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CLASSICAL STRAIN IMPROVEMENT

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1.0 INTRODUCTION

Improving complex phenotypes, which are typically multigenic in nature, has been a long-standing goal of the food and biotechnology industry well before the advent of recombinant DNA technology and the genomics revolution. For thousands of years, humans have (whether intentionally or not) placed selective pressure on plants, animals, and microorganisms, resulting in improvements to desired phenotypes. Clear evidence of these efforts can be seen from the dramatic morphological changes to food crops since domestication (1). These improvements have been predominantly achieved through a "classical" approach to strain engineering, whereby phenotypic improvements are made by screening and mutagenesis of strains that use methods naive of genome sequences or the resulting genetic changes. This approach is well suited for strain optimization in industrial microbiology, which commonly exploits complex phenotypes in organisms with poorly defined or monitored genetics. As a recognition of importance, Arnold Demain and Julian Davies begin their *Handbook of Industrial Microbiology and Biotechnology* with "Almost all industrial microbiology processes require the initial isolation of cultures from nature, followed by small-scale cultivations and optimization, before large-scale production can become a reality" (2). The classical approach is concerned

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with the central steps in this process—between isolation and large-scale production. Hence, the methods and techniques utilized in this approach amount to "unit operations," that is, standard procedures that can be generically applied to any desired strain of interest.

A variety of approaches are used to force genetic (and hence phenotypic) diversity including naturally occurring genetic variation and genetic drift, mutagenesis, mating/sporulation, and/or selective pressures. These methods have garnered large successes across a wide range of host organisms owing mostly to the absence of required sophisticated genomic information or genetic tools (3). Thus, the classical approach can be applied to both model organisms (such as *Escherichia coli* and *Saccharomyces cerevisiae*) and newly isolated or adapted industrial strains. As a result, the classical approach has seen wide adoption in industrial fermentations due to its proven track record in alcohol and pharmaceutical production. Finally, strains developed in this manner are currently accorded non-genetically modified organism (GMO) status, removing significant barriers to their acceptance by both regulatory agencies and consumers. This chapter will highlight several of the approaches and successes that exemplify the classical approach for improving complex phenotypes of industrial cells as well as indicate its limitations and potential interfaces with emerging technology.

1.1 THE APPROACH DEFINED

The classical approach is characterized by the introduction of random mutations (either forced or natural) to a population of cells followed by screening and/or selection to isolate improved variants. The defining quality of classical strain engineering (as opposed to other evolutionary engineering methods) is genome-wide mutagenesis. This approach utilizes techniques that introduce variation across all regions of the genome, in contrast to other techniques that specifically target the mutations to single genes (or subsequences thereof). To date, this approach has been successful in improving complex phenotypes because of the global nature of classical methodologies (see Box 1.1 in this chapter and case study in Chapter 6). Complex phenotypes such as tolerance to environmental stress, altered morphology, and improved flocculation characteristics are often influenced by the interactions between multiple (often uncharacterized) genes. In contrast, without significant prior understanding, variants generated through mutagenesis of specific genomic subsections are unlikely to gain proper coverage of the genotype. Indeed, as will be discussed later, this approach has continuously yielded improved variants for a wide variety of complex biotechnological applications. The theory and techniques for the two major steps of classical strain improvement (CSI) (mutagenesis and screening) are the focus of this chapter, including practical recommendations for their implementation as well as brief discussion of examples of each method's industrial application.

BOX 1.1: APPLICATION OF CSI IN SAKE FERMENTATION

The Japanese-brewed sake is produced from rice mash using *Aspergillus oryzae* to saccharify the rice and strains of sake yeast (genus *Saccharomyces cerevisiae*) to ferment the sugars to ethanol. The ideal process imposes a number of complex traits on the sake yeast, including high fermentation capacity over the 20- to 25-day process at low temperatures (typically 10°C), high ethanol tolerance (ethanol levels can approach 15–20%), minimal foaming, resistance to contaminating microbes, and the ability to create the correct proportion of flavor components including higher alcohols and esters (82). Many of these traits have been approached using methods of the classical approach including mutagenesis, selection, and cell mating. Specifically, UV and chemical mutagenesis have dominated as a means of retaining GRAS status for this yeast. Moreover, difficulty in sporulation has limited genetic dissection and a more rational approach until recently (83). Natural selection and isolation from hundreds of years of fermentation has resulted in the series of commonly used strains named the Kyokai series, with Kyokai no. 7 and Kyokai no. 9 as the main fermentation strains used industrially. Due to the superior brewing capacity of Kyokai no. 7, many attempts have been made to improve this strain through the classical approach as well as dissect the underlying genetic changes. Recently, it has been demonstrated that the breeding and selection process of this strain resulted in heterozygosity of many alleles responsible for ethanol production and aromatic compound synthesis (84,85) as seen by sporulation analysis. Many attempts have been made to improve the characteristics of Kyokai no. 7. Non-foaming mutants have been isolated from spontaneous clones as well as UV-induced mutants using selection methods such as cell agglutination and froth floatation (86). Improved strains have also been isolated through chemical mutagenesis (e.g., by EMS) to select for improved flavor profiles. In this case, mutant Kyokai no. 7 strains more resistant to cerulenin were thought to produce more ethyl caproate, an important flavor component. This approach was successful in improving this flavor component; however, the complete portfolio of complex phenotypes was not fully assayed (47). Finally, prevention of contaminants has been explored through mating sake yeast strains with strains exhibiting the killer phenotype (56), which would ward off contaminating yeasts. Collectively, these examples of complex phenotype engineering highlight the difficulties of the process, specifically; it is often hard to create all traits at once. The evolution of the sake yeast demonstrates the power of the classical approach. More recent attempts have been made to use the rational or evolutionary approach for this strain; however, Kyokai no. 7 remains the industrial favorite for sake production.

1.2 MUTAGENESIS

A fundamental parameter dictating success in classical strain engineering is the frequency and type of mutation applied to the parent cells. Typically, this rate is determined by the dose and type of mutagen delivered. To test mutagen specificity and rate, it is common to generate an inactive (mutant) form of some easily assayable gene (e.g., *LacZ* in *E. coli*) that differs from the wild-type gene by a single base-pair change, and test the frequency of reversion. For example, Cupples et al. generated six variants of *LacZ* to show that many common mutagens (EMS, NTG, 2-aminopurine, and 5-azacytidine) are in fact quite specific for certain base-pair changes in *E. coli* (4). Hampsey undertook a similar approach in *S. cerevisiae* and found similarly that mutagens were highly specific. However, the mutation frequencies and specificities were significantly different from those observed in *E. coli* (5). Frameshift and deletion frequencies can also be detected through analysis of a cleverly mutated marker (6). Through analyses of reversion frequencies or high-throughput sequencing, a detailed picture of a treatment's mutagenic profile may be ascertained. This detailed information can be then be used to compute several useful quantities, such as the average number of mutations per genome or the expected number of distinct variants among a mutated population. Knowledge of these frequencies and landscapes are especially useful when designing a selection program, as detection of rare variants (e.g., individuals possessing certain particular mutations and no more) will require many individuals to be screened, whereas more probable patterns of mutagenesis (e.g., if additional silent or neutral mutations are tolerable) will not. At the same time, more focused patterns of mutation inherently limit the search space.

1.2.1 Numerical Considerations in Screen Design

Although in general every possible base substitution will occur at a different frequency (and vary nonuniformly throughout the genome), it is instructive to neglect deletions or insertions and assume all base changes at each site are equiprobable (i.e., occur at the same frequency) to make use of the binomial distribution, to obtain approximate probabilities of any desired mutagenic outcome. If the probability of a single base being mutated to any other base is p, then the probability that a genome of size g has n mutations after mutagenesis is:

$$P(g, n, p) = \frac{g!}{n!(g-n)!} p^n (1-p)^{g-n}.$$

By using well-known properties of the binomial distribution, the average number of mutations per genome is gp with variance $gp(1-p)$. Random genetic drift results in mutation rates of 10^{-10} to 10^{-5}, while forced mutagenesis can elicit rates upwards of 10^{-3} as described below, so this will restrict the range

of p. It is apparent that if p is too low (that is, less than 1/g), there will be many variants with few or no mutations and a vanishingly small population of highly mutated individuals. Furthermore, the binomial coefficient indicates that libraries with low mutation rate (and thus a high population of slightly mutated individuals) are very likely to be redundant, that is, have many individuals of the same genotype. Thus, it is of interest to know the expected number of *distinct* variants in a mutant library to guide screen design. Patrick et al. developed a suite of algorithms to compute many quantities of interest for screening a mutant pool derived from a mutagenic procedure of arbitrary specificity, including the expected number of distinct mutants following mutagenesis (7,8). If the library is highly redundant, then screening of the entire mutated population may not be necessary to ensure complete coverage. As diversity increases, however, the required screening fraction will approach unity. Since complex phenotypes are controlled by the action of multiple genes, high mutation rates are often employed, generally resulting in high library diversity and a strong incentive to screen the entire mutated pool.

To choose the correct rate of mutagenesis and screening, it is important to know the rarity of the phenotype of interest. In the worst and most restrictive case, an improved phenotype will be acquired by mutants containing only a certain set of mutations. For example, consider a particular phenotype that only manifests itself when n-specific mutations are present and no more. In this case, one must determine the mutation rate $\hat{p}$ which maximizes the fraction of n-mutant variants in the mutated population (using one of the tools mentioned earlier) and screen until a reasonably high probability of complete coverage is achieved. For a genome of g base pairs, we can take the derivative of the binomial distribution with respect to mutation rate and set it equal to zero:

$$\frac{d}{dp}\left(\frac{g!}{n!(g-n)!}p^n(1-p)^{g-n}\right)=0.$$

Eliminating constants and taking the derivative, we have:

$$\hat{p}^{n-1}(1-\hat{p})^{g-n-1}(n-g\hat{p})=0.$$

The obvious interesting candidate for a solution is:

$$\hat{p}=\frac{n}{g}.$$

Taking the second derivative of the binomial distribution yields:

$$\frac{g!}{n!(g-n)!}\hat{p}^{n-2}(1-\hat{p})^{g-n-2}\left(n(-2(g-1)\hat{p}-1)+(g-1)g\hat{p}^2+n^2\right).$$

Because

$$\frac{g!}{n!(g-n)!}\hat{p}^{n-2}(1-\hat{p})^{g-n-2} > 0,$$

we can substitute our candidate solution into the remaining portion of the second derivative to determine its sign:

$$\left(n\left(-2(g-1)\frac{n}{g}-1\right)+(g-1)g\left(\frac{n}{g}\right)^2+n^2\right)=\frac{n^2}{g}-n,$$

which is clearly negative for $g > n$. Hence, the likelihood of attaining n mutations in a genome of size g is maximized when the mutation rate is n/g. This maximum likelihood is:

$$r \equiv \frac{g!}{n!(g-n)!}\left(\frac{n}{g}\right)^n\left(1-\frac{n}{g}\right)^{g-n}.$$

It is generally necessary to screen more than the number of possible mutants to ensure coverage of the diversity. To obtain, on average, F fractional coverage of all n-mutant variants, it will be necessary to solve

$$F = 1 - e^{-r^*a^*L}$$

for L, where a is the probability of selecting the correct n-mutant variant ($1/V$ in this case, where V is the number of possible n-mutant variants [given by the binomial coefficient]) and L is the library size (7). For a small-sized genome (10^6 base pairs) and a phenotype requiring two specific mutations (hence at an optimal mutation rate of $2*10^{-6}$), L works out to be $5.5*10^{12}$ to obtain 95% coverage, on average, which is outside the scope of most screening programs (Assuming a standard yeast cell density of 10^7 per mL and an average cell sorting rate of 10^3 per second, screening this many individuals would require 550 L of culture (for growth-based selections) or 241 years of cell sorting [for fluorescence-based screens]!)

Luckily, most complex phenotypes can tolerate the existence of additional silent/neutral mutations. To account for a small number of allowable neutral mutations, let us assume that the desired n mutations may be found in any variant containing up to $m > n$ total mutations, but no more. The analysis for this case proceeds in much the same way as before with one minor alteration resulting from the fact that a variant with $m > n$ mutations contains

$$\frac{m!}{n!(m-n)!}$$

instances of n mutations. Therefore, maximizing the quantity

$$\sum_{i=n}^{m} \frac{i!}{n!(i-n)!} * P(g, i, p)$$

with respect to p will yield the mutation rate $\hat{p}$ which maximizes the number of n-mutant combinations encountered in the randomized pool. This rate may be used to find the probability that a variant selected at random will have between n and m mutations:

$$r \equiv \sum_{i=n}^{m} P(g, i, \hat{p}).$$

Given that a variant has between n and m mutations, the probability that it contains the mutations of interest can be obtained by summing the probabilities of finding the mutations of interest at each particular mutational level:

$$a = \sum_{i=n}^{m} \frac{P(g, i, \hat{p})}{\sum_{i=n}^{m} P(g, i, \hat{p})} * \frac{i!}{n!(i-n)!} * \frac{1}{V}$$

where

$$\frac{P(g, i, \hat{p})}{\sum_{i=n}^{m} P(g, i, \hat{p})}$$

is the probability that a variant with a mutation rate i is selected, and

$$\frac{i!}{n!(i-n)!} * \frac{1}{V}$$

is the probability of finding a particular combination of n mutations within that variant. r and a can then be substituted to the equation for F, which is solved for L as before. Continuing with the example stated above, if the search is expanded to allow desired mutations to occur in a background of up to 5 mutations, then the mutation rate can be increased to $4.2*10^{-6}$, requiring screening of $4.3*10^{11}$ individuals, which, though an order of magnitude less than in the previous case, is still rather unmanageable.

The property that allows strain engineering programs to be feasible is the additivity of the effects of mutations; that is, even if a particular combination of 12 point mutations is optimal, a couple of them, even in isolation, will be beneficial. This allows engineering to proceed in several single mutation steps as opposed to a single multiple-mutation bound. Because any given single mutation is much more probable than a particular double mutation, the probability of isolating improved variants is greatly increased. Even if a phenotype could only be improved by a single base-pair change in the absence of any

others, the number of mutants that must be screened is $8.2*10^6$, which is attainable from a fraction of a milliliter of culture under growth selection or from less than an hour of cell sorting (9).

These probabilities guide strain selection. In the following sections, multiple mutagenesis techniques and screening strategies will be discussed. Particular attention should be paid to mutation rate and throughput, respectively, so that screening programs are designed and carried out efficiently.

1.2.2 Random Genetic Drift

Natural mutations due to errors in replication take place at frequencies between 10^{-5} and 10^{-10} , depending on the strain and organism. Given this frequency, it is not surprising that large-scale selections are required to isolate any improved mutant. These frequencies are supported by a meta-analysis of phenotype occurrence frequencies for the basic yeast *S. cerevisiae* (10). Phenotype reversions requiring single base-pair changes including amino acid auxotrophy reversion and resistances occurred, on average, at a frequency of 10^{-8}. On the basis of this low mutation rate alone, it would appear that random genetic drift may be most suited for the optimization of phenotypes under the control of nonepistatic factors, despite the prevalence of epistatic interactions in nature (11). However, single point mutations are not the only genetic change to take place in evolving cells. Specifically, Lenski et al. found that the majority of the genotypic changes observed through the course of a long-term natural evolution experiment in *E. coli* resulted from transpositions and rearrangements as opposed to single base-pair substitutions (12). These large-scale genetic changes have a much higher probability of generating mutants on distant peaks than do point mutations. The variety of possible genetic changes resulting from a natural evolution program points to its versatility in optimizing a wide variety of complex phenotypes. However, generation of mutants with this method requires a significant amount of time, during which individuals are subjected to growth-based selective pressures. If the phenotype of interest is at odds with growth, then this mutagenic procedure may not be optimal with respect to library size and screening. Natural mutagenesis, however, does lend itself very well to growth phenotypes, as no additional effort on the part of the strain engineer is required to generate mutants and compare them against the fittest variant.

1.2.2.1 Tracking Evolution through Neutral Phenotypes Since natural evolution experiments are often accomplished in continuous liquid culture, new variants are constantly being generated and compared against the fittest variant. Therefore, evolution does not proceed in rounds or stages like most forced evolution experiments, and so it is unclear when fitness increase has ceased or when a population has stopped evolving. To overcome this limitation, one can make use of neutral markers to detect a mutation event. Neutral markers are genotypes that confer no alteration in growth rate yet whose

phenotype is easily detectable. Common neutral phenotypes include resistance to phage T5 or reversion to lactose fermentation (when the appropriate selective pressure is not present in the screening medium, of course). In a strain normally deficient in either of these phenotypes, it is expected that over the course of evolution these neutral mutations will become fixed in the evolving population at a slow but steady rate. Due to the low probability of attaining the neutral marker and the fact that it does not pose a selective pressure on those who carry it, it is assumed that neutral markers are never predominant in the culture and that over time the prevalence of this phenotype will attain a steady state as the rate of incidence becomes balanced by mutational losses. When a variant of high competitive fitness is generated (which in all likelihood does not possess the neutral marker), it steadily outcompetes the existing population, driving the proportion of the neutral marker down. After this new variant becomes predominant in the culture, the fraction of mutants containing the neutral marker again increases at a slow but steady rate as before. As a result, these sharp declines in the presence of a neutral marker signal the appearance of a new adaptive mutation, and the jagged graph of the neutral marker over time is called a periodic selection curve (see Figure 1.1). However, if the adaptive mutation happens to occur on a variant containing the neutral marker, the fraction of the neutral marker will approach unity in the selective medium, although it confers no selective advantage. This phenomenon is known as "hitchhiking," and it is predicted to occur often in evolving systems (13,14). In addition, the appearance of an additional adaptive mutation does not necessarily imply any significant improvement in phenotype as desired by the strain engineer, as fixation of mutations may simply result in a competitive advantage quite unrelated to the phenotype of interest. Therefore, it is necessary to assay for improvement as selection proceeds, to ensure that progress is still occurring.

1.2.2.2 Genetic Determinants of Mutation Rate Although a wide range of chemical and physical agents can serve to mutate a microbial population (discussed later), a variety of more natural factors can contribute to an increase in the mutation rate of naturally evolving populations, including ploidy, genotype, and environmental conditions. Diploid strains have been shown to more quickly fix adaptive mutations than haploids. Since diploid strains make copies of genes at twice the rate of haploid strains, adaptive mutations are generated at twice the rate. Assuming adaptive mutations are dominant, this increased generation of adaptive mutations should result in an increase in the rate of fixation of adaptive mutations. Indeed, Paquin and Adams showed that diploid strains accumulated advantageous mutations at 1.6 times the rate of haploid strains (15). This implies that diploid strains may achieve maximum phenotypic increase much faster than haploid strains, allowing further selection programs to be undertaken.

In addition to diploid strains, a number of additional "mutator" genotypes are known to increase the mutation rate in bacteria (16,17). These genotypes

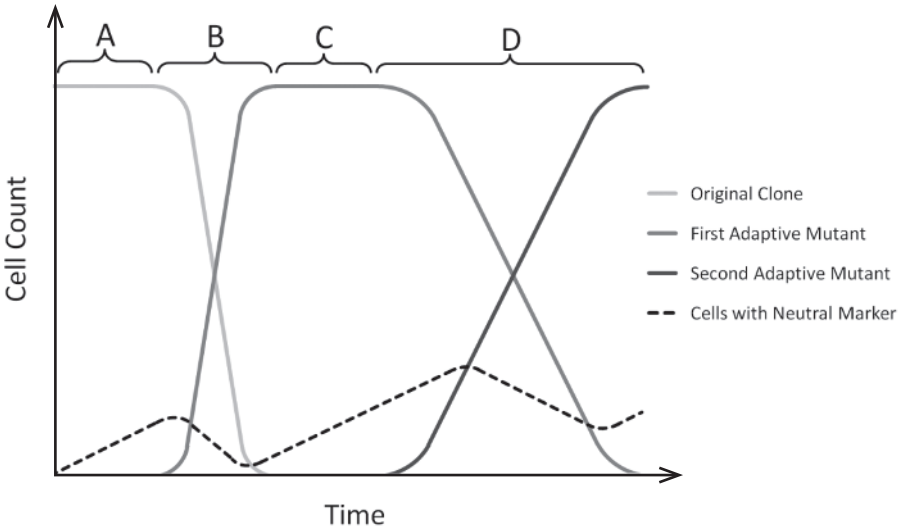

FIGURE 1.1. Periodic selection in an asexual population. The numbers of successive adaptive clones and the number of clones possessing the neutral marker are tracked over time. (A) The prevalence of the neutral marker increases in the wild-type population at a rate determined by natural mutation frequency. (B) The first adaptive mutant (with a large selective advantage) appears in the neutral marker-null population and quickly outcompetes the original clone, causing a sharp decline in the number of cells containing the neutral marker. (C) The prevalence of the neutral marker increases in the first adaptive mutant at a rate determined by natural mutation frequency. (D) The second adaptive mutant (with a relatively small selective advantage) appears in the neutral marker-null population and gradually outcompetes the original clone, causing a slower decline in the number of cells containing the neutral marker than in time interval (B). Reproduced with permission of Annual Reviews, Inc., from Reference (14); permission conveyed through Copyright Clearance Center, Inc.

may encode for enzymes that are naturally mutagenic or may confer mutagenic activity upon an existing enzyme. Although the presence of a gene conferring a high mutation rate would appear detrimental, it has been theorized that a gene conferring a 1000-fold increase in mutation rate in a particular individual can cause a population to increase in fitness quite quickly while remaining in but a small fraction of individuals (18). The ability of this genotype to confer a selective advantage without becoming ubiquitous can be understood through the high rate of reversion of the mutator genotype, due to its high mutation rate. This implies that mutator strains may be an excellent starting point for a variety of evolution experiments, as isolates from the resulting culture are likely to be genetically stable. However, it should be noted that most known mutator genes only achieve a 100-fold increase in mutation frequency, at which level they have been theorized to attain a much larger

fraction of the population (5–10%) (16,17). In addition, the mutator phenotype may be amplified by certain chemicals such as thymidine, allowing for increased control over evolutionary rate over the course of the experiment. Mutator genes are also unique in that they may have a very specific mutational spectrum. In *E. coli*, mutY increases the frequency of GC->TA transversions, mutT results in TA->GC transversions, and the mutD5 mutation appears to increase the rate of mutation in a nonspecific manner. The specificity of mutator genotypes allows a great deal of control over the spectrum of mutants generated, possibly enabling preservation of a counter-selected genotype that is necessary for the application of interest.

1.2.2.3 Applications of Random Genetic Drift Continuous culturing and serial transfers have been successfully used to select for fast-growing strains generated through a natural evolution program (19–22). Included in this list are improvements of basal-level growth rate as well as improvements in growth rate on alternative sugars such as xylose. Accumulated mutations in a yeast strain selected on xylose over time resulted in greatly altered xylose transport kinetics, doubling V_{max} (15.8 to 32 mmol/[g dry weight]/h) and reducing K_m by 25% (132 to 99 mM) (22). A second highlighted example involves a study on the bacterium *E. coli*, where 10,000 generations were studied via serial culturing (12). The resulting strains exhibited a 50% improvement in fitness as well changes in other complex phenotype such as cell size and morphology. Moreover, this study highlighted that the mutations regulating these phenotypes were indeed quite rare and diverse. In some cases, this change could be accomplished by point mutations; however, genomic rearrangements were also seen. Furthermore, most of the change occurred during the first 2000 generations, with improvements slowed over the last 8000. This highlights the importance of screening high levels of mutants, a prime difficulty with natural selection-based mutations. Both of these examples are highly relevant because growth improvement is a highly complex process. Not only does metabolism need to be regulated and carried out more efficiently, but a number of additional factors such as substrate uptake, metabolite tolerance, and reproductive machinery also need to be optimized in a fast-growing strain. This breadth obtainable by classical strain engineering would be unfeasible in more directed approaches. As a final example, Wiebe et al. used a glucose-limited chemostat to select for mutants of *Fusarium graminearium* with delayed onset of colonial morphology, further illustrating the power of natural evolution to enrich for highly complex phenotypes (23). Delayed onset of a particular phenotype requires alteration of a wide range of regulatory factors, especially for a trait that is carried out by a plethora of cellular machinery. Furthermore, because many factors controlling morphology are unknown, directed approaches would be ineffective at generating highly improved variants.

Industrially, natural genetic drift is always under way in large-scale fermentations. As an example, naturally improved strains of yeasts (both

Saccharomyces and *Pichia* sp.) have been isolated from a sulfite liquor fermentation plant. These strains demonstrated the complex phenotype improvement of increased tolerance to acetic acid and enhanced galactose fermentation capacity (24). Samples from ongoing fermentations, especially long-term culturing, will present a diverse genetic population. The continuous sampling and analysis for these cultures can give rise to novel, complex phenotypes. However, other methods such as forced mutagenesis can improve the frequency of improvements as well as the prospect for success.

1.2.3 Forced Mutagenesis

Mutagenesis by ultraviolet (UV) or chemical treatment is a widely used approach for obtaining point mutations to create auxotrophic markers and improve strains. Cellular exposure to UV radiation can disrupt DNA structure, leading to a dose-specific occurrence of mutations. In addition, certain chemicals such as ethyl methane sulphonate (EMS) and nitrosomethykguanidine (NTG) have been known to cause DNA damage. It should be cautioned that all of the agents described in this section are mutagenic and hence carcinogenic and thus extreme care must be used during handling to prevent damaging exposure. In addition, certain chemicals carry orthogonal risks. For instance, NTG is explosive (25). Therefore, the benefits of increased mutation rate must be weighed against increased safety costs when working with these compounds.

The attractiveness of chemical and physical mutagens is the increased mutational capabilities compared with natural variation. Mutation frequencies are often measured as a function of auxotrophic marker development or gene mutation reversion. While basal-level, natural drift mutations can result in average reversion frequencies of nearly 1 in 10^{-8} (as described above), induced mutations by methods such as UV radiation can increase this value upwards of 10^{-3} to 10^{-5}, depending on the organism used and the intensity/duration of irradiation (26–28). Similar results and mutation frequencies can be seen with chemical mutagenesis using reagents such as EMS and NTG. Given these rates, it is still expected that the frequency of obtaining auxotrophic mutants in diploids by direct mutagenesis should be rare without prior selection. However, it has been demonstrated that auxotrophic mutants due to forced mutagenesis of diploid industrial strains can occur at frequencies of 10^{-4}, illustrating that the mechanism of mutations is still unknown (29). Therefore, improved mutants will occur at rates higher than those suggested by the probabilities. In terms of fitness landscapes, this higher mutation rate allows a further exploration of genotypic space. Thus, generating mutations with this method may yield variants located on more distant peaks, possibly at higher levels of fitness. When the phenotype of interest is influenced by a significant number of epistatic interactions, the resulting landscape will be more rugged, making forced mutagenesis more desirable for isolating improved variants than natural evolution.

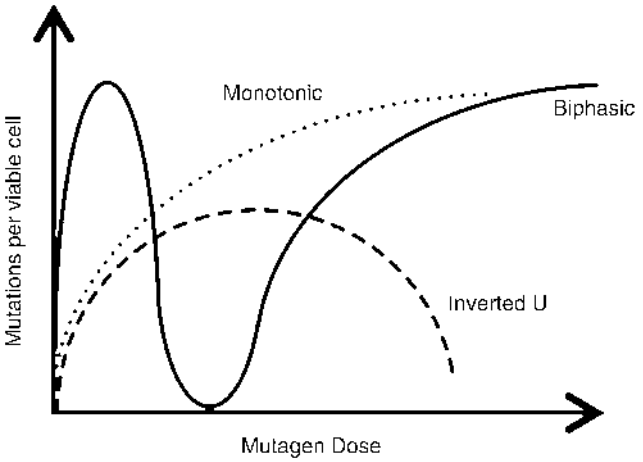

FIGURE 1.2. Common dose–response curves. Determining the optimal mutagen dose is critical for success in identifying altered mutants. This optimal level is dependent on the type/doses of mutagen and on the trait of interest. In general, three types of response curves are seen: reversion to prototrophy or resistance mutations normally follow the monotonic curve, whereas titer-increasing or decreasing mutations normally have an inverted-U shape. If the culture contains a subpopulation of radiation-sensitive individuals, biphasic behavior may be observed.

1.2.3.1 Optimal Mutagen Dose Mutation rate and cell survival are both strongly affected by mutagen dose. Thus, it is necessary to determine optimum mutagen dose. If the mutation rate is too low, variants with mutations (and especially improved phenotypes) will be rare compared with unmutated cells, making detection difficult even in high-throughput screens. In contrast, if the mutation rate is too high, the effects of deleterious mutations will swamp those of beneficial mutations, yielding poorly performing (or even nonviable) variants. Since the goal of the strain engineer is to maximize the number of beneficial mutations per variant, plots akin to Figure 1.2 are often constructed to evaluate the effects of different types or doses of mutagen on the trait of interest. A crude measure of phenotype on a small number of variants is preferred to minimize the resources spent at this preparatory stage. Curves similar to those in Figure 1.2 are often seen, depending on the phenotype of interest. Reversions to prototrophy or resistance mutations are normally monotonic, whereas titer-increasing or decreasing mutations normally follow an inverted U curve (30–32). It is important to note that as titer is improved, the likelihood of finding further beneficial mutations is reduced, making the statistic of population variance as important as the average for selection of optimal mutagen dose (33). Indeed, Lenski, et al. found a hyperbolic decline in fitness increase

over time in a population of *E. coli* undergoing natural selection, suggesting that a small number of mutations of large effect were fixed in the population during early times whereas a larger number of mutations of smaller effect were fixed in subsequent generations, assuming a constant mutation rate per generation (34). Hence, the optimal mutagen dose is likely to change as superior variants are isolated. The dose that results in the highest enrichment in desirable variants is then applied and a large number of variants are screened more accurately.

Although plots akin to Figure 1.1 are useful for single-round selection programs, selecting the optimal mutagen dose based on the maximum observed phenotypic increase may cause problems for prolonged selection experiments. Although this dose will maximize the single-round phenotypic increase, subsequent rounds of improvement will have to contend with any deleterious mutations that will have occurred, possibly limiting maximal improvement in phenotype. For cases where improved titer is important, it is generally accepted that low mutation rates are better than high, even though high rates will yield a more immediate benefit (35). The rationale behind this choice is that the small number of mutations selected in a low mutation rate program will have a much higher probability of being beneficial than the high number obtained in a more error-prone program. Therefore, any subsequent beneficial mutations will not have their effects attenuated by the presence of deleterious mutations. Only after low levels of mutagen fail to yield improved variants is it desirable to increase mutagen concentration, thus expanding the evolutionary search to reveal epistatic peaks in the fitness landscape. By alternating low and high mutation rates in this manner, the prevalence of deleterious hitchhikers may be minimized.

1.2.3.2 Determination of Mutagen Specificity and Frequency A wide variety of chemical mutagens have been used to introduce DNA damage. Not only do individual chemicals produce different mutation profiles as mentioned above, but the environmental context and strain in which these mutagens are applied can also have a large effect on the changes observed (36,37). Chemical mutagens have been found to delete large (~1 kbp) sections of an organism's genome as well as generate mutations at the single base-pair level (38). Furthermore, the advent of high-throughput sequencing technology allowed the identification of mutagen-specific "hotspots" in *E. coli*, emphasizing the nonrandom nature of the induced changes (39). In addition, it has been noted that NTG acts upon the DNA replication forks, causing the resulting mutations to be tightly clustered (40). Therefore, it is highly recommended to change mutagens as a strain improvement program proceeds, not only to avoid development of resistance, but also to allow fuller sampling of genomic sequence space. Alternatively, it is possible to apply multiple mutagens in the same dose; however, the mutagens must act on different DNA repair pathways in order for this approach to be beneficial (41). In general, unless mutagenesis rates

and specificities have been previously characterized for the strain of interest, characteristics of mutagens may be known only approximately, especially if the organism's cellular repair pathways are unusual.

1.2.3.3 Mechanisms of Mutagenesis Most of the mutagens introduced in this section serve to make DNA repair machinery more error prone, in addition to damaging DNA directly. Therefore, it is plausible that over the course of a selection program a mutation that confers resistance to a particular mutagen will arise. In this situation, no change in phenotype will be observed upon mutagenesis. To distinguish this case from cases where further phenotypic improvement is unlikely, some easily observable reversion phenotype may be used to confirm efficacy of the mutagenic treatment. In cases where resistance has developed it will be necessary to attempt different types of mutagens in order to introduce mutations via an alternate repair pathway (35).

UV light has been extensively studied in terms of its mutagenic frequency, specificity, and mechanism, in large part due to its ease of implementation (27,42–44). Cells may be mutated simply by exposing them to UV light for some length of time (analogous to the dose of a chemical mutagen). In the case of *E. coli*, it is thought that UV radiation causes DNA damage but that these initial lesions are not converted into base-pair changes until activation of the SOS repair pathway, a global response to DNA damage. For this reason, mutation frequency initially increases according to the square of UV dose, confirming that two distinct DNA lesions are required for mutagenesis to occur: one to induce the SOS repair pathway, and the second to cause a detectable phenotypic change. After this initial stage, mutations increase linearly with exposure as lesions continue to accumulate after SOS induction. A regime of higher order response to radiation indicates the appearance of mutations dependent on the presence of two DNA lesions in proximity (43). Finally, the mutation rate reaches a steady value as lethal mutations accumulate. Treatment with UV radiation is analogous to chemical mutagenesis in that UV has its own mutagenic specificity and frequency. However, studies have shown UV to be slightly broader in its action than other mutagens (4).

1.2.3.4 Effects of Environment Since each of the mutagens previously described require the action of cellular repair machinery, "recovery" of mutated cells in rich medium has been found to increase the mutation rate realized in the surviving cells. Not only does this treatment allow sufficient time for SOS repair to convert DNA lesions into base-pair changes, it also allows mutated proteins to be synthesized, which is important if screening occurs directly after mutagenesis. Also, certain additives to this recovery medium may promote or inhibit recovery of mutated cells. For example, addition of caffeine and acriflavine following UV mutagenesis will increase the mutation rate (35), whereas addition of manganese II, purine nucleosides, and

inhibitors of protein synthesis will decrease the mutation rate (45). Interestingly, 8-methoxypsoralen is antimutagenic when present before UV irradiation, but is mutagenic if introduced after UV, illustrating the complexity of the repair mechanisms involved (46). It should be noted, however, that any treatment that increases the mutation rate will also increase lethality; therefore, it should be ascertained whether such treatments actually increase the number of mutants per survivor before mutation-amplifying additives are introduced (35).

1.2.3.5 Applications of Forced Mutagenesis Forced mutagens have seen wide use in development of complex phenotypes. For example, UV mutagenesis was used to generate auxotrophic mutants of sake yeast (29). This is significant because it allows this yeast to be used in breeding programs and in metabolic engineering efforts utilizing molecular cloning techniques. The ability of mutagens to introduce variation in a wide variety of organisms is a major benefit to this approach, as standard genetic manipulation techniques are only established for a handful of (possibly industrially suboptimal) strains. Furthermore, EMS was used to generate sake yeast mutants with improved flavor profiles, clearly indicating the ability of forced mutagenesis to improve industrially relevant complex phenotypes whose molecular basis may be largely unknown (47,48). In addition, a forced mutagenesis/selection scheme was used to screen for improved microalgae capable of producing L-ascorbic acid. By screening over 10^5 mutants, a greater than 50-fold improvement in specific productivity was achieved (49). These results illustrate the large size of libraries necessary to achieve metabolic phenotypes. Many processes (including the penicillin production process (50)) also rely on this method to continuously enhance strains. As an example of this process improvement, penicillin titers are over 40,000-fold higher in improved strains than the original isolated wild-type strain (51). Improved antibiotic production is a prime example of a complex phenotype, as strains must evolve not only the enzymes responsible for antibiotic synthesis but also any factors involved in nutrient transport and chemical tolerance. The organisms normally responsible for high production of a compound of interest are often genetically uncharacterized; thus, the genome-blind nature of the classical approach becomes an asset. This approach of mutagenesis and screening has even been used to improve the activity of baker's yeast for bread making purposes (52). As a result of the ease of operation and selection, this method continues to be used to generate complex phenotypes in industrial cells.

1.2.4 Strain Mating

Strain mating represents an effective tool for generating a population with a high number of non-detrimental mutations. One of the main limitations of random mutagenesis is the high probability that the changes induced in a daughter cell will be detrimental, and this probability increases as the mutation

rate increases. However, high mutation rates are required to escape local optima on the fitness landscape. Strain mating allows recombination to occur between two divergent (yet functional) genotypes, generating a library of highly mutated individuals. However, because meiotic recombination operates at the level of the gene, as opposed to the base pair, any mutations introduced are likely to be in the genomic context in which they were found in one of the parents, decreasing the likelihood that such mutations are lethal. In essence, this technique allows exploration of distant regions of the fitness landscape without the associated high probability of failure, thus allowing generation of mutants located specifically on regions of high fitness. It is obvious that strain mating will have its maximum effect when applied to two highly divergent members of the same species, allowing a high rate of mutation with a low probability of lethality. This technique allows the possibility of running multiple mutagenesis programs in parallel (utilizing perhaps different mutagenic techniques and screening strategies) and mating the most successful individuals from each program, especially if mutagenesis has been conducted to minimize the occurrence of deleterious mutations. In fact, it has been theorized that the accumulation of deleterious mutations causes evolving populations to gradually reach a maximum fitness. If this is the case, then strain mating should greatly improve the potential for phenotypic increase (53). It should be cautioned, however, that if significant epistatic interactions exist between genes, there will be a high likelihood of disrupting them upon mating, possibly leading to inferior individuals (54).

Protoplast fusion is a distinct method of strain mating that does not involve recombination. Instead, the cell walls of two individuals are digested away and their genetic material is combined to form a new individual with both sets of chromosomes (55). This technique allows the characteristics of both organisms to be combined (forming a heterokaryon) without the risk of recombination loss. Heterokaryons are often verified by nutrient complementation. Therefore, it is imperative that each parent be auxotrophic for a different compound. Removing the cell wall while preserving the cell membrane is a delicate process. Therefore, reliable isolation of heterokaryons is dependent on a number of factors, including protoplast isolation from exponentially growing cells, maintenance of isotonicity in the protoplast media, and the addition of polyethylene glycol as a fusogenic agent. Since both genomes are isolated from viable individuals, the probability of deleterious interactions is small. Further, since enzymatic deficiencies are recessive, any lack of functionality in one parent will be complemented by the genome of the other. An added benefit of generating a polyploid strain is the doubling of the effective mutation rate for each gene, allowing evolution of improved phenotypes to proceed at a faster pace, as mentioned earlier.

Strain mating can be used to combine two distinct functionalities into one organism. For example, a common problem in fermentations is the evolution of a “killer” phenotype, whereby a nonproductive individual gains the ability to secrete a toxic compound, thus outcompeting the organisms of desirable

phenotype and resulting in a failure of the fermentation. However, strain mating can be used to generate a productive "killer" phenotype, whereby the resulting population is able to both secrete the product of interest and kill any contaminants that may be introduced (56). Indeed, Bortol et al. were able to fuse strains of *S. cerevisiae* possessing the "killer" phenotype with traditional baker's yeast, producing competitive variants that retained the ability to make dough rise (57). Clearly, strain mating has enormous potential for generating mutants improved in a variety of complex phenotypes.

1.3 GENOTYPIC LANDSCAPES

If phenotype and genotype are graphed such that related genotypes are close together, the resulting landscape is ripe with series of peaks and valleys, with peaks representing genotypes of high fitness and valleys representing genotypes of low fitness. Natural selection dictates that individuals residing on higher peaks are more likely to reproduce, and upon reproduction, a new generation arises at some genotypic distance away from their parents, depending on the mutation rate. As evolution proceeds, the population will tend toward peaks and away from valleys if the selection pressure is toward improved phenotype. As a result, understanding aspects of this landscape helps develop proper mutagenesis and selection strategies. For example, what magnitude of fitness differential is observed when moving from a peak to a valley? Are there many different peaks, or just one? Do there exist mountain ridges connecting each peak, or are each separated by deep chasms? A good understanding of the topography of this fitness landscape will allow prediction of the evolutionary trajectories of a population under selection. For the strain engineer, this understanding will allow comparison of different selection programs in terms of their ability to generate a mutant residing on the tallest peak of this evolutionary landscape.

One important quality of fitness landscapes is their "ruggedness" as developed by Kauffman (58). This quality indicates the correlation in phenotype observed between related genotypes. In the limit of no ruggedness (i.e., perfect correlation), it can be shown that there is only one peak in the fitness landscape and that this peak is accessible from any genotype by progressing through successively more fit one-mutant neighbors. However, in the limit of maximal ruggedness (no correlation between related genotypes), the landscape is essentially random, with many local optima and a very small chance of encountering the global optimum by progressing through successively more fit one-mutant neighbors. This quality is therefore extremely important for evaluating *a priori* which mutagenic and selection treatments are likely to yield improved mutants. Treatments that result in small genotypic changes (such as single base-pair changes) may only be able to proceed in small steps through the fitness landscape. If selection is operated such that the fittest

mutant is selected for subsequent mutagenesis and selection, then repeated rounds of generation and screening are only able to yield a local maxima. However, as mutagenic treatments become more severe, the possibility of generating a mutant on a more distant peak becomes higher at the expense of mutant generation on the current peak. Naturally, more severe mutagenic treatments become more desirable as the number of peaks in the evolutionary landscape increases. Alternatively, selection regimes that preserve a nonzero fraction of suboptimal mutants may also have an advantage in detecting more distant peaks. However, the costs associated with running many selection programs in parallel may prove too great (59). It has been shown that phenotypes that exhibit a high degree of epistasicity resemble more rugged fitness landscapes, whereas phenotypes under the control of genotypes whose effects are perfectly additive resemble the gradual "Fujiyama" type of landscape (58).

1.4 SCREENING

The success of classical strain engineering is due in large part to the ability of researchers to search through a large number of variants to isolate a few improved individuals: a process called screening. As more mutants are screened, the probability of isolating an improved variant increases linearly (assuming the number of mutants generated is large compared with the number of mutants screened). Therefore, significant effort has been spent to develop improved techniques and technologies to allow larger numbers of variants to be assayed per unit time. Screens can be classified into one of two broad categories: rational screens and random screens (35). Rational screens are defined by their exploitation of knowledge about the system of interest, whereas random screens are of more general applicability. Ideally, the quantity being measured during the screen will correspond exactly to the phenotype of interest, but in cases where this is difficult to measure several orthogonal correlates of phenotype may be assayed to decrease the rate of false positives. It is important to keep in mind that the optimal screening strategy will depend in large part on the phenotype of interest and any prior knowledge of the system. In addition, resource limitations may restrict which screens may be performed as well as the number of mutants that may be assayed. The error rate of a screen is also of critical importance, as poorly designed growth screens may yield false positives and noisy assays will necessitate screening replicates to increase confidence. Finally, the importance of selecting individuals that exhibit true phenotypic improvements cannot be overemphasized. The powerful techniques of classical strain engineering often generate individuals that may perform well in a particular screen yet do not produce the phenotype of interest. Although detection of these "screening artifacts" can be largely eliminated through careful experimental design,

further characterization of isolates at the conditions of interest is often required.

1.4.1 Rational Screens

In general, there are at least as many ways to screen for a particular phenotype as there are measurable phenotypes. However, a handful of rational screening strategies stand out due to their popularity and generality. It should be noted that all rational screening procedures assume at least a crude knowledge of the mechanism by which a phenotype is manifested. For instances in which this is not well known, it may be necessary to proceed first with a random screen to identify improved variants, followed by a study to determine which screens are most selective for the isolated individuals. However, it should be noted that none of the techniques mentioned below assume a *molecular* knowledge of the biochemistry involved, which is the minimum requirement for a directed approach to succeed, indicating this approach's generality for a wide variety of uncharacterized microbial strains and complex phenotypes.

Phenotypic titer depression is a common way of shifting the "detectable range" of a random or rational screen. It is often the case that one is interested in mutants exhibiting a high rate of product secretion or growth rate. However, the method used to detect phenotypic changes may not be accurate at the range of interest, especially when the population under selection is derived from a highly improved parent. Therefore, by artificially decreasing titer, differences among high-producing variants may be discerned. This is often accomplished by altering media composition so that a particular nutrient is limiting product formation, or through introduction of a metabolic inhibitor. It is assumed that individuals proficient under these limiting conditions will maintain their superiority in a production setting (60).

Toxic analogs of metabolic precursors can be used to select for variants with improved metabolic qualities. When a metabolic precursor is synthesized intracellularly, mutants resistant to its toxic analog may be overproducing the nontoxic compound, diluting the poisonous effects of the analog and increasing flux through the pathway of interest. This method has been applied successfully for bioproducts derived from amino acids (61). For compounds provided as nutrients in the growth medium, however, sensitivity to their toxic analog may indicate improved transport properties for that class of molecule, thus increasing metabolic flux toward the pathway of interest. One potential drawback of this method (and assaying for sensitivity in general) is that it must be accomplished through replica plating, which has much lower throughput and is more labor-intensive than screening in liquid culture (62).

In instances where the product of interest is known to inhibit the activity of a toxic compound, selection for resistant mutants may result in isolates of improved production (62). This screening method, called selective detoxification, is most applicable to solid media due to its ability to provide each mutant

with a unique chemical environment; liquid cultures allow the product of interest to diffuse and provide resistance to nonproducers, confounding results. It should be cautioned, however, that if alternate pathways to resistance are present, the possibility of encountering screening artifacts may be unavoidable. This method has seen success in generating *Acremonium chrysogenum* variants proficient in detoxifying metallic ions through production of Cephalosporin C (61).

Desirable concentrations of the product of interest may be infeasible for a number of reasons. First, the desired compound may be directly toxic to the cell. Second, the product of interest may participate in an inhibitory feedback loop, which limits its production. The first bottleneck may be alleviated simply by screening for individuals resistant to high concentrations of the desired compound. Mutants deficient in feedback inhibition may be isolated by screening for mutants resistant to a toxic analog of the end product. It is expected that survivors will be deregulated, overproducing the compound of interest and thus diluting the effect of the toxic analog (63).

A particularly clever screening strategy involves the mutagenesis of nonproducing strains, isolated through mutagenesis of a productive parent strain. In theory, productive mutants isolated after this second round of mutagenesis will have had at least two mutations in the relevant biosynthetic genes: an inactivating mutation followed by a mutation that restores productive ability to levels that are (hopefully) higher than the parent strain. An added benefit of this method is the low level of background activity observed, enabling more rapid screening techniques to be employed. Furthermore, revertants are more likely to contain mutations in genes directly related to product synthesis, as opposed to genes whose effect is epistatic (62). This technique has seen success in overproduction of the antibiotic aurodox in *Streptomyces goldiniensis* (63).

As a strain of interest becomes more highly optimized, the likelihood of generating phenotypic changes of large magnitude steadily decreases. Hence, the maximum expected improvement in phenotype may be within the error of the screen. To increase the probability of detecting variants with low (but significant) improvement, a rapid recycling scheme can be implemented (35,59). In this statistical approach, a large rake-off (~10–50%) of mutants are immediately rescreened. This process is repeated multiple times to enrich the fraction of genuinely improved variants, the rate of enrichment corresponding to the magnitude of phenotypic increase. Mutagenesis can be undertaken between rounds of recycling or after isolation and characterization of improved individuals. Due to the power of this technique, screening artifacts can become a major concern if the selective conditions are poorly designed. Although such statistical rigor is recommended throughout the optimization process, it becomes critical to continued isolation of improved variants as phenotypic increases become more marginal and rarer.

A significant number of phenotypes cannot be linked to microbial growth, necessitating the development of alternate screening methodologies. Colorful

or fluorescent phenotypes may be detected spectrometrically (9,64), but for phenotypes that do not exhibit obvious color or fluorescence, a substantial amount of creativity is often required. Identification of a suitable colorimetric assay may be relatively simple for popular phenotypes, but in more specialized cases a solution may have to be developed in-house. In any instance where a large amount of processing is necessary before a phenotype can be measured, screen throughput will be significantly diminished and optimization of assay protocols becomes of paramount importance.

1.4.2 Random Screens

In the absence of any knowledge about the causative factors of the phenotype of interest, a random screen is often the only option for isolating desirable variants. However, the conditions of the screen must be very similar to those of the final production setting of these strains or else screening artifacts will be encountered. The major concern with random screening is the immense library size and screening effort required.

A common way of quickly reducing this library size and isolating interesting variants is known as preselection. In this approach, a crude growth-based correlate of the phenotype of interest is used to eliminate any variants that are not superior to the parent strain. This scheme is especially useful in cases where accurate measures of phenotype are difficult to achieve, thus precluding their use in the entire mutated population. In cases where the phenotype of interest naturally confers a growth advantage, preselection can simply consist of a crude growth-based random screen. Otherwise, when interested in the production of a secondary metabolite, any of the rational screens discussed above may be used (61). Since the aim of a prescreen is to increase the throughput of a selection program, the time savings conferred by the prescreen must be sufficient to make its inclusion worthwhile (59).

1.4.3 Screening Platforms

When the phenotype of interest can be directly coupled to growth, selection based on growth rate offers a simple, high-throughput method for isolating improved variants. Growth conditions are of critical importance in such schemes, as poor choices will result in a high incidence of screening artifacts. In addition to the chemical environment in which selection takes place, the physical environment will also have a significant impact upon which mutants exhibit a growth advantage. The physical environments most commonly used include agar plates, batch culture, and continuous culture.

1.4.3.1 Solid Media The defining feature of solid media for microbial growth is its resistance to diffusion. Not only are individual variants spatially separated, but also any diffusible metabolites remain localized to their parent colony. As mutants are spatially separated, they do not compete with one

another for nutrients, allowing individuals to be isolated, maximizing the phenotype of interest, as opposed to those who use energy to decrease the fitness of other mutants. Additionally, mutants exhibiting significant growth differences are easily discernible by eye or by image processing software. Also, differential secretion of a colorful or bactericidal compound can be identified by the size of "halos" surrounding each colony. However, since colony diameter increases as the cube root of population and halo diameter as the square root of secretion capability, differences among high-producing individuals may not be discernible. To overcome this limitation, phenotypic titer depression, as discussed earlier, may be implemented (60). However, phenotypic advantages in these artificial conditions may not translate to an advantage in a production setting. Indeed, growth conditions on agar plates in general are significantly different from those present in a bioreactor, and as such, testing under more realistic conditions is often necessary to refine the pool of promising individuals. An additional consideration when screening on solid media is the maximum allowable throughput. Although 1 mL of liquid media may contain upwards of 10^8 individuals, a 100-mm plate may only contain 10^3–10^4 in order to allow sufficient time for the phenotype to be expressed before colonies become indistinguishable. Furthermore, high plating densities on selective media may decrease the recovered fraction of mutants due to the Grigg effect (65,66). Briefly, plating a high density of nonviable cells may inhibit the growth of viable ones due to nutrient consumption or secretion of a toxic compound. Therefore, the benefit of colony separation must be weighed against increased throughput when designing such a growth-based screen. Screening programs incorporating agar plates have been used effectively to select for a variety of highly complex phenotypes, including antibiotic production (67), amino acid auxotrophy (68), ethanol production (69), as well as numerous improved tolerance applications.

1.4.3.2 Batch Culture Batch culture is characterized by repeated cycles of exponential and stationary phase growth. Therefore, variants under selection are alternately subjected to rich and starvation conditions. Those mutants that can reproduce the fastest under rich conditions will be preferentially selected as colonists of the next batch culture. Given the exponential nature of bacterial growth, mutants with even a slight growth advantage will come to dominate the final population. Hence, this environment is best suited for isolating strains with reduced lag time and higher growth rates (70). However, growth is essentially the only phenotype that may be selected for using this approach. Since variants are not spatially separated and secreted compounds are freely diffusible, mutants cannot be distinguished based upon their secretory characteristics. Additionally, any mutant that secretes a toxic compound to which the mutant itself is immune will have a selective advantage unrelated to the screen's intended phenotype. These "killer" phenotypes, although seemingly inconvenient, offer the ability to confer a selective advantage to a production strain (through a technique such as protoplast fusion), extending the time over

which a fermentation may take place before contamination occurs (56). It cannot be overemphasized that the selective environment encountered in liquid culture is highly dependent on the microbial ecology. Unlike in solid media, individuals in liquid culture continuously compete for the same nutrients. Hence, selective conditions will change with time as microbial populations change and the superiority of selected variants will, in general, be dependent upon the microbial environment in which they were grown. In other words, the fittest variant among a competitive population may not be superior when considered in isolation. On the other hand, liquid media provides an excellent environment for optimization programs using an organism's natural mutation rate, as improved variants are continuously being generated and taking over the existing mutant pool. Perhaps the best known example of such a long-term evolution experiment comes from Lenski et al., who subjected *E. coli* to batch conditions for 10,000 generations. It was found that individuals present at the end of the experiment had a shorter lag phase and higher growth rate than the strain used to start the experiment. In addition, it was found that most of the competitive advantage was obtained within the first 2000 generations of the culture (34). Although Lenski et al. were not interested in generating an industrially useful phenotype, these results imply that similar techniques would be very effective at generating improved isolates of industrial relevance. Indeed, through cycled batch cultivations of *S. cerevisiae* in glucose, xylose, and arabinose, a variant that obtained the ability to completely ferment all three sugars in almost half the time as the parent was isolated (71). Such an improvement would require a highly detailed understanding of the bottlenecks limiting the consumption of each sugar, including transport, metabolism regulation (to alter diauxie) and carbon metabolism. Characterization of each of these components (not to mention analyzing their interaction) would be an enormous undertaking if a directed approach were to be followed. However, by simply allowing faster-growing mutants to outcompete less fit individuals, a highly desirable solution to this complex problem can be achieved.

1.4.3.3 Continuous Culture Chemostats, in contrast to batch cultures, operate at steady state, with a steady outflow of culture balanced by a corresponding influx of media (at a level below that which would wash out all of the cells). Those individuals that are best able to utilize these low levels of nutrients will have a selective advantage under this condition. Hence, instead of selecting for mutants with a high μ_{max}, as is the case in batch cultures, chemostats select for variants with a low K_s, that is, the concentration of a limiting nutrient (such as glucose) at which the growth rate of an organism achieves half its maximal value. Thus, chemostats tend to select for specialists who can make maximum use of a limiting nutrient instead of selecting for general opportunists of high growth rate, as for batch cultures. In addition, because all individuals share the same nutrient pool, the possibility of forming stable ecologies exists, with the unused nutrients and excreted metabolites of one

population providing nutrients for a second (72). This situation, though problematic for instances requiring monoclonal cultures (e.g., when protoplast fusion with another variant is desired), may be acceptable in other cases (e.g., remediation of a toxic compound). When the chemostat population is largely monoclonal, however, evolution in a chemostat follows a strictly sequential process, with fitter variants deriving from the most populous clone and subsequently replacing it. Since fitness differences are not transitive (due to epistatic effects), it is possible for the fitness of a population to decrease with time, as measured by pairwise comparisons between isolates that are not immediately related to one another (73). In these cases, there is no "best" variant for a particular selective environment due to fitness's dependence on the microbial composition of the chemostat culture. In addition, since chemostats select for populations with high residence time, adherence to bioreactor walls can become a major concern (70). Finally, in comparison to agar plates and batch cultures, the chemostat apparatus can be quite expensive. Nevertheless, chemostats have been quite successful in the development of a wide variety of very complex phenotypes, with results such as altered morphology (23), increased plasmid stability (74), and increased xylose uptake rate (22).

Although the steady-state operation of the chemostat is desirable for some selection programs, it is often necessary to control a particular variable as growth proceeds. The combination of a chemostat with an online controller is referred to as an auxostat. The increased versatility of this instrument (reflected in its increased cost) allows a wide variety of schemes to be implemented during screening. For example, by controlling media flow rate to maintain a constant low cell density, a strong pressure can be applied to select mutants with high growth rate in rich media. In essence, this setup results in a batch reactor with infinite volume, which is useful when the ability to adapt to stationary phase is not required (75). Alternatively, instead of varying the media flow rate to control cell density, the concentration of a toxic compound may be steadily increased, resulting in selection for a tolerant phenotype. The process of continuously changing selective conditions in real time as improved variants emerge is known as interactive continuous selection (ICS) (76). This method has been employed to select for *Streptomyces* mutants tolerant to increasingly high levels of streptomycin, resulting in strains that produce large quantities of this antibiotic (76). Finally, it is possible to simulate a continuous culture through serial batch subculturing in which a fraction of a batch culture is reinoculated into a fresh culture. The growth state of the inoculum and frequency of transfer will dictate how similar the process will be to either batch or continuous.

1.4.3.4 Modern Screening Platforms Although liquid culture allows for a much higher numbers of variants to be screened than solid media, it allows crosstalk between individuals, altering the selective pressure applied to the cells. To overcome this limitation, Naki et al. developed a microtube-based screening system that allows a growth-based selection to be applied in liquid

media while preventing crosstalk (77). It is estimated that this method can provide an order of magnitude increase in throughput as compared with solid media.

When the phenotype of interest results in a visible change at the single-cell level, microfluidic techniques allow multiple orders of magnitude improvement in throughput over other non-growth-based assay systems. In particular, 10^8 mutants per hour may be assayed via flow cytometry, which queries individual cell size and fluorescence. One issue unique to such a sensitive instrument is the ability to detect significant variability among a monoclonal population. Hence, it is possible to isolate what seems like improved variants that reproduce to yield an unimproved population average. Thus, characterization of average cell-to-cell variability is of paramount importance in designing a precise flow cytometric screen. Nevertheless, mutations that increase cell variability may arise, necessitating the use of a rapid recycling scheme allowing cell division between each measurement. Of course, the phenotype of interest must result in a visible difference at the single-cell level, but nevertheless flow cytometry has been successfully employed to enrich for a wide variety of phenotypes. For example, carotenoids exhibit a characteristic fluorescence and are localized to cellular membranes, thus allowing An et al. to select for yeast variants with improved carotenoid production capabilities (9). Furthermore, Tyo et al. implemented a product-specific stain to select for overproducers of poly-3-hydroxybutyrate, a thermoplastic of commercial relevance (64).

Despite efforts to adapt the selection of many phenotypes to high-throughput platforms such as growth cultures or solid media, it is often necessary to screen variants individually in liquid media. For example, secretion of a particular compound may not occur on solid media, or it might be desirable to test isolates obtained via another method under more industrially relevant conditions. In addition, a large cell count may be necessary for more accurate phenotype quantification. In instances where development of a phenotype in 50-mL shake flasks would be too resource-intensive, deep 96-well plates offer a reasonable compromise. Isolates may be grown in up to 2 mL of media in plates especially designed to maximize aeration and prevent cross-contamination (78). Depending on the phenotype of interest, up to 10^4 variants may be assayed per day per technician. Finally, the 96-well format has gained wide acceptance in industry, prompting the development of a plethora of equipment specifically designed for running experiments in this setting.

The development of robotics and microcontrollers during the past 50 years has greatly enhanced the efficiency of selection programs, especially for cases when variants must be kept separate. Screens based on solid media can greatly benefit from automated colony pickers equipped with image analysis software. In addition, more specialized systems exist for inoculating a lawn of bacteria with "plugs" from a plate containing antibiotic-secreting variants to determine inhibition zones and hence product secretion ability. Furthermore, a wide variety of robotic systems designed for manipulation of cultures in the 96-well

plate format have been developed, including media handlers, plate movers, plate storage systems, and plate readers. One important consideration when operating a robotic system is maintenance of sterility, as robotic components comes into regular contact with a large number of cultures. Additionally, robotic screening systems are only as good as their software; interesting or unexpected phenotypes will not be selected unless their characteristics have been programmed into the detection routine. Despite the added complexities associated with operating a robotic system, expenditure of a reasonable amount of care will make the operation of a high-throughput, statistically rigorous screening program much more efficient. (For more information on high throughput fermentation techniques, see Chapter 5.)

1.5 CONCLUSIONS

Complex phenotype optimization via the classical approach is well established in the food and pharmaceutical industry. Improvement of yeast strains for alcohol fermentations has long taken the classical approach due to "generally recognized as safe" (GRAS) classification and ease of selection. This approach has been quite successful in improving complex phenotypes such as complex metabolite profiles, flocculation, and chemical tolerances (79). The success of this approach can be seen in the evolution of the sake fermentation yeast (See Box 1.1). In addition, since the advent and discovery of antibiotics, a long-standing goal has been the increase of titer. The significant improvements seen in these processes have mostly been due to the use of the classical strain engineering approach (see case study in Chapter 6).

The genome-wide mutations induced by classical strain engineering are not as efficient when the desired mutations occur in a single gene. However, when it is desirable to obtain mutations across many genes in the cell (as is often the case with complex phenotypes), the global nature of this approach is an asset. Moreover, there is no need to understand the underlying genetic and regulatory network to direct mutagenesis, as the "space" of possible mutations covers the entire genome, in contrast to rational methods, which require more intimate knowledge of influential genes to be successful. Classical strain engineering, therefore, may return mutants that exploit previously unknown regulatory mechanisms or metabolic pathways, making this approach applicable not only to organisms that are poorly characterized, but also to model organisms. Furthermore, techniques of classical strain engineering can induce previously dormant sections of a genome to become active. Exploitation of these "cryptic genes" would be unlikely in a rational approach to strain improvement, demonstrating the ability of classical strain engineering to find novel and nonintuitive solutions to a design goal (80). An important disadvantage of this method is that the incurred changes are not easily traceable or movable to another host strain. Recently, advances in whole-genome resequencing and

"omics" technologies are beginning to evaluate these strains in hopes of identifying the underlying changes (11) (see Chapter 3). However, this sort of inverse metabolic engineering is seen to be a new frontier at the interface of the classical and rational approaches for complex phenotype engineering (81).

The classical strain engineering approach has long stood the test of time in the fermentation industry due to its ability to consistently generate improved phenotypes using simple techniques. By starting from single base-pair changes and progressively increasing the rate of mutation, the strain engineer can explore ever-more distant reaches of the fitness landscape, eventually traversing wide valleys in single bounds as optimized strains are combined to create individuals for further mutagenesis and improvement. The power of these techniques to improve complex phenotypes lies in the lack of assumptions made in their application. No hypotheses about rate-limiting steps or flux imbalances are needed to generate improved variants, just a well-designed assay and patience. Luckily, with the continued introduction of cost-effective robotic and microfluidic systems, the length of time required for isolation of improved variants will steadily decrease. Further, the use of this technique is readily accepted by both regulators and consumers for the improvement of food organisms. The generality of this approach, however, is often its major downfall. Rational metabolic engineering, with its ability to precisely alter the function of specific genes, is often able to generate improved variants in much less time than classical strain engineering when such detailed knowledge is available (see Chapter 2). Furthermore, the directed nature of such rational techniques allows inferences to be made about the mechanism underlying a phenotype, even when such techniques do not work. On the contrary, successful variants isolated through classical techniques cannot yield any information about underlying causes. As genome sequencing continues to increase in speed and affordability, however, the ability to uncover and rationalize the causes of phenotypic increase in classically engineered variants will increase. Thus, classical techniques promise not only to continue to yield improved strains, but also to elucidate the hidden bases of complex phenotype display in microorganisms.

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