CHAPTER
9

Quantifying Fitness and Gene Stability in Microorganisms

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Fitness represents the combined effects of all other phenotypic properties on the capacity for survival and reproduction by a particular genotype in a particular environment. Many genetically engineered microorganisms will be more fit than their wild-type counterparts under environmental conditions corresponding to their intended biotechnological application. For example, microorganisms engineered to degrade some environmental toxin may increase in frequency after their introduction into the contaminated environment if they can use that toxin as a growth substrate. The efficacy of the intended application may often be enhanced by the increased frequency of the engineered genotype.

On the other hand, genetically engineered microorganisms may often be *less* fit than their wild-type counterparts under other environmental conditions, owing to the "excess baggage" associated with carriage and expression of the recombinant genes (Regal 1986; Lenski and Nguyen 1988). If so, then microorganisms engineered for biodegradative functions, for example, will decline in frequency, and may eventually be lost (but see Chapter

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10) after they have detoxified a site or if they are transported to an uncontaminated site.

This "excess baggage" hypothesis has been frequently invoked as an argument for the safety of deliberate release of genetically engineered microorganisms, because it implies that unintended spread and any consequent adverse effects are unlikely (Brill 1985; Davis 1987). It is my opinion that this hypothesis will hold true in many cases, perhaps most, but that there may also be some exceptions (e.g., Bouma and Lenski 1988). Alternative points of view on this subject can be found elsewhere (Brill 1985; Colwell et al. 1985; Sharples 1987; Davis 1987; Lenski and Nguyen 1988; Regal 1988), and further discussion of this hypothesis is not the purpose of this chapter.

However, reports sponsored by the Ecological Society of America (Tiedje et al. 1989) and by the U.S. National Research Council (Committee on Scientific Evaluation of the Introduction of Genetically Modified Microorganisms and Plants into the Environment 1989) have emphasized the importance of confining a modified organism after its introduction into the environment in order to minimize the duration and scale of any potentially adverse ecological effects. Both reports also provide frameworks for assessing ecological risk or uncertainty, wherein the fitness of a genetically modified organism relative to its unmodified counterpart in the appropriate environment is an important criterion for evaluating confinement. In this chapter, I will present methods that can be employed to measure selection (i.e., a difference in fitness) that arises from the carriage and expression of engineered genes by microorganisms.

Selection is also important because its effects may be confounded with losses of an engineered gene due to the infidelity of replication or transmission of the recombinant DNA. Imagine, for example, that the level of expression of some engineered function diminishes with time in a population of recombinant microorganisms, thereby hindering the efficacy of the intended biotechnological application. If one assumes that this instability is due to genetic infidelity, and not to selection, then it would be reasonable to increase the number of copies of the recombinant gene in each individual. If, however, the primary cause of instability is selection against expression of the intended function, then an increase in copy number (and a concomitant increase in the level of expression) may actually aggravate the instability of the recombinant gene at the level of the population. In this chapter, I will also present methods that can be used to distinguish the effects of selection from the effects of genetic infidelity.

9.1 GENERAL PRINCIPLES

I will use the term segregation to refer to any losses of an engineered gene that are due to the infidelity of replication or transmission, including mutation. I will assume that these losses are irreversible, although models incorporating reversible events (e.g., back-mutation) can be readily developed. I will use the term selection to refer to changes in the relative abundance of two clones that result from genotypic properties that cause them to differ in their capacity for survival or reproduction.

I use the terms wild-type, parental, and segregant to refer to clones that lack the recombinant gene or genes. I assume that wild-type and engineered clones are isogenic except with respect to the recombinant gene or genes of direct interest. I will discuss exceptions to this assumption, both deliberate and unintentional, in Sections 9.3.1.3 and 9.4.1.

Dykhuizen and Hartl (1983) and Kubitschek (1970) provide excellent discussions of selection and mutation in microbial populations, which the reader is encouraged to consult.

9.1.1 Effect of Selection

Let p be the frequency of an engineered clone, and let $q = 1 - p$ be the frequency of the parental clone. The change in the frequency of the engineered clone is governed by the following differential equation (Kimura and Ohta 1971):

$$dp / dt = -s\,p\,q = -s\,p\,(1 - p) \tag{1}$$

where s is the selection coefficient, or the difference in fitness between the two clones, which we are assuming to be constant. If $s>0$, then selection favors the parental clone; if $s<0$, then selection favors the engineered clone. It is possible to integrate equation 1 and thereby obtain an expression for the frequency of the engineered clone:

$$p_t = \frac{p_0}{(1 - p_0)\,e^{st} + p_0} \tag{2}$$

where p_0 is the frequency of the engineered clone at time zero, and p_t is the frequency of that clone at some later time t. Equation 2 can be linearized, but one must first convert the *frequencies* of the two clones, p and q, to the *ratio* of one clone to the other: $R = p / q = p / (1 - p)$. With the relative abundance of the clones thus expressed, a natural logarithmic transformation yields (Dykhuizen and Hartl 1983):

$$\ln R_t = \ln R_0 - s\,t \tag{3}$$

where R_0 is the ratio of the engineered clone to the parental clone at time zero and R_t is that ratio at some later time t. In Section 9.3.1, I will discuss the use of linear regression to estimate the selection coefficient from empirical data.

9.1.2 Effect of Segregation

Segregation is typically an exponential decay process, whereby individuals with the engineered genotype are converted to segregants at a constant rate. The rate of change in the frequency of the engineered genotype is therefore

described by the following differential equation:

$$dp / dt = - u p \tag{4}$$

where p is the frequency of the engineered genotype and u is the segregation rate. Integrating equation 4 yields:

$$p_t = \frac{p_0}{e^{ut}} \tag{5}$$

where p_0 is the frequency of the engineered genotype at time zero, and p_t is that frequency at some later time t. Equation 5 can be linearized by a natural logarithmic transformation of the frequencies of the engineered genotype:

$$\ln p_t = \ln p_0 - u t \tag{6}$$

In contrast, recall that under the model of selection, genotypic frequencies must first be converted to ratios before a logarithmic transformation linearizes the dynamics.

In Figure 9–1, the dynamics of an engineered genotype subject to *segregation* of the recombinant gene (dashed line) are contrasted with the dynamics of an engineered genotype subject to *selection* against carriage of the recombinant gene (solid line). With segregation, the rate of decline in the frequency of the engineered genotype is greatest when that frequency is near one, and it declines continuously as the frequency approaches zero. In contrast, the rate of change under selection is greatest when the frequency of

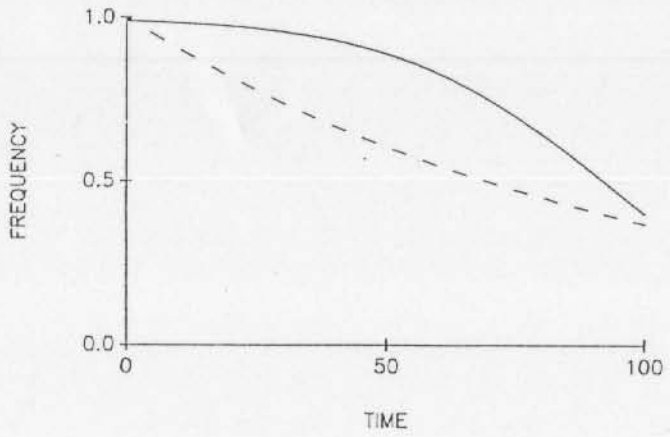

FIGURE 9–1 The dynamics of loss of the engineered genotype as a function of time. Solid line: model of loss of engineered genotype by selection (equation 1). Selection coefficient s is 0.05 per unit time; initial frequency p_0 is 0.99. Dashed line: exponential decay model of loss of engineered genotype by segregation (equation 4). Segregation rate u is 0.01 per unit time; initial frequency p_0 is 1.

the engineered clone is near 0.5, and is lowest when that frequency is near one (or near zero).

9.1.3 Combined Effects of Segregation and Selection

The joint effects of segregation and selection are described by the following differential equation:

$$dp / dt = - s p q - u p = - s p (1 - p) - u p \tag{7}$$

where p is the frequency of the engineered genotype, u is the rate of segregation, and s is the selection coefficient. I will now consider the case in which segregants are more fit (i.e., $s>0$), so that both segregation and selection drive the frequency of the engineered genotype downward.

It is possible to integrate equation 7 and thereby obtain an expression for the frequency of the engineered genotype remaining at time t (Charles et al. 1985; Lenski and Bouma 1987):

$$p_t = \frac{p_0 (u + s)}{[u + s (1 - p_0)] e^{(u+s)t} + s p_0} \tag{8}$$

There is no transformation that can linearize equation 8 over the entire parameter space and range of frequencies (Cooper et al. 1987). However, one can use nonlinear regression to estimate simultaneously the segregation rate and the selection coefficient (Lenski and Bouma 1987), as I will show in Section 9.3.2.

9.2 METHODOLOGICAL ISSUES

9.2.1 Selection

Koch (1981) clearly presents various methods for measuring the growth rate of a microbial population in pure culture. In principle, one could determine the difference in fitness of two genotypes simply by measuring the growth rates of each in isolation, and then calculating the difference. In practice, however, it is often difficult or even impossible to estimate fitness differences in this way. For example, imagine two clones, each grown separately in a chemostat. Provided that each clone is capable of growing at a rate sufficient to offset washout, then each clone will become established. At equilibrium, each clone has a growth rate equal to the rate of flow through the culture vessel. Therefore, if both clones can become established, then they must have the same equilibrium growth rates when grown in isolation. Yet one clone may be more efficient than the other at exploiting some limiting resource, or one clone may produce a substance that inhibits the growth of the other. By reducing the level of resources, by the production of growth inhibitors, and so on, populations modify their environment. Thus, to compare the growth rates of two populations grown in isolation is to ignore the

effects that each population would have on the other, as mediated by their effects on the environment.

There are other limitations to traditional measurements of growth rate obtained for pure populations. Most measurements of growth rate are made on exponentially growing populations. Starting from even a single cell, there are only a relatively few generations of exponential growth before nutrient limitation or metabolic by-products limit growth, and still fewer generations during which one can accurately quantify a population's density from measurements of light scattering using a spectrophotometer. If the difference in fitness between two clones is small, then it is unlikely that a comparison of exponential growth rates will reveal any significant difference. But small differences in fitness, when compounded over hundreds and thousands of generations, may be quite important. Also, exponential growth rates are typically measured only under conditions of superabundant resources, a situation that is ephemeral in nature.

Differences in fitness inferred from measurements of growth rates for pure populations may require a high degree of replication, especially if sampling error or variation among replicates is large. Direct measurements of selection, on the other hand, invariably have an internal control; whether a particular sample density is high or low, or whether a particular replicate is nutrient rich or poor, the same is true for both clones. This internal control makes direct measurements of differences in fitness more accurate than those calculated indirectly from separate measurements of growth rate for two populations, each of which inevitably contains its own independent error of estimation.

Dykhuizen and Hartl (1983) provide an excellent review of studies in which measurements of selection have played a central role; their review focuses on chemostat studies that were undertaken to test a variety of evolutionary hypotheses. Many of the studies reviewed by Dykhuizen and Hartl relate directly to the issue of whether or not there is measurable selection associated with expression of unused functions or associated with carriage of accessory genetic elements, including plasmids, prophages, and transposons (see also Cooper et al. 1987; Bouma and Lenski 1988; Lenski and Nguyen 1988; Nguyen et al. 1989).

It should be emphasized that measurements of selection can be made not only in highly simplified laboratory systems, such as chemostats, but also in more complex natural or semi-natural systems, such as microcosms (Pritchard and Bourquin 1984); all that is required is the ability to monitor the relative abundance of two clones sharing a common environment. Whether the total number of individuals in a population is increasing, constant, or decreasing, the relative frequencies of genotypes may be changing systematically as the result of differences in their fitness.

9.2.2 Gene Instability

The instability of a gene is often described in terms of the frequency of segregants in a population after a specified period of time. Implicit in this simple description is the assumption that the dynamics of gene loss are

governed solely by an exponential decay process, so that the segregation rate can be calculated from equation 6. However, the dynamics of instability are altogether different if selection is acting in concert with segregation, as shown in Section 9.1.3. In fact, any number of combinations of segregation rate and selection coefficient can be chosen to pass through a particular pair of initial and final frequencies. Thus, it is essential to have a series of frequencies and to analyze the dynamics of instability using mathematical models that allow one to determine the effects of both segregation and selection.

Several studies have attempted to distinguish between the effects of segregation (including mutation) and selection on the dynamics of loss of some genotype from a population (Nordstrom et al. 1980; Charles et al. 1985; Boe et al. 1987; see also additional citations in Cooper et al. 1987; Lenski and Bouma 1987). In all of these studies, the frequency of individuals possessing the gene of interest was determined at several points in time, thereby permitting inferences regarding the importance of both segregation and selection.

While the authors of the papers cited above clearly recognized the significance of selection as well as segregation for the dynamics of instability, they did not present explicit procedures for estimating both the segregation rate and the selection coefficient from empirical data. Two recent papers (Cooper et al. 1987; Lenski and Bouma 1987) have filled this void, and although the details of their procedures differ, both permit an investigator to estimate simultaneously the segregation rate and the selection coefficient from a series of genotypic frequencies. Both procedures were first applied to experimental studies of bacterial plasmids, but they are equally applicable to any recombinant gene, whether chromosomal or extrachromosomal.

Cooper et al. (1987) identify alternative transformations that linearize genotype frequencies, at least over a portion of their range; the success of a particular transformation depends upon the relative magnitudes of the selection coefficient and the segregation rate. Standard linear regression techniques can be used to estimate the relevant parameters, but some of the experimental data invariably must be excluded from the analysis because frequencies fall outside the range over which a particular transformation successfully linearizes the dynamics.

Lenski and Bouma (1987) use nonlinear regression to estimate the segregation rate and selection coefficient; while nonlinear regression procedures are less familiar, they are widely available in statistical packages. One advantage of this approach is that it does not require identification of an appropriate transformation to linearize the dynamics, but instead utilizes a single mathematical expression to describe the dynamics expected for all parameter values and over the entire range of genotype frequencies. Another advantage of this approach is that it uses all of the experimental data. For these reasons, and because of my greater familiarity with my own work, I will present the approach used by Lenski and Bouma in Section 9.3.2.

9.3 SPECIFIC METHODS FOR ESTIMATING PARAMETERS

9.3.1 Measuring Selection

The basic logic of an experiment designed to estimate the difference in fitness between two clones is very simple. Two clones are mixed together in some initial ratio (usually about 1:1) in any environment of interest. At subsequent points in time, additional samples are obtained and the ratio of the two clones in each sample is determined. If the ratios shift upward or downward in a systematic fashion, then one can infer that one clone is more fit than the other in that particular environment; if the ratios remain essentially unchanged with time, then one must conclude that the two clones are equally fit, at least within the limits of one's statistical resolution.

The natural logarithm of the ratio of two clones is expected to exhibit a linear relationship with time (see equation 3). Therefore, the selection coefficient, s, can be estimated by the slope of the regression of the natural logarithms of these ratios against time and has units of inverse time. These units may be universal units, like hours, or they may require additional knowledge of the particular experimental system, like generations.

Data from one experiment are presented in Table 9–1. In that experiment, a clone of *Escherichia coli* resistant to the bacteriophage T4 was grown in mixed culture with its T4-sensitive progenitor (see Lenski and Levin 1985, for further details). A steady decline in the ratio of resistant to sensitive cells was observed, and a linear regression of the natural logarithm of the sample ratios against time yields a selection coefficient (here, equal to the negative value of the slope) of 0.15 per hr. The correlation coefficient gives a measure of the fit of the data to the regression line and is equal to 0.99 for this data set, which is significant at $p<0.01$ (Rohlf and Sokal 1981). Hence, one can conclude that there was selection against the T4-resistant clone.

In the sections that follow, I discuss the conditioning of clones prior to measurements of selection; several methods for enumerating clones, including the use of neutral genetic markers; and alternative approaches to making statistical inferences about estimates of selection coefficients.

TABLE 9–1 Effect of Selection on the Ratio of T4-Resistant to T4-Sensitive Genotypes of *Escherichia coli*

Time of Sample (hr)	R[1]	*ln* R[1]
0	1.6082	0.475
21.0	0.2972	−1.213
45.5	0.0040	−5.521
68.5	0.0001	−9.210

[1] R, ratio of resistant to sensitive cells; ln R, natural logarithm of R.
Source: Lenski and Levin 1985.

9.3.1.1 Preconditioning. One important consideration for measuring the difference in fitness between two clones is the conditioning that both clones receive prior to their introduction into a common environment. In general, it is desirable to meet the following two conditions: (1) each clone should be preconditioned in the same environment as the other; and (2) the environment used for preconditioning should be the same as the environment in which the selection coefficient is to be estimated.

If (1) is not met, then subsequent changes in the ratio of two clones may reflect their physiological states rather than their genetic composition. For example, imagine that clone A possesses a recombinant gene that enables it to utilize some novel substrate for growth, while clone B is the parental wild-type. Let us say that A has been preconditioned on the novel substrate, while B has been preconditioned on the standard medium in which the two clones will compete. When the two clones are mixed together in the standard medium, it is observed that the ratio of A to B declines significantly. Does this indicate that carriage of the recombinant gene reduces fitness in the standard medium? It may, but it may indicate instead that there is a physiological effect of switching from one medium to another that has inhibited the growth of clone A for a few generations.

If (1) is met, but (2) is not, then significant changes in the ratio of two clones do reflect genotypic differences, as their physiological states should be identical. However, failure to meet (2) may alter one's interpretation of the environment in which selection was measured. For example, imagine that clones A and B have both been engineered to be resistant to some toxin; A expresses the resistance function constitutively, while B requires induction of the resistance function by the toxin. Both clones are preconditioned in the same toxin-free medium, so (1) is met. The selection coefficient is then estimated in medium supplemented with the toxin, so (2) is not met. It is observed that the ratio of A to B increases significantly. Does this indicate that A is more fit than B in medium supplemented with toxin? Once again, the interpretation is ambiguous. The data might indicate instead that A is able to respond more rapidly to the toxic challenge by virtue of its constitutive expression of the resistance function; had the two clones been preconditioned in the presence of toxin, thereby inducing the resistance function in B, there might have been no significant selection. Thus, when (2) is violated, one cannot infer whether a difference in fitness was characteristic of the experimental medium or whether the difference resulted from the *transition* between the preconditioning and experimental media.

In some instances, it may be difficult or impossible to ensure that (1) and (2) are met. If one clone has a highly unstable genotype, then it may be necessary to maintain that clone in a selective medium during the pre-

conditioning step. If selection is to be measured in a natural or semi-natural environment, then it may be necessary to precondition clones in the laboratory. However, it is important to be aware of (1) and (2), and to interpret one's results with appropriate caution when either of these conditions has been violated. In general, violations of (1) or (2) are less likely to affect one's interpretation when the selection coefficient is estimated from a long-running experiment, because the alternative interpretations depend upon transitions between environments. However, the reader is cautioned that there is also a potentially serious complication that arises when these experiments are carried out too long. Longer experiments increase the likelihood that additional genetic changes will arise in one or both clones, confounding interpretation of the genetic basis of any observed difference in fitness (see Section 9.4.1).

There is one other important consideration regarding the treatment of clones prior to measurements of selection. It is essential that clones be stored properly, and that experiments be conducted with clones taken directly from this storage. If this is not done, then once again additional genetic changes may occur that confound interpretation of the genetic basis of differences in fitness (see Section 9.4.1). For example, imagine that one measures the difference in fitness between two clones, one carrying some recombinant gene and the other the parental clone lacking that gene. The parental clone has been stored in a low temperature freezer ever since its acquisition. After transformation with the recombinant DNA yielded an engineered clone of possible interest, a number of that clone's properties were determined, which required that it be propagated for several weeks in the standard laboratory medium. Only after these properties were determined was the engineered clone properly stored. Let us imagine that a subsequent experiment indicated that the engineered clone was significantly more fit than the parental clone when measured in the standard laboratory medium. Does this result mean that the engineered gene enhances fitness in the standard laboratory medium? It may, but it is also likely that the additional exposure of the engineered clone to the standard medium led to one or more genetic substitutions, unrelated to the engineered function, that rendered that clone better adapted to the medium. Evolution by natural selection does not stop when microorganisms are brought into the laboratory, and precautions must be taken to ensure that it does not confound interpretation of fitness differences.

In summary, the following steps are recommended prior to measuring the difference in fitness between two clones.

1. Clones should be properly stored (e.g., in a low temperature freezer) as soon as possible after their initial isolation. If a clone is subsequently determined to have inappropriate properties, then it can be discarded at that time.
2. Clones should be removed from storage and their relevant properties confirmed as directly as possible, preferably by a single round of selective plating.

3. The two clones should normally be preconditioned in the same medium as one another, and that medium should normally be the same as that in which the ratio of the clones will be monitored.

9.3.1.2 Enumeration. Clones cannot usually be distinguished by optical methods, such as microscopy or spectrophotometry, but their colonies can often be distinguished by some means on agar plates. (In the next section, I discuss the use of genetic markers to facilitate distinguishing colonies belonging to different clones.) There are three distinct plating procedures for determining the relative abundance of two clones during the measurement of selection, which I call *direct*, *replicative*, and *selective*. For all procedures, samples taken from the experimental environment should be processed immediately or stored in such a way that no further change occurs in the abundance of either clone.

For selective plating, a sample from the experimental environment, after appropriate dilutions, is plated on both selective and nonselective medium. The nonselective medium gives an estimate of the total density of both clones ($A + B$), while the selective medium gives the density of one of the clones (A). One calculates the ratio of A to B from the ratio of selective to (nonselective minus selective) plate counts. With selective plating, one must calibrate the plating efficiency of the selectable clone A on both selective and nonselective medium, and adjust the estimated ratios accordingly. Selective plating is quite useful when A is a small fraction of the total, but it is essentially useless when A is numerically dominant.

With both direct and replicative plating, a sample from the experimental environment, after appropriate dilution, is plated only on nonselective medium. In the case of direct plating, colonies are distinguished by their visual appearance, as for instance when a genetic marker causes differences in colony color (see Section 9.3.1.3). In the case of replicative plating, the relative abundance of the two clones is determined by testing all (or some randomly chosen subset) of the colonies isolated on the nonselective medium for some distinguishing genetic characteristic, usually by transferring the colonies to selective plates with toothpicks (colony by colony) or with velveteen (whole plate imprints). If only direct or replicative platings are used, then there is no need to determine relative plating efficiencies, as these efficiencies do not affect the estimate of a selection coefficient. This is so because a difference in plating efficiency between two clones will affect the intercept of the linear regression of the logarithmic ratios, but not the slope of that regression. Both direct and replicative platings are far preferable to selective plating when the selectable clone A is near fixation; both are also quite effective when the frequencies of the two clones are of a similar magnitude. However, if one clone is much rarer than the other, then selective plating is usually preferable.

9.3.1.3 Neutral Markers. I use the term marker to refer to a gene that facilitates identification of a particular clone, but is not itself of special interest. This marker should have little or no effect on fitness in the environment of interest; i.e., it should be effectively neutral. If the marker is not neutral, then it may obscure or confound interpretation of fitness effects associated with the genes of primary interest. Clearly, the putative neutrality of a marker must be established by a control experiment.

For example, imagine that one is interested in determining whether or not there is a difference in fitness between an engineered clone *A* and its parental clone *B*. If the two clones can be distinguished by selective or replicative plating, but not by direct plating, then one might use a marker to create an additional clone that permits direct plating, perhaps by causing differences in colony color on a certain nonselective medium. Alternatively, if clones *A* and *B* cannot be distinguished by replicative or selective plating, then any additional marker (e.g., antibiotic resistance) that permitted such platings would be quite useful. In either case, one would produce a third clone *C* that carried the marker but not the engineered gene. One then could estimate the selection coefficient acting on clone *B* relative to clone *C*, which indicates any effect of the marker, and the selection coefficient of clone *A* relative to clone *C*, which indicates the combined effects of the marker and the engineered gene. If the selection coefficient for *B* relative to *C* is not significantly different from zero, then one may conclude that the marker is neutral, within the limits of one's statistical resolution. If the selection coefficient of *A* relative to *C* is significantly different from the selection coefficient of *B* relative to *C*, then one must conclude that the engineered gene has a significant effect on fitness.

When using neutral markers, it may be necessary to determine that the marker and the engineered gene have retained their presumed linkage during the course of an experiment. If the engineered gene is on a plasmid, for example, it may segregate, thereby altering the linkage of the marker and the engineered gene. In such cases, it is important to confirm the presumed linkage by appropriate replicative or selective platings. If dissociation has occurred, then it may be necessary to use the methods presented in Section 9.3.2 to estimate both the segregation rate and the selection coefficient.

In *Escherichia coli*, genotypes with and without the ability to use certain sugars (including arabinose, lactose, and maltose) can be readily distinguished by their colony color on broth plates supplemented with the sugar and 2,3,5-triphenyltetrazolium chloride (Levin et al. 1977; Carlton and Brown 1981; Nguyen et al. 1989). In several studies, these markers have been shown to be neutral (or nearly so) in medium in which some other nutrient is limiting. Other types of markers include resistances to viruses and antibiotics, but many genes conferring resistance have large effects on fitness under "nonselective" conditions. In *E. coli*, resistance to virulent phage T5 is often neutral (or nearly so), whereas resistance to virulent phage T4 engenders a substantial reduction in fitness (Lenski and Levin 1985; see also Table 9–1). The neutrality of a particular marker in a particular clone in a particular environment is an empirical question that can be addressed only by an appropriate control.

9.3.1.4 Statistical Inference. The purpose of statistical inference is to formalize the degree of confidence (i.e., the significance level) in some conclusion. I wish to review briefly one important issue that relates to the statistical interpretation of selection coefficients. There are two distinct ways of assigning significance to an estimated selection coefficient. According to one, a single measurement of the selection coefficient is calculated from the slope of the linear regression of the natural logarithm of the ratio of two clones against time. One then asks: Is that slope significantly different from zero? The procedures for determining the answer to this question were briefly illustrated for the data in Table 9–1; further details can be found in almost any statistics book (e.g., Sokal and Rohlf, 1981) and need not concern us. Basically, the more sample points one has and the closer these points lie to the fitted line, the more confidence one has in the slope of that line. (Many statistics books also describe the calculations necessary to ask whether two slopes are significantly different from one another.) The resulting significance level is based upon the assumption that each point is a truly independent observation, which seems quite reasonable at first glance. But what if some chance event impinges upon our single measurement, and that event affects the relative fitness of the two clones? Imagine, for example, that a single colony used to found one of the two clones just happened to have contained a deleterious mutation; one would wrongly ascribe that clone's reduced fitness to its intended genotype.

An alternative approach is to obtain many estimates of the selection coefficient, each based upon an independent experimental replicate. A slope would be calculated for each experiment as before, but no significance level would be attached to any single slope. Instead, one would use a *t*-test to ask whether the *mean* of many slopes was significantly different from zero (e.g., Nguyen et al. 1989). (One could also use a *t*-test to ask whether two means differed significantly from each other.) This second approach is usually preferable, because statistical inferences based on many independent replicates are more robust than those based on a single experiment; chance events are not inadvertently ascribed a stronger association with the intended comparison than is appropriate. Hurlbert (1984) discusses the importance of proper replication in ecological experiments. Koch (1981) also illustrates the importance of independently replicated measurements in making statistical inferences.

9.3.2 Analyzing Instability

It is simple to estimate both the segregation rate and the selection coefficient, at least in principle. A population is founded in which the frequency of the recombinant gene is near one. The population is propagated and sampled

at intervals, and the frequency of individuals possessing the recombinant gene is enumerated. From these dynamics, nonlinear regression can be used to estimate simultaneously the segregation rate and the selection coefficient, as illustrated in the next section. In practice, it may sometimes be difficult to distinguish between the effects of segregation alone and the combined effects of segregation and selection. In such cases, it is desirable to run a parallel experiment in which the population is deliberately "seeded" with segregants lacking the recombinant gene, as discussed in Section 9.3.2.2.

9.3.2.1 Nonlinear Regression. The precise mathematical methods used in nonlinear regression analysis need not concern us, as programs are widely available in statistical packages to perform the actual calculations (e.g., Dixon 1985). These programs can be used to find the intercept (p_0), segregation rate (u), and selection coefficient (s) in equation 8 that minimize the sum of the squared deviations about the fitted model for a set of frequencies of the engineered genotype obtained at different points in time (p_t's).

Table 9–2 presents a set of data corresponding to the dynamics of the loss of plasmid pACYC184 from *Escherichia coli* (Lenski and Bouma 1987); it includes the time at which the sample was taken, the number of individuals scored, and the frequency of the plasmid-bearing genotype. For each sample frequency, the approximate 95% confidence interval has been calculated from the binomial distribution, which is available in many statistical tables (e.g., Rohlf and Sokal, 1981). These confidence intervals are neither always symmetric about the sample frequency, nor are they always of equal breadth, despite the equal sampling effort at all points in time; this is characteristic of the binomial distribution.

The data from Table 9–2 are plotted in Figure 9–2, along with two fitted models. The solid line represents the fitted model when the intercept, the

TABLE 9–2 Dynamics of Instability of Plasmid pACYC184 in *Escherichia coli*

Time of Sample (hr)	*Number Scored*	*Frequency*[1]	*C.I.*[2]
0	300	0.963	0.94–0.98
4.5	300	0.810	0.76–0.85
10.5	300	0.560	0.50–0.62
16.5	300	0.133	0.10–0.18
22.5	300	0.020	0.01–0.04
28.5	300	0.000	0.00–0.01

[1] Frequency, p, of the plasmid-bearing genotype.

[2] C.I., the approximate 95% confidence interval for the sample frequency (from Rohlf and Sokal 1981).

Source: Lenski and Bouma 1987.

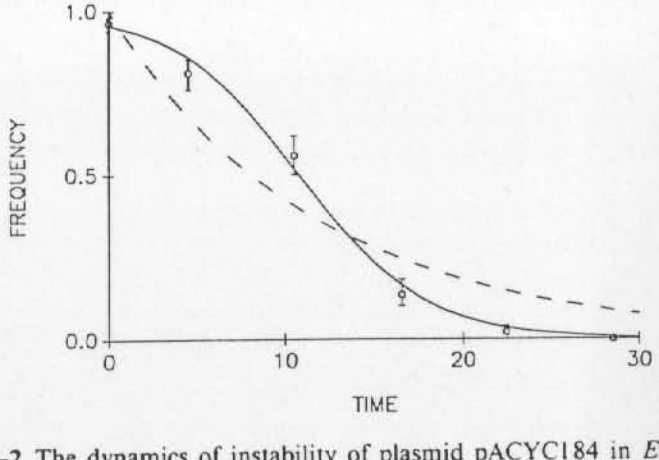

FIGURE 9–2 The dynamics of instability of plasmid pACYC184 in *Escherichia coli*. Raw data and 95% confidence intervals for each sample frequency are presented in Table 9–2. Solid line: model fit with both segregation and selection parameters. Dashed line: model fit with segregation parameter only. From Lenski and Bouma 1987.

segregation rate, and the selection coefficient are allowed to vary. The dashed line indicates the fitted model when the intercept and the segregation rate are allowed to vary, while the selection coefficient is held to zero. The dashed line, which ignores selection, is clearly inconsistent with the data; there are too few segregants in the early samples, and too many in the later samples, to be explained by segregation alone.

9.3.2.2 Manipulation of Initial Frequency. In some cases, it may be difficult to distinguish between the effect of segregation alone and the combined effects of segregation and selection. This is especially likely to be true when the frequency of segregants remains low over the entire course of an experiment. On the one hand, it is more parsimonious to accept the simpler model of segregation alone. On the other hand, it is possible to test more rigorously the null hypothesis that the selection coefficient is zero.

The most obvious solution to this problem is to continue the experiment for a longer period of time, thereby obtaining a greater range of sample frequencies. However, longer experiments may be complicated by additional genetic changes that go undetected, and that have effects on fitness that may be erroneously associated with the genotypic property of interest (see also Sections 9.3.1.1 and 9.4.1). A preferable approach is to perform a parallel experiment in which the frequency of segregants at the start of the experiment is deliberately increased by the inoculation of the appropriate wild-type clone, which is presumed to be identical to a segregant. This manip-

ulation of the initial frequency of the wild-type will increase the range of frequencies subsequently observed, and thereby will permit better distinction between the effect of segregation alone and the combined effects of segregation plus selection (Lenski and Bouma 1987).

9.4 CAVEATS AND ASSUMPTIONS

No methods are without assumptions, and it is important to be aware of those situations in which assumptions may be violated. In this section, I will discuss several important assumptions and the consequences of their violation. The reader is encouraged to consult the references cited below for further discussion.

9.4.1 Fitness-Enhancing Mutations (Periodic Selection)

One of the most critical assumptions of the analyses presented in this chapter is that any relevant genetic differences between genotypes are known to the investigator. If this is not true, then these hidden genetic differences may confound one's interpretation of the effect on fitness caused by the known genetic factors.

Secondary mutations invariably arise such that any population actually contains many genetically distinct subclones. However, these secondary mutations are usually hidden to the investigator. Each subclone is perceived as belonging to one of the two intended clones, whose average fitness is thereby affected. Rare subclones have very little effect on the average fitness of the clone, and mutations that reduce fitness should remain rare. But if a mutation enhances fitness, then it will spread through the population, a process that has been termed *periodic selection* (Atwood et al. 1951; Kubitschek 1974; Dykhuizen and Hartl 1983). Neutral and even disadvantageous genes can be dragged along with the adaptive mutation, owing to the inevitable linkage that exists in clonal organisms (e.g., Helling et al. 1981).

The likelihood of confounding effects caused by periodic selection can be minimized by storing clones properly just after their isolation (see Section 9.3.1.1) and by limiting the length of experiments (see Sections 9.3.1.1 and 9.3.2.2). One can test whether anomalous results are due to the effects of periodic selection by isolating subclones derived from each original clone at the end of an experiment, and performing additional measurements of selection coefficients. If heritable changes in fitness have occurred, then the fitness of a derived subclone will have increased relative to its progenitor. Periodic selection is probably most frequent when a microorganism has been recently introduced into a novel environment, so that certain genetic variants, previously neutral or disadvantageous, are suddenly favored.

The likelihood that a fitness-enhancing mutation will enable an otherwise competitively inferior microorganism to persist after it has been introduced into the environment will be discussed in the next chapter.

9.4.2 Gene Transfer

The methods presented in this chapter assume that there is no gene transfer. The effects of gene transfer have been incorporated into mathematical models (Stewart and Levin 1977; Levin and Rice 1980), but I am unaware of any published papers that present explicit methods for simultaneously estimating the rate of gene transfer, the segregation rate, and the selection coefficient. I suspect that such methods can be developed, although this will not be trivial for the following reason. Segregation and selection can be distinguished because the rate of change due to the former depends on the frequency of the engineered genotype, whereas the rate of change due to the latter depends on the *product* of the frequencies of the engineered and segregant genotypes (see equation 7). The rate of change due to gene transfer (including conjugation, transduction, and transformation) depends on the product of two populations, and so it may be more difficult to distinguish its effects from the effects of selection. Of course, gene transfer can also break apart any presumed linkage of a marker and an engineered gene, as can segregation (see Section 9.3.1.3).

When a recombinant gene reduces fitness, segregation and selection act in concert to reduce the frequency of the engineered genotype. Under the continued action of these forces, the recombinant gene will eventually be lost completely from a population. However, transfer can maintain a gene or extrachromosomal element in a population despite the purging effects of segregation and selection (Stewart and Levin 1977; Levin 1980; Levin and Rice 1980; Levin and Lenski 1983).

9.4.3 Constancy of Parameters

Although perhaps obvious, it is important to emphasize that analyses of the models presented in this chapter are predicated on the assumption that the segregation rate and the selection coefficient are constants. However, there may be heterogeneity among cells even within a single genetically uniform subclone with respect to the effects of segregation or selection. For example, in continuous culture, cells attached to the walls of the vessel are subject to a reduced rate of turnover and consequently a reduced selection coefficient (Dykhuizen and Hartl 1983; Chao and Ramsdell 1985). An environmental refuge such as this may generate the appearance of an equilibrium, although in fact the rate of change due to selection may only have slowed.

In some cases, the selection coefficient may vary in direct response to the relative frequencies of the competing clones. If ecological feedbacks are such that one clone has a selective advantage when it is scarce, but it is

selectively disadvantaged when common, then frequency-dependent selection can promote a stable equilibrium whereby the two clones coexist indefinitely (e.g., Lenski and Hattingh 1986).

9.5 SUMMARY AND CONCLUSIONS

Fitness represents the combined effects of all other phenotypic properties on the capacity for survival and reproduction by a particular genotype in a particular environment. For most environmental applications of genetically modified microorganisms, efficacy will be enhanced if the engineered genotype is more fit than its wild-type counterpart in the target environment. However, inadvertent spread of the engineered genotype will be less likely if it is less fit than the wild-type. Thus, the fate of a population of genetically engineered microorganisms, and the likelihood and magnitude of any environmental effects (whether beneficial or detrimental), will be strongly influenced by the relative fitnesses of modified and unmodified genotypes. In this chapter, I have presented theoretical principles and empirical methods for determining the relative fitnesses of engineered and wild-type clones. Selection coefficients were used to provide a quantitative measure of the difference in fitness between the two clones in a particular environment.

Many engineered genotypes are unstable, such that their frequencies decline with time. Instability may be caused by infidelity of replication or transmission of a particular gene (which is termed segregation), or it may be caused by a difference in the fitness of genotypes that retain or have lost that gene (selection). In this chapter, I have also presented theoretical principles and empirical methods for distinguishing the effects of selection and segregation.

Finally, it should be emphasized that selection coefficients and segregation rates can be estimated not only in highly simplified laboratory systems, but also in more complex natural or semi-natural systems, such as microcosms. All that is required is the ability to monitor the relative abundance of two clones (e.g., engineered and wild-type) that share a common environment.

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CHAPTER
10

Quantifying the Risks of Invasion by Genetically Engineered Organisms

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The rate of evolutionary (genetic) change in most organisms is sufficiently slow so that it does not effect ecological dynamics over the time span that usually interests us. But, at certain times, the specific characteristics of the organisms under question require us to include or even to concentrate our efforts on their evolutionary properties in order to solve ecological problems. An important example is encountered in attempts to conserve rare species where, due to the very small size of the populations, the lack of genetic variability becomes an important issue, although the original problem was primarily demographical (Simberloff 1988; O'Brien and Evermann 1988). Interestingly, at the other end of the spectrum, when the population size is extremely large, new advantageous mutations may arise in the population every generation. If the generation time of such organisms is short, these advantageous mutants can be rapidly selected, and ecological parameters could change very fast.

Most genetically engineered organisms proposed for release into field are microorganisms. Microorganisms have precisely these characteristics of

Assessing Ecological Risks of Biotechnology

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