection should disappear in chemostats limited for something other than a source of energy, such as nitrogen or phosphate. In addition, several predictions of the energy-economy hypothesis have not been confirmed (33). First, reasonable calculations of the amount of energy saved under repressed conditions by not making the tryptophan-synthesizing enzymes and producing excess tryptophan suggest that the savings are on the order of 0.01% of the total energy budget; this is 500 times smaller than the observed amount of selection and is, indeed, below the limit of resolution of present techniques. Second, a polar nonsense mutation is not favored over a missense mutation, contrary to expectation. Third, the selection for a Trp^- mutant is the same in chemostats supplied with indole as it is with anthranilate, although energetic considerations imply that there should be a difference. Dykhuizen suggests that the selection for auxotrophs may be a consequence of some general metabolic effect of tryptophan intermediates in the wild-type strain. In short, although selection for auxotrophs is dramatic and most likely an important phenomenon in microbial evolution, the metabolic basis for the selection can be disputed. The work of Collins et al. (23) cited earlier in connection with proton symport in alanine transport provides a counterexample to metabolic economy inasmuch as the mutants selected in the chemostat have evolved an energetically less efficient transport mechanism. Although energetic economy must be important in some overall, general sense, examples such as this one imply that it cannot be uncritically invoked.

The importance of metabolic economy has also been tested explicitly by Andrews and Hegeman (5), using a set of five isogenic strains carrying various F'*lacO*^{c} (lactose-constitutive) episomes grown in competition in turbidostats, which provides a comparison of growth rates near μ_{max}. The amount of *lac* operon proteins produced by these constitutives ranged from 0.6 to 3.6% of total cellular protein, but no simple direct relationship between the level of *lac* operon expression and selective disadvantage was found. Cultures were grown in 0.2% glycerol at 20°C, corresponding to $\mu_{max} = 0.10\ h^{-1}$. All strains differed from the F'*lac*$^+$ strain by the amount 0.02 selection per generation or more, which was attributed to possible genetic divergence between the strains arising from 12 generations of preadaptation in monoculture turbidostats. Three strains producing <0.91% *lac* protein had a selective disadvantage of 0.021 or 0.022 per generation, and two strains producing

1.6 and 3.6% *lac* protein had a selective disadvantage of about 0.026 per generation. Clearly there is no linear relationship, and the correlation between selection rate and percentage of wasted protein is not statistically significant. If anything, there is a sort of threshold effect in which strains producing a small amount of unneeded protein are selected against mildly if at all, whereas strains producing much more than several percent of unneeded protein are at a detectable disadvantage. Andrews and Hegeman (5) suggest this explanation and provide additional evidence that the amount of protein synthesis is not limiting to growth under their conditions. Dykhuizen and Davies (36) have suggested that the selection against high-level *lac* operon expression can be explained in part by indirect metabolic effects.

Another argument for selection based on energy economy has been put forward in the case of a petite mutant (*rho*^{o} no. 7) of *S. cerevisiae* relative to its parental strain (C86-2-15B *a*) in anaerobic chemostats. This petite mutant has <5% of the normal complement of mitochondrial DNA as assessed by density gradient centrifugation and fluorescent staining. James (67) has shown that the petite mutant is selectively favored over the wild type in anaerobic chemostats limited for glucose at a variety of generation times. Two curious aspects of the selection were noted. First, there is a lag that occurs before the onset of selection, which is particularly pronounced at a short generation time. Second, the displacement of the wild type does not go to completion. Nevertheless, the petite strain is favored at a rate $s = 0.03$ ($D = 0.04$) to $s = 0.07$ ($D = 0.16$) per h depending on the growth conditions, and James (67) speculates that this might be due to a smaller size of petite cells leading to a smaller energy expenditure in maintenance and reproduction. On the other hand, smaller cells also have a more favorable surface area/volume ratio, and this might account for at least some of the selection.

Gibson et al. (51) stimulated an interesting series of experiments by their finding that strains carrying the *mutT1* mutator gene were favored over their otherwise isogenic counterparts in glucose-limited chemostats. In these experiments, the selective advantage of *mutT1* strains ranged from $s = 0.004$ ($D = 0.46$) to 0.032 ($D = 0.28$), with an average of 0.014. However, it was originally unclear whether the selective advantage was due to some unknown metabolic effect of *mutT1* or to *mutT1* strains having a greater chance of undergoing a favorable mutation. This problem was investigated further by Nestmann and Hill (99), who found a similar effect of *mutH1* (map position, 61 min). The selection was as high as $s = 0.07$ ($D = 0.10$) in a few cases, but interpretation is complicated in this case because the Gal^{-} marker used in the strains was not itself neutral. In any event, if the selective advantage is due to new favorable mutations, then the selection should be frequently dependent in the sense that the initial inoculum of mutator cells should be able to be made small enough that the mutational advantage of the mutator could be overcome by the numerical preponderance of the normal strain. Under these conditions the mutator strain should be eliminated from the chemostat. Nestmann and Hill (99) did observe this expected frequency dependence in two cases out of four, but the expected lag preceding the onset of selection caused by a favorable mutation becoming fixed in the nonmutator strain was not observed.

Further evidence on the mechanism of selection for mutators was provided by Cox and Gibson (26). They studied mutator strains that had survived competition in chemostats and found that they were competitively superior in at least three ways. First, they had an enhanced tendency to stick to glass powder, suggesting a greater ability to stick to the walls of the chemostat vessel. Second, they could utilize citrate, a chelating agent in the chemostat medium, as an energy source. And third, they were more resistant to starvation for glucose than were wild-type clones taken from the same chemostat. These results strongly support the mutational origin of the selective advantage of *mutT1*.

Chao and Cox (20) have recently reinvestigated the kinetics of *mutT1* selection in chemostats, and they find the characteristics expected from the mutation hypothesis. There is indeed a lag before the onset of selection, averaging about 70 generations and evidently due to the time required for a favored mutation to become nearly fixed in the *mutT1* population. Remarkably, the degree of favorability of the favored mutant was quite consistent from chemostat to chemostat, which was interpreted as meaning that the mutations could be genetically similar if not identical. Moreover, the kinetics of selection were consistent with the view that the favored mutations were present in the original inoculum. This conclusion that early periodic selection events involve preexisting mutants was also reached by Helling et al. (62) relative to nonmutator strains. In any case, the mutator strain is not alone in having the favored mutation. The normal strain has it, too, but in lower frequency. This results in the normal strain not being completely replaced by reaching a plateau in its frequency, which persists until still more favorable mutations occur.

Chao and Cox (20) also observed the expected frequency dependence of selection. Where the initial ratio of *mutT1*/*mut*$^{+}$ is 7×10^{-5} or great-

er, the *mutT1* strain increases in frequency after the lag, but when the ratio is less than 7×10^{-5}, the *mutT1* strain decreases in frequency after the lag. This is tolerably in agreement with what would be expected based on the ratio of mutation rates in the competing strains, and indeed the entire course of selection is in good agreement with the theoretical predictions put forward by Painter (105).

ACCESSORY DNA ELEMENTS: GENERAL ASPECTS

Campbell (18) has discussed the evolutionary significance of accessory DNA elements in microorganisms. Accessory DNA includes such elements as plasmids, viruses, transposons, and insertion sequences and can be distinguished from typical chromosomal DNA by two properties. The first is that the elements generally contain no genes that are unconditionally required for growth and reproduction of the organism in its usual environment. Second, these elements are able to replicate autonomously and in some cases increase their copy number relative to the chromosomal DNA. Campbell argues that these elements should show coadaptation with the host and that they might indeed benefit the host in some manner under particular environmental conditions. If this is correct, then it will be important to determine the conditions under which these elements provide a growth advantage. These conditions will be relevant to the mechanisms leading to the persistence of such elements in populations. The relevance of chemostats to these issues has been argued by Woods and Foster (121): "When the host is complex, and has differentiated tissues, it will have some form of circulatory system. The bacterium will therefore be in an environment which is constantly renewed in the sense that there will be, due to the activities of the host, both a continual replenishment of growth metabolites and a continual removal of waste products of metabolism. The situation is essentially similar for organisms living in an intestinal tract. The system is therefore analogous in many ways to continuous culture and less like the batch cultures that are the source of so much of the bacterial material used for metabolic studies *in vitro*." See Mason and Richardson (84) for a general discussion of presumed conditions in the mammalian intestinal tract and Mason and Richardson (85) for the use of chemostats to simulate the human gut. Freter and collaborators (47, 48, 50) have recently developed an anaerobic continuous-culture system containing a set of 95 strictly anaerobic strains isolated from mouse cecal contents. This synthetic indigenous microflora can simulate many of the bacterial interactions observed in the mouse large intestine.

Plasmids

A substantial literature has developed concerning the behavior of plasmids in continuous culture, motivated in part by the as yet largely unexploited potential of these techniques in industrial application. The sort of problem that has to be overcome is exemplified in the case of plasmid pSC101 *trp*.I15.14 in *E. coli* W3110 as host. The host in this case has a deletion of the *trp* operon and of the tryptophanase gene, and it has an inactive *trp* repressor; the plasmid carries tetracycline resistance and a *trp* operon containing a mutant *trpE* gene that is insensitive to feedback inhibition. In batch culture, this strain produces 0.23 g of tryptophan per liter per h, which would warrant industrial production of L-tryptophan by fermentation (3). However, in continuous culture, the plasmid is unstable in that deletions and other mutational alterations of the *trp* operon are selected (32). Clearly, an understanding of the factors promoting plasmid stability would be an important achievement.

Many general considerations of plasmids are based on the supposition that the maintenance of a plasmid represents a significant burden on the economy of the cell. In some cases this is demonstrable. Many, but by no means all, plasmids increase host generation time. Zund and Lebek (127) have studied 101 R factors from this point of view. About one-quarter of these increased host generation time by more than 15%. Among R factors of >80 kilobases in size, the majority increased generation time. Beyond this general observation there appears to be no consistent relationship between plasmid size and effect on growth rate under nonlimiting conditions.

In many cases a plasmid-free host readily outcompetes its plasmid-bearing competitor in chemostats. This is true of plasmid RP1, a conjugative R factor carrying resistances to ampicillin, carbenicillin, tetracycline, and kanamycin/neomycin, in *E. coli* host W3110 (91). Interestingly, the plasmid-free cells take over only in phosphate-limited chemostats in this case; under glucose or magnesium limitation the plasmid-free cells are gradually lost from the chemostats. Even in phosphate-limited chemostats when the plasmid-free strain takes over, takeover is not complete and the plasmid-bearing cells are stably maintained at a low frequency. This persistence of plasmid-bearing cells has been noted in several other cases (52, 69). This could be a trivial artifact of wall growth of the plasmid-bearing strain, or it might represent some unrecognized mechanism of maintaining plasmids at low frequency that would be highly relevant to the persistence of plasmids in natural populations. Godwin and Slater (52) have pointed out

that the selection against plasmid-bearing strains, when it occurs, is sometimes much greater than would be expected from energetic economy. In a related context Helling et al. (62) have defined plasmid interference as those metabolic effects resulting from a plasmid that are distinct from plasmid maintenance effects.

Helling et al. (62) have also studied the dynamics of competition between plasmid-bearing and plasmid-free strains. The host in this case was *E. coli* RH204 and the plasmid was one of several non-colicin-producing derivatives of RSF2124, which also carries transposon Tn*3* (*amp*). When mixed chemostats are initiated at a sufficiently high frequency of the plasmid-bearing strain, its frequency at first declines, then increases again, and then decreases. These oscillations are due to periodic selection. They are mediated by mutations of chromosomal genes because the favorable mutations do not cotransform with plasmid DNA. The nature of the oscillations is quantitatively in agreement with the expectations of an extension of the Monod model to periodic selection. In a related study, Adams et al. (2) examined selection involving the original ColE1-producing plasmid RSF2124, which is 11.3 kilobases in size and present in 10 to 30 copies per cell. In this case there is a pronounced frequency-dependent effect: when the colicin-producing cells are at a sufficiently high frequency initially, they produce enough colicin to inhibit growth of their competitors and increase in frequency to near fixation; when the initial frequency of the colicin producers is low, the inhibition of competitors is insufficient to counteract the intrinsic growth rate disadvantage of the plasmid-bearing strain, and the plasmid-bearing strain decreases in frequency. In the longer term, after 100 cell generations or more, plasmid-free but colicin-resistant cells begin to take over the chemostat. However, in structured habitats (soft agar), the conditions are more favorable for selection for colicinogenic bacteria (21).

Competition between a toxin-producing strain of *S. cerevisiae* and a sensitive strain in chemostats has been studied by Young and Philliskirk (124). Strains NCYC738 and NCYC235 are killer yeasts producing a glycoprotein toxin associated with a cytoplasmically inherited double-stranded RNA. These strains and their killer-cured derivatives all displace a sensitive nonisogenic competitor (NCNY1006) in glucose-limited chemostats, but displacement by the killer strains occurs more rapidly, particularly under conditions of temperature, pH, dilution rate, and other factors that promote production of the killer substance. This phenomenon is relevant in the brewing industry where, in one case of continuous fermentation, a killer contaminant displaced the resident and dominated the fermentor (88).

The fate of a monoculture chemostat containing a plasmid-bearing strain depends on the plasmid, the host, the limiting nutrient, and the dilution rate. A key factor is segregational loss of the plasmid, theoretical aspects of which have been studied for F factors by Anderson and Lustbader (4). Jones et al. (69) found that plasmids RP1, pDS401 (a ColK derivative), and pDS1109 (a ColE1 derivative) were maintained at least for 120 generations in glucose- or phosphate-limited chemostats. Interestingly, the copy number of pDS1109 fell by fivefold during 80 generations but was restored to its original level after a single cycle of batch growth. In contrast, plasmids pMB9 and pBR322 generate plasmid-free segregants after approximately 30 generations. Jones et al. (69) suggest that fidelity of segregation at low copy number determines stability and argue that the *par* locus of pBR322 (90) might well be involved. Similarly, in studies of RP1 (91), the plasmid was stably maintained in chemostats limited for carbon, magnesium, or phosphate and at all dilution rates between 0.05 and 1.0 h^{-1}. A similar stability of R6 has been reported by Wouters and van Andel (123).

Factors influencing plasmid stability have been studied by Roth et al. (109), Noack et al. (100), and Roth and Noack (110). The importance of the plasmid and conditions is exemplified by the difference between pBR322 and pBR325. Under the glucose- and nitrogen-limiting conditions of Noack et al. (100), pBR322 is stably maintained, whereas pBR325 is lost under limiting glucose at low dilution rates (0.15 h^{-1}). Temperature effects are apparent in the work of Wouters et al. (122), who found conditions under which pBR322 was lost from chemostats at 37, 40, and 42°C but stably maintained at 30°C. Host genotype is also important, as pBR322 is maintained in strains *E. coli* GY2354 and GM31 but not in an *E. coli* K-12 *recA recB sbcB* host (100).

The fate of a plasmid in chemostats is frequently not simply a matter of retention or loss. In certain cases when pBR325 is lost, for example, the tetracycline resistance phenotype is lost very rapidly, whereas the ampicillin and chloramphenicol resistances cosegregate and are lost more slowly (100). Perhaps unexpectedly, the tetracycline-sensitive derivative of the plasmid exhibits no detectable change in molecular weight. An even more complex situation regarding marker loss from TP120 (=R46), which carries resistances to tetracycline, ampicillin, streptomycin, and sulfonamide, has been studied by Godwin and Slater (52). In carbon-limited chemostats, only the tetracycline resistance is lost. By contrast, in phosphate-limited chemostats,

the tetracycline or the ampicillin resistance, or both, may be lost. The resistances lost and their pattern of loss vary from experiment to experiment, and various mutant plasmids may be found in the same chemostat. The molecular basis of these changes is unknown.

The transmissibility of conjugative plasmids in chemostats has also been studied with an eye to developing satisfactory models for the maintenance of plasmids in nature (76). Levin et al. (79) have estimated the transfer rates in glucose-limited chemostats of three plasmids: F'*lac pro*; R1 (kanamycin, chloramphenicol, ampicillin), a naturally occurring plasmid; and R1*drd-19*, a derivative of R1 derepressed for transfer functions. Rates of transfer for F'*lac pro* and R1 were 3.26×10^{-12} and 2.55×10^{-14} ml/cell per h, respectively, whereas that for R1*drd-19* was 1.83×10^{-11} ml/cell per h. The rate of transfer in chemostats is thus low but by no means negligible and is markedly influenced by derepression of transfer functions. Moreover, rates of transfer in chemostats are smaller than in exponential cultures by roughly 2 orders of magnitude (79).

Freter et al. (49) have also studied the transfer of plasmids R1 and R1*drd-19* among *E. coli* strains maintained in anaerobic continuous cultures along with 95 strictly anaerobic strains constituting a synthetic large intestinal microflora which can simulate bacterial interactions observed in the mouse gut. The density of *E. coli* maintained in this complex community is much less than in cultures containing no competing microflora, and since the plasmid-bearing donor strain is added to the culture after the culture is equilibrated and cannot establish a permanent population of its own, plasmid transfer occurs in two distinct ways: first, via direct transfer from donor to recipient while the donor is passing through the culture vessel; and second, via transfer from transconjugants (plasmid-infected recipients) to uninfected recipients within the stable population. Remarkably, the donor-to-recipient transfer rate constant for plasmid R1 in this complex community was 2.3×10^{-14} ml/cell per h (average of 12 experiments), which is nearly identical to the estimate of Levin et al. (79); transfer of R1*drd-19* seems to be somewhat impaired by the presence of indigenous microflora, its donor-to-recipient transfer rate being 3.1×10^{-13} ml/cell per h (average of 5 experiments). However, the rate constants for transconjugant-to-recipient transfers were 5 to 6 orders of magnitude higher than the donor-to-recipient rates, being 3.9×10^{-8} (average of 12 experiments) in the case of R1 and 1.5×10^{-7} (average of 5 experiments) in the case of R1*drd-19*. This large difference occurs in part because of derepression of R1 transfer functions in the transconjugants but also because the donor strains are near stationary phase whereas the transconjugants are growing, and stationary cultures have lower transfer rates (79).

Long-term continuous cultures studied by Freter et al. (49) revealed that plasmid transfer often does not go to completion. Rather, the populations of transconjugants and potential recipients achieve and maintain constant levels. This phenomenon was analyzed by means of a series of increasingly complex mathematical models incorporating such features as repression of transfer functions (relevant to plasmid R1 only), segregational loss of the plasmid, reduction in growth rate of plasmid-bearing bacterial hosts, competition for nutrients, and bacterial attachment to the wall of the gut or culture vessel. The long-term results were best approximated by a model assuming an inferior growth rate and a small wall-attached portion of the population of the plasmid-bearing bacteria. Plasmid transfer efficiencies were also studied in gnotobiotic mice carrying a synthetic indigenous microflora resembling in their functions the normal indigenous microflora of the mouse large intestine. On the whole, plasmid transfer in vivo did not differ from that occurring in vitro in the presence of gut-simulating anaerobic strains, and any peculiarities noted in vivo could be referred to the population density of *E. coli* in the gut.

Levin and Rice (78) have also studied the transfer rates of a nonconjugative plasmid (pCR1) when mobilized by an F'*lac pro* mobilizing factor. In glucose-limited chemostats they estimated an overall transfer rate of 6.4×10^{-12} ml/cell per h, with about 26% of the transfers involving only the nonconjugative plasmid, 68% involving only the conjugative plasmid, and 6% involving both. Again, the rate of transfer in chemostats was about 2 orders of magnitude smaller than in exponential cultures. Such "triparental matings" have been studied theoretically by Freter et al. (49), who find in computer simulations that high-efficiency transfer of the conjugative plasmid is actually detrimental to the transfer of the nonconjugative plasmid, the reason being that the conjugative plasmid rapidly sweeps through the population of potential recipients and so renders this population immune to subsequent cotransfers of the nonconjugative plasmid. They suggest that the low level of fertility of wild-type *E. coli* strains may actually be optimal for maintaining an ecological balance among diverse types of plasmids.

Temperate Phage and Transposons

Chemostats have also been used to examine the selective forces impinging on lysogens and on transposon-bearing strains. Theoretical considerations relating to this point have been orga-

nized and cogently discussed by Campbell (17, 18) and Campbell et al. (19).

Paynter and Bungay (107) have studied the temperature induction of bacteriophage λ in strain *E. coli* 159T⁻ in continuous culture. A culture initially inoculated with the lysogen and maintained at 30°C rapidly achieves a steady-state cell density of lysogens. Shocking the culture briefly at 42°C leads to a rapid 100-fold decline in cell density due to induction of the prophage. The density then gradually increases for about 24 h and achieves another steady-state level slightly greater than the original, presumably because there are fewer sensitive, nonlysogenized cells in this population. Another temperature shock leads to repetition of this cycle of induction and recovery.

More directly relevant to evolutionary considerations are studies on the relative growth rates of phage lysogens and isogenic nonlysogens in chemostats. Edlin et al. (42) and Lin et al. (80) have shown that λ lysogens (λ *c*I857 *ind- susJ*) are favored at an approximate rate of $s = 0.03$ per h in glucose-limited chemostats independent of the initial frequency of the lysogen. This phenomenon occurs with limitation by glucose, glycerol, lactose, or acetate, but it occurs only in aerobic chemostats; under anaerobic conditions the lysogen is disfavored. Such selective differences are not observed in batch cultures, and they have been attributed to a generally higher metabolic rate of lysogens under carbon source limitation as judged by a tetrazolium assay of NADH level in aerobically grown cells (80).

In any event, similar selective effects have been reported for lysogens of bacteriophage P1 (P1cm), P2 (P2-186p), and Mu (Mu *c*ts) (41). Edlin (40) has studied certain outer membrane proteins of λ lysogens and nonlysogens by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. He emphasizes two abundant proteins, the relative proportions of which are altered in lysogens or in nonlysogens grown anaerobically. The gels show other differences as well, and it is not necessarily the case that any of the differences are associated with the selection observed in the chemostat. Conceivably, there could be many differences between lysogens and nonlysogens that are real but not involved in the observed selection.

Lin et al. (80) have attributed the selective effect of λ to the λ *rex* function because a presumptive λ *rex*⁻ lysogen was selectively neutral or nearly neutral. Since the mutation in question was obtained as a mutant lysogen sensitive to phage T4 *r*II and not characterized further, it could have been mutated in *rexA* or *rexB* (87). Indeed, the mutant lysogen could have involved phage genes in addition to *rex* or might even have been a bacterial mutation overriding the T4 *r*II exclusion function of *rex*⁺ and therefore not *rex*⁻ at all. The situation has been clarified somewhat by Dykhuizen et al. (35). In addition to confirming the selective effect of λ lysogens, they observed an initial slight decrease in the frequency of the lysogen before the favorable selection commenced. However, studies with *rex5a*, which is in the *rexB* cistron (87), revealed the same favorable selection as occurs with wild-type λ. The *rexB* function does not seem to be involved in the selective effect, although there is a curious and unexplained interaction with *malT* such that *malT* λ *rex5a* lysogens are favored in chemostats whereas *malT* λ *rex*⁺ lysogens are disfavored. On the other hand, the *rex am*301 (= *rexQ*) mutation was associated with a much reduced amount of selection. The *rex am*301 mutation is in the *rexA* cistron near the carboxyl end of the coding sequence (87), and the small amount of selection observed could perhaps be attributed to residual activity of this truncated polypeptide. In any event, if *rex* is involved in the λ lysogen growth rate advantage, it would seem to be the *rexA* function. The effect of λ, whatever its cause, seems to be quite general among lysogens but, unfortunately, its detailed mechanism is unknown.

The same features that make chemostats suitable for studies of the evolution of lysogeny make them suitable for studying the evolution of transposable elements. A novel in vivo effect of the transposable element Tn*5* (kanamycin/neomycin resistance) has been described by Biel and Hartl (9, 10). Strains carrying Tn*5* are favored over their otherwise isogenic counterparts in chemostats limited for glucose, lactose, glycerol, or proline at various densities and dilution rates. The effect is independent of frequency and is substantial, ranging from 2 to 8% per h ($D = 0.35$) in various experiments, but it is not observed in all *E. coli* strains. Derivatives of CSH12 consistently fail to exhibit a detectable effect of Tn*5* in chemostats. In other strains such an effect is observed, and it is independent of site of insertion, having been observed with random insertions, with *lac*::Tn*5*, *ilvD*::Tn*5*, and even with an F′*lacP*::Tn*5*. The selection does not involve actual transposition of the element, as strains selected in chemostats retain Tn*5* in its original position without change in copy number. However, whereas the selection does not involve actual transposition, it may involve a transposase-related function because the selection is not observed with Tn*5*-112, a transpositionless deletion mutation missing the transposase-coding region of the right-hand insertion sequence flanking the element. A similar growth rate advantage, less thoroughly studied, seems to occur with Tn*10* (10). This Tn*10* effect has

been confirmed and studied further by Chao et al. (L. Chao, C. Vargas, B. B. Spear, and E. C. Cox, submitted for publication), who provide evidence that the effect might be due to new favorable mutations induced by transposition of one or both flanking insertion sequences without transposition of the intact element. Altogether, these observations demonstrate a subtle physiological effect of transposons that may be significant in the fluctuating environmental conditions that bacteria experience in nature, and they suggest a model for the proliferation and maintenance of insertion sequences and transposable elements in the absence of other identifiable selection pressures.

The selective advantage associated with Tn*5* appears to be a temporary physiological effect occurring immediately upon carbon or energy limitation or both. This is indicated in part by the course of selection. The Tn*5*-bearing strain never becomes completely fixed. Selection may increase its frequency from 50 to 95 or 99%, but the competing strains are maintained at approximately constant frequencies thereafter. This constant frequency is maintained even after the culture has been transferred to a new chemostat, eliminating the possibility that this constancy is due to wall growth (Dykhuizen, unpublished data). This effect can also be observed when the Tn*5*-bearing strain and its isogenic counterpart are maintained in monoculture chemostats before they are mixed in the experimental chemostat. With such preadaptation the selective advantage of Tn*5* declines and by 72 h of preadaptation has disappeared almost completely (10). This effect of preadaptation is the reverse of that reported for λ lysogens. When a λ lysogen and its isogenic nonlysogen are preadapted to limiting glucose, selection in a mixed chemostat in favor of the lysogen is even greater than that observed otherwise (42).

NATURALLY OCCURRING GENETIC VARIANTS

A very recent use of chemostats is in the study of functional effects of genetic variants that are found in natural populations. Natural populations of *E. coli* and probably many other microorganisms contain an astonishing amount of genetic variation. Extensive genetic variation associated with electrophoretically distinct enzyme variants (allozymes) among natural isolates of *E. coli* has been described by Milkman (92, 93) and Selander and Levin (111). The use of chemostats has permitted a study of the functional effects of these variants. Two extreme views regarding the functional effects of allozyme-associated alleles have come to be known as the selectionist hypothesis and the neutralist hypothesis. The selectionist view holds that such genetic variants are adaptively significant and that they are maintained in natural populations by virtue of their functional effects on the organism. The neutralist view holds that such alleles have so little functional effect that they are not acted upon directly by natural selection, their frequencies being determined largely by a balance between mutation and random genetic drift. In spite of their large actual population size, bacterial populations can be susceptible to random drift because of frequent extinction and recolonization of local populations (83). Of course, both extreme views of genetic variation are to some extent caricatures, and many intermediate positions are possible.

In one series of experiments, Dykhuizen and Hartl (38, 60) studied the functional effects of six alleles of *gnd* (map position, 44 min) coding for electrophoretic variants of 6-phosphogluconate dehydrogenase that had been found in wild isolates of *E. coli*. By a series of P1-mediated transductions, these alleles were transferred into an isogenic genetic background of *E. coli* K-12. Appropriately marked strains were then placed in pairwise competition in chemostats limited for gluconate, glucose, or a mixture of ribose and succinate. With gluconate limitation, a *gnd* missense mutation is selected against at the rate $s = 0.09$ ($D = 0.35$) per h; this experiment serves as a positive control. This same missense-bearing strain shows no selective effect in chemostats limited for a mixture of ribose and succinate; this medium establishes a negative control. Computer simulations of the sampling procedure indicated that a rate of selection as small as $s = 0.005$ per h could be detected in the procedures.

The situation regarding selection of *gnd* alleles proved to be unexpectedly complex. Four of the alleles had no detectable selective effect (i.e., $s < 0.005\ h^{-1}$) in gluconate-limited chemostats; these are evidently neutral or nearly neutral under these conditions. One allele (S1) was found to be detrimental ($s = 0.03\ h^{-1}$) in gluconate-limited chemostats but was neutral in glucose-limited chemostats. The remaining allele (S8) had an interaction with the *tonA* ($T5^r$) neutral marker such that the *tonA* gnd^+(S8) combination was selected against ($s = 0.02\ h^{-1}$). These results imply a great deal of functional diversity among allozyme-associated alleles. Many are neutral or nearly neutral under normal conditions but could have a potential for selection that could be significant under the appropriate conditions of environment or genetic background. Indeed, two alleles that otherwise seemed neutral could be shown to be selectively important in a genetic background containing an *edd* mutation that cuts off the alternative metabolic route of gluconate metabolism, and the

selection could be related to the K_m of the allozymes (38).

Similar experiments with naturally occurring alleles of *pgi* (map position, 91 min), which codes for phosphoglucose isomerase, have also been carried out (34; D. E. Dykhuizen and D. L. Hartl, submitted for publication). Five allozyme-associated alleles were studied in this case. In chemostats limited for glucose, selective differences, if they occur at all, are below the limit of resolution of the technique. Four of these alleles are also neutral or nearly neutral in chemostats limited for fructose, which is also a substrate for phosphoglucose isomerase. One of the alleles does exhibit a small but consistent selective effect ($s = 0.002\ h^{-1}$; $D = 0.35$) in fructose-limited chemostats, illustrating again a potential for selection among naturally occurring allozymes that becomes important under the appropriate conditions.

ENGINEERED MUTATIONS

Recombinant DNA techniques have made possible the in vitro creation of mutations with defined nucleotide sequence by means of site-directed mutagenesis. For the study of gene structure-function relationships, particularly as regards growth rate, the methods of site-directed mutagenesis represent a quantum advance over more traditional techniques because the site-directed mutations can be produced and studied independently of any a priori assumptions concerning their effects on phenotype. Traditional methods of mutagenesis require detectable phenotypic effects for identification of the new mutants. These methods yield a biased sample of all possible mutations that can occur because those having minimal phenotypic effects go undetected. This is the underlying reason why such methods typically yield mutations having large effects on, for example, enzyme activity. Studies of naturally occurring genetic variants also involve a biased sample of mutations, but here the bias is in the opposite direction. Naturally occurring genetic variants will include only those that have survived the screen of natural selection. Near-neutral mutations will be among this group, as will those mutations that are selectively favored in particular environments or genetic backgrounds. Although such studies are important for an understanding of microbial population structure and evolution, they do not involve a random sample of mutations.

Site-directed mutagenesis can in principle be used to generate a random set of mutations independent of their phenotypic effects. Several methods are currently available for specific in vitro mutagenesis and have already provided important information on which parts of genes are essential for function. On the other hand, the functional assays currently in use are admittedly rather crude, typically involving the ability of appropriately transformed cells to give rise to colonies on a plate or on their maximum growth rate. Chemostats would provide a useful tool for finer-scale functional assays and could be used to focus on the "fine tuning" of gene function or regulation. Chemostats are rapid, convenient, and reproducible and have a fine limit of resolution of differences in growth rate. Many mutations that have growth rate effects that are easily detectable in chemostats have no discernible effects on such gross characteristics as maximal specific growth rate. When more than first-order information about the efficacy of gene function is desired, some continuous-culture technique such as chemostats would seem to be the method of choice.

CONCLUSIONS

Studies of selection in chemostats have revealed a number of important phenomena, and it may be well to summarize them here. First, the selective events referred to as periodic selection are not as haphazard as commonly assumed. At least in some cases (e.g., *lac* constitutives), the mutants selected in replicate chemostats are highly predictable and have a defined genetic and physiological basis. This opens the prospect of controlling or perhaps eliminating undesirable genetic changes that commercially important strains sometimes undergo in prolonged continuous culture. Second, the widely invoked appeal to energy economy does not account for all cases of selection in chemostats. Even the prototype case of selection in favor of amino acid auxotrophs is not proven critically and warrants further investigation. Third, the selection observed in favor of lysogens or transposon-bearing strains is an important phenomenon in search of a molecular mechanism, as it undoubtedly plays a role in the natural history of these elements. Fourth, the behavior of cloning vectors in chemostats is a subject of great interest because of the potential uses of continuous culture for the study of the functional effects of defined, engineered mutations and for potential applications in industrial microbiology.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant GM30201 from the National Institutes of Health.

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