4

Enzyme Engineering by Directed Evolution

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4.1 Introduction

Previous chapters have outlined the huge potential of enzymes as tools for organic synthesis. However, this potential is only slowly being realized in large-scale industrial applications. The main reason for this is that enzymes are often incompatible with the specific requirements of a synthesis, especially under economic constraints. Enzyme behaviors such as substrate or product inhibition, stability, and catalytic efficiency ($k_{cat}/K_m$) are all finely tuned by natural evolution to support efficient reproduction of the organisms that make them. Product inhibition can be useful in a living cell, where it prevents the accumulation of undesired or even toxic products. But it is highly undesirable in a synthesis requiring high substrate concentrations and complete conversion into products. Similarly, an enzyme may naturally be highly substrate specific so as to prevent undesired side reactions with other chemically similar metabolites. But such an enzyme can only be used to synthesize a very limited range of products. Other properties that are highly desirable for chemical applications, such as long-term stability and activity in organic solvents, are simply not required in nature and are therefore not found in natural enzymes. While it is possible to devise effective bioprocess engineering solutions to some of these problems, it will often be necessary or more effective to engineer the catalyst itself.

The previous chapter reviews methods for structure-guided enzyme engineering. A prerequisite for this approach is knowledge of the enzyme structure and detailed insight into how this structure determines function. Then we must be able to predict how specific amino acid changes affect the desired properties. Despite rapid growth in the numbers of enzyme structures solved and the considerable progress made in computational methods, our understanding is still very limited and in most cases insufficient to obtain the desired features with an acceptable probability of success.

The strategy nature uses to adapt organisms to new demands is evolution. According to Darwinian theory, the fantastic diversity of life was created by random mutation and natural selection. The power and simplicity of the evolution
algorithm has tempted scientists and engineers to try to implement this same approach for biomolecular design. In 1984, long after Eigen’s pioneering work on the theory of evolution\(^{[2-3]}\), Eigen and Gardiner suggested the following procedure that “should allow a new type of evolutionary biomolecular engineering”\(^{[4]}\):

10 PRODUCE A MUTANT SPECTRUM OF SELF-REPRODUCING TEMPLATES
20 SEPARATE AND CLONE INDIVIDUAL MUTANTS
30 AMPLIFY CLONES
40 EXPRESS CLONES
50 TEST FOR OPTIMAL PHENOTYPES
60 IDENTIFY OPTIMAL GENOTYPES
70 RETURN TO 10 WITH A SAMPLE OF OPTIMAL GENOTYPES

Scientists wishing to design useful proteins, peptides, or nucleic acids have picked up this evolutionary approach, which is now known as directed evolution, applied molecular evolution, in vitro evolution, or molecular breeding\(^{[5-13]}\). Directed evolution combines a high probability of success (the possibility of obtaining an improved catalyst within months) with no requirement for detailed knowledge of structure, function, or even mechanism. The basic evolutionary engineering approach outlined in Fig. 4-1 has generated impressive results in a few short years, from enzymes that function in organic solvents\(^{[14]}\) and at high temperature\(^{[15]}\) to enzymes that are active towards non-natural substrates\(^{[16]}\) or even carry out whole new reactions\(^{[17]}\). It is now clear that directed evolution will drive biocatalysis into a growing number of commercial settings, including many synthetic applications.

The aim of this chapter is to explain the concepts underlying directed evolution and to describe its application to engineering useful enzymes. In Sect. 4.2, we describe the principles of an evolutionary optimization algorithm. The tools and their implementation in different working strategies of directed evolution are then described in Sections 4.3 and 4.4. The intention is to highlight the main practical and conceptual differences among the various approaches and to compare their strengths and limitations. Section 4.5 discusses specific examples of directed evolution, with a focus on enzymes and properties that are of interest in organic syntheses. Many other important and highly successful applications of directed evolution, such as the design of catalytic antibodies and nucleic acids (ribozymes) or peptides and proteins of pharmaceutical interest, are covered in recent reviews\(^{[13, 18-26]}\).

4.2 Evolution as an Optimizing Process

Without an understanding of the theory of evolution, one may be tempted to consider in vitro evolution an irrational, trial-and-error approach to protein design. However, the beauty of the structural architectures and sophisticated functions that nature has created attest to the power of the evolutionary design strategy. Many theoretical studies of evolution explain this process based on physical principles. The
principles that emerge are very different from those important in traditional "rational" design. Rather than trying to fully understand how mutations affect the structure and function of the enzyme (which is very difficult), the physics of evolution aims to understand the forces that make systems and problems evolvable. That is, what makes proteins so apt for evolution? Moreover, how can this be used to advantage in enzyme design?

4.2.1
The Search Space of Chemical Solutions

To describe evolution as a search process, it is necessary to define the search space. It is convenient to define sequence space as the connected network of all possible amino acid combinations (for a fixed sequence length)\(^\text{[27]}\). For a protein composed of \(A\) different amino acids and a sequence length of \(N\) residues, there are \(N\) sequences, connected by an \(N(A-1)\)-dimensional network. Each point in this vast space has an associated fitness, representing the combination of properties undergoing selection. Together, sequence space and a fitness description construct a fitness landscape on which an enzyme walks towards higher peaks under the influence of mutation and selection\(^\text{[28–30]}\).
Exhaustively searching all possible solutions is impossible as sequence space is extraordinarily large. The mass of all amino acid combinations for 285 residues (about $10^{370}$ possible sequences) would be $10^{300}$ times the mass of the universe, thus creating sequence spaces of a size greater than the power of our imagination\textsuperscript{[31]}. Nature could have explored only a small fraction of the sequence space of proteins during the age of earth. Nevertheless, excellent solutions to biological and environmental challenges have been found. Similarly, \textit{in vitro} evolution experiments have been successful in finding improved molecules, although \textit{a priori} success probabilities may seem to be prohibitively small. There are many successful examples where only a very small fraction of the many possible sequences have to be explored to find mutants with improved properties. The key is to develop experimental algorithms that optimize exploration subject to practical limitations.

4.2.2

The Directed Evolution Algorithm

Spontaneous mutations, recombination and selection are the tools of evolutionary design. Plant and animal breeders who influence properties of the offspring by choosing parents with the desired traits have been successfully using these tools for millennia. Following their lead, molecular biologists first employed selection strategies that acted on spontaneously generated bacterial mutants with the goal of developing new metabolic pathways\textsuperscript{[32, 33]}. Solutions found by these approaches often resulted from complicated changes in regulatory genes, transport proteins, or the activation of silent genes. Whole pathways are targeted by the evolution experiment when such solutions are desired. However, if the target is a specific enzyme, it is not desirable to produce solutions that are not directly related to the enzyme.

The milestone techniques of cloning and \textit{in vitro} recombination of genetic information\textsuperscript{[34]} and other advances in molecular biology, such as the development of the polymerase chain reaction (PCR)\textsuperscript{[35]}, allow carefully controlled directed evolution experiments. Researchers are now able to specifically engineer the enzyme of interest and control the rate of mutagenesis and focus mutations towards specific regions within the gene. Furthermore, methods are now available to reconstruct "sexual" recombination \textit{in vitro}\textsuperscript{[36–38]} as well as in recombinant cells\textsuperscript{[39, 40]}. In addition, using screening and \textit{in vitro} selection methods, we can control the selection pressure independently of constraints in living cells, thus allowing acquisition of properties never required in nature. Technologies are available to create protein libraries of up to $10^{13}$ molecules and select them within a few hours or days\textsuperscript{[41]}.

All of these tools allow us to implement the evolutionary design algorithm \textit{in vitro} and accelerate it to create molecules with desired properties in a fraction of the timescale of natural evolution. The success of evolutionary protein design is highly dependent on the thoughtful combination of methods for creating diversity and searching the mutant population that is generated. Optimizing an evolutionary search has been well studied in the computer science genetic algorithm literature\textsuperscript{[42, 43]}. Some of these results can be applied to directed evolution, including
determining appropriate mutation and recombination rates, optimal recombination parameters, and the appropriate screening effort\textsuperscript{[44]}.

### 4.3

Creating a Library of Diverse Solutions

Given the high cost (both in terms of money and time) of analyzing a mutant library, the goal of the diversity-creating step is to produce mutant libraries that are rich in variants with improved properties. To achieve this, the few positive mutations that might occur on a gene cannot be diluted with many neutral or deleterious mutations. The level of mutant redundancy also affects the quality of the molecular diversity. Redundancy must be low because screening or selection efforts are wasted on testing identical mutants. In this section, we will first describe different approaches to creating mutant libraries, including mutation and recombination.

#### 4.3.1

Mutagenesis

A commonly used strategy to create mutant libraries is to target the whole gene for random point mutagenesis. Nucleotide mutations are typically introduced by error-prone PCR, mutator strains, or by treatment of the isolated DNA with chemicals or UV light. The success of this approach depends critically on using an appropriate error rate. If the error rate is too low, inadequate diversity is created and screening is wasted on large numbers of redundant parent enzymes. On the other hand, if the error rate is too large, the fraction of positive mutants also becomes very low and the search for improved mutants is wasted on screening inactive clones.

A serious limitation of the random mutagenesis approach comes from the degeneracy of the genetic code and the biases of available methods, for example the preference for transitions over transversions. Together, these effects limit the amino acid substitutions that are accessible by DNA point mutations. A combination of a stepwise random mutagenesis approach with methods of focused mutagenesis and recombination can circumvent some of these limitations. The different requirements, limitations, and advantages of the most commonly employed methods are summarized in Table 4-1. In practice, a good strategy is to use a combination of methods.

#### 4.3.1.1

Random Point Mutagenesis of Whole Genes

Before the introduction of the polymerase chain reaction (PCR)\textsuperscript{[35]}, point mutations were usually produced by UV radiation, by chemical treatment\textsuperscript{[45]} or by using mutator strains that have an increased mutation rate compared to normal strains because of defects in their DNA-repair mechanisms\textsuperscript{[46]}. Chemical mutagenesis\textsuperscript{[47]}, mutator strains\textsuperscript{[48, 49]}, and even spontaneous mutations coupled with selection in a
Table 4.1. Comparison of methods for creating genetic diversity for directed evolution.

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Advantage</th>
<th>Limitation</th>
</tr>
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<tbody>
<tr>
<td>Random point mutagenesis</td>
<td>None</td>
<td>Exhaustive</td>
</tr>
<tr>
<td>Focused mutagenesis</td>
<td>Structural information or knowledge from previous generations</td>
<td>Reduced library size; multiple simultaneous mutations possible</td>
</tr>
<tr>
<td>Recombination</td>
<td>Recombine positive mutations; remove neutral and deleterious ones</td>
<td>No multiple simultaneous mutations; recreates large number of already known sequence</td>
</tr>
<tr>
<td>– single gene</td>
<td>None</td>
<td>“Functional diversity”; large jumps in sequence space</td>
</tr>
<tr>
<td>– family shuffling</td>
<td>Homologous genes</td>
<td></td>
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</tbody>
</table>

chemostat culture\[^{50}\] are still used for random point mutagenesis in directed evolution experiments. However, the dominant method is now error-prone PCR because the protocols are very straightforward, safe, fast and versatile, and allow simple adjustment of the error level. Point mutations are introduced by PCR because of erroneous incorporation of a nucleotide during the amplification of the target gene. Under normal reaction conditions, the error rate of the Taq DNA polymerase is about 0.001% to 0.02% per nucleotide per replication cycle\[^{51, 52}\]. Although this error rate is sufficient to create libraries of large genes\[^{53}\], it is too low for efficient mutagenesis of small genes. However, the fidelity of the PCR can be reduced by changing the reaction conditions (e.g., increasing the concentration of MgCl₂, adding MnCl₂ to the reaction mixture, increasing and unbalancing the concentrations of the four dNTPs, adding deoxynosine triphosphate (dITP), increasing the concentration of Taq polymerase, or increasing the extension time and cycle numbers\[^{54–58}\]). These methods can result in error rates as high as 2% per nucleotide position. The possibility of using of low fidelity Taq mutants has been described\[^{59}\], but has yet to be explored experimentally.

By changing some of the PCR conditions, the error rate can be adjusted according to the gene length to produce the desired average number of amino acid substitutions. A frequently used method to estimate the level of mutation (a quality check of the diversity) is to determine the fraction of inactive clones from small samplings of the generated mutant libraries\[^{60, 61}\]. However, the statistical distribution of muta-
tions (which should be narrow) cannot be estimated by this method, and the relation between the fraction of inactive clones and average number of amino acid substitutions can differ for different enzymes. Therefore, the statistical distribution of mutations and the relationship between inactive clones and the number of mutations are determined by sequencing randomly picked mutants.

Another consideration is the distribution of mutation type. Typically, there is a strong bias for transitions (A→G or T→C) over transversions (C→G or G→C), which limits the accessible amino acid substitutions. There are protocols to reduce this bias, but they do not completely eliminate it\(^{[55, 60, 62]}\). In addition, the structure of the genetic code limits the accessible amino acid substitutions. Depending on the specific codons, only 24–40% of the possible amino acid changes are accessible by single base substitutions\(^{[63]}\). Furthermore, the accessible substitutions are more likely to be conservative with similar physicochemical properties. For large genes and small error rates in whole-gene mutagenesis, it is very unlikely that two DNA mutations will occur in the same codon, dramatically reducing the possible amino acid substitutions.

Although little is known of the cost of these constraints in directed (and natural) evolution, several studies have shown that the best mutations at specific sites required multiple substitutions in a single codon\(^{[64, 65]}\). Methods that introduce diversity at the codon level might therefore be preferable to methods that create point mutations at the nucleotide level\(^{[63]}\). Methods available for codon-level mutagenesis of a few amino acid positions are unfortunately very cumbersome and expensive for mutagenizing whole genes, leaving room for future developments of improved mutagenesis methods.

### 4.3.1.1.1 Optimal Mutation Rates: Experimental

One rule-of-thumb has been to adjust the error rate according to the number of targeted amino acid residues and the size of the screen, such that a significant fraction of the total number of combinatorial possibilities can be sampled\(^{[66]}\). For an enzyme of 300 amino acid residues, there are about six thousand possible single mutants, sixteen million double mutants, and thirty billion triple mutants. It is generally within our ability to exhaustively screen a single-mutation library, whereas double-mutation libraries already exceed the throughput of most screening methods (typically < 10\(^6\)). Standard selection methods allow a throughput of about 10\(^8\) mutants and can therefore sample most double mutants. In vitro selection methods might push this limit somewhat higher (10\(^12\)). The assertion that it is desired to sample most of the combinatorial possibilities is based on the assumption that beneficial mutations are rare and the probability is very small that multiple random amino acid substitutions are beneficial.

An error rate resulting in single or double mutation libraries has been found to be a good compromise between creating adequate diversity while limiting the screening effort. In practice (e.g., Table 4-3), significant improvements in enzyme activity, stability, selectivity, folding, and expression have been achieved by the stepwise accumulation of single amino acid substitutions. Even large changes in substrate
specificity\textsuperscript{[62]} and inversion of enantioselectivity\textsuperscript{[65]} can be achieved by this conservative approach. The obvious disadvantage of this approach is that some properties will benefit from simultaneous mutation of multiple amino acids. These solutions will not be found.

Arguments for low error rate mutagenesis were recently challenged by several researchers. Zaccolo and Gheradi produced $\beta$-lactamase libraries with error rates that they claim generated 5–16 amino acid changes per mutant enzyme per generation\textsuperscript{[67]}. The libraries ($\sim 10^4$–$10^5$ clones) were selected for increased resistance to the antibiotic cefotaxime over three generations. However, the best mutant differed in only three effective amino acid substitutions from wild-type and is therefore much less mutated than would be expected from three generations of $>5$ amino acid changes per generation. This indicates that the applied average error rate was actually much lower, and a more suitable average mutation rate would have been three amino acid substitutions. Another recent study by Georgiou and coworkers indicates that a high mutation rate was appropriate in improving the affinity of a single-chain antibody\textsuperscript{[68]}. When screening for improved affinity, they found that the most improved mutants were observed in libraries created with moderate to high mutation rates (3.8–22.5 mutations/gene). In the next section, we discuss the circumstances under which a higher mutation rate is advantageous.

4.3.1.1.2 **Optimal Mutation Rates: Theory**
In nature, the spontaneous mutation rate is tightly controlled. During one replication round, about $3 \times 10^{-3}$ mutations occur in the genome of human cells, which is about the same level as in *Escherichia coli*\textsuperscript{[69]}. RNA viruses, such as HIV, have a much higher mutation rate, typically on the order of one mutation per genome per replication round\textsuperscript{[70]}. Such a high mutation rate is crucial for the viruses to survive the attacks of the immune system. However, there is a maximum mutation rate, above which the requirement of inheritance for evolutionary optimization breaks down (referred to in quasi-species theory as the error threshold\textsuperscript{[71]}). At the mutation rate just before this threshold, the speed of evolution is highest\textsuperscript{[3, 72]}. Interestingly, it was demonstrated that the mutation rate of the replication machinery of fast evolving RNA viruses is indeed close to this error threshold\textsuperscript{[73]}. For directed evolution of enzymes, the optimal mutation rate maximizes the speed of the adaptive walk and is influenced by the number of mutants that can be screened and the structure of the fitness landscape. As discussed in the previous section, the general rule has been to use a mutation rate for which the permutations can be effectively sampled during screening. However, if the fitness landscape is amenable to a high mutation rate, fewer mutants must be screened in order to achieve the benefit of a higher mutation rate. For example, it would require a smaller library than all double mutant permutations to benefit from a mutation rate of two per sequence. In this section, we explore how the optimal mutation rate is influenced by the number of mutants that can be sampled, the fitness of the parents, and the ruggedness of the fitness landscape.

The optimal mutation rate depends on fitness of the parental sequence. For a sub-
optimal sequence, a large mutation rate allows a greater sweep of sequence space. However, because the probability of finding improved mutations decreases as the fitness of the sequence increases, adaptation via a large mutagenesis rate is rapid at first, then slows. If the parent is highly optimized, the probability that a mutation is deleterious is higher. The accumulation of deleterious mutations is more rapid and these mutations quickly erode the few positive mutations that occur. By using a Markov chain analysis to study genetic algorithms, Mühlenbein found that there should be approximately one amino acid substitution per sequence for highly optimized sequences\textsuperscript{[74]}. His analysis also suggested that the optimal mutation rate should decrease as the fitness of the parent increases. In several independent studies, it was demonstrated that the solution of an evolutionary search is improved when the mutation rate was decreased over time\textsuperscript{[75–78]}.

A higher mutation rate dramatically increases the fraction of mutants in the library that contain stop codons, requiring a larger screening effort\textsuperscript{[79]}. For instance, if the average number of DNA mutations per gene is five, over 20% of the resulting library will contain stop codons. The quality of the mutant library can also be degraded by the accumulation of deleterious mutations, an effect that is exacerbated by the landscape ruggedness\textsuperscript{[79]}. For the mutation of a highly coupled residue to generate a fitness improvement, it is necessary to optimize all the other residues to which it is coupled. Ideally, the optimal mutation rate equals that of the maximum number of residues involved in a single coupled interaction, thus assuring that the sequence will not become trapped in a local optimum. However, the finite number of mutants that can be screened imposes an upper limit on the mutation rate. Therefore, the optimal mutation rate decreases as the landscape ruggedness increases. This observation is similar to the long-jump mutagenesis strategy suggested by Kauffman\textsuperscript{[80]}. By making moves that are larger than the correlation length (smoother landscapes have larger correlation landscapes), more space can be explored. Quasi-species theory also predicts that smoother landscapes have higher optimal mutation rates\textsuperscript{[81]}.

Because real protein fitness landscapes are undoubtedly highly anisotropic, they contain many correlation lengths, and different regions of the sequence will have different optimal mutation rates\textsuperscript{[44, 82]}. A highly coupled region (such as the catalytic site) has a small correlation length; thus a smaller mutation rate is allowed with a limited mutant library. Based on some simplified simulations, it was found that the probability of picking a mutant that has a highly coupled mutation decreases significantly as the sequence increases in fitness\textsuperscript{[44]}. This effect intensifies as the number of interactions that are coupled to the mutated residue increases. From this observation, it follows that when the screening effort is limited, uncoupled regions of the protein should be targeted for mutation. More highly coupled residues require a larger rearrangement of amino acids than is likely given the limited mutation rate. Avoiding the regions of high coupling decreases the total number of residues undergoing mutagenesis. To utilize this observation, it is necessary to have experimental techniques to target specific positions as well as methods that can be used to predetermine the coupling of each residue. These goals are the subject of the following two sections.
Focused mutagenesis strategies are used with the intention of enriching a library for desired mutants. To reduce screening efforts, the targeted region can be reduced from 300 to only a few residues (Fig. 4-2). The library of quintuple mutants has a theoretical size of only $-10^6$ mutants, compared to $10^{16}$ if the entire gene is targeted. This reduced library can be searched exhaustively with currently available methods. Focused mutagenesis significantly reduces screening requirements for libraries of mutants with multiple amino acid substitutions and eliminates the codon bias of PCR. However, it imposes obvious limitations on the possible solutions and can fail to explore the most effective mutations.

Targeting single amino acids ("saturation mutagenesis") is straightforward because of available strategies that eliminate laborious subcloning steps. Several commercial kits are available, such as the Transformer™ (CLONTECH Laboratories, Palo Alto, CA, USA), Altered Site® II (Promega, Madison, WI, USA), and QuickChange™ (Stratagene, La Jolla, CA, USA) site-directed mutagenesis systems, which can produce targeted mutant libraries in one day. This approach has been used to target amino acid positions that random point mutagenesis identified as important for the targeted enzyme properties. Variants with improved proper-

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**Figure 4-2.** Plot showing the number of possible protein variants ($\gamma$) that can be created given the number of amino acid positions ($M$) changed simultaneously and the sequence length ($N$) ($\gamma=19^M(N-1)!/(N-M)!M!$). From a given standard screening throughput ($<10^6$ clones) one can calculate that it is not possible to exhaustively screen protein libraries of all possible double mutants of an average sized protein.
ties were identified in saturation mutagenesis libraries that contained mutations not accessible by random point mutagenesis.

Methods for randomization of specific regions of a gene typically employ randomized oligonucleotides or PCR mutagenesis of small stretches of the gene. The required diversity is introduced by randomized oligonucleotides that are produced by an automatic (programmable) DNA synthesizer. If only one amino acid position is targeted, the specific codon can be completely randomized (saturated) by adding an equal amount of all four bases (A, T, G, C) during the oligonucleotide synthesis at the first two positions of the triplet and with a mixture of G and C at the third position to exclude stop codons (referred to as NN(G,C)-mutagenesis).

To target multiple amino acid positions simultaneously within a small region of the gene, oligonucleotide-cassette mutagenesis is often used. This method was among the first applied for in vitro evolution of DNA sequences [86] and has also been used successfully for the evolution of various enzymes [59, 87-90]. When suitable restriction sites are not already present, they are introduced by site-directed mutagenesis adjacent to the targeted region. These restriction sites are then used to substitute the wild-type region with the synthetic randomized DNA duplex (cassette) [91]. Depending on the number of targeted amino acid positions, randomization strategies are used to facilitate an exhaustive search of the library. Complete randomization of all targeted codons is preferred if few amino acid residues are targeted and can be done as described above for the randomization of single sites by using NN(C/G) during oligonucleotide synthesis. It was also reported that trinucleotide analogs such as 9-fluorenylmethoxycarbonyl (Fmoc) trinucleotide phosphoramidites can be used during DNA synthesis to achieve a codon-based mutagenesis [92].

If several distant positions are targeted simultaneously, cassette mutagenesis is technically cumbersome. Other methods allow the efficient assembly of several randomized oligonucleotides to whole genes. For example, recursive PCR [93], the ligase chain reaction [88, 94], or in vitro assembly of whole genes [95] can be used to construct targeted mutant libraries. Partial randomization of many positions most frequently employs spiked oligonucleotides, which are produced by DNA synthesis using a mixture of the wild-type base and equimolar amounts of all four bases [96]. The oligonucleotides can also be produced by error-prone PCR. It is possible to calculate the appropriate compositions of the nucleotide mixture in order to encode whole sets of amino acids at certain positions [97].

4.3.1.3
Calculation of Mutagenesis Hot-Spots

In focused mutagenesis experiments, the challenge is to identify the residues where mutagenesis is likely to be beneficial. Indeed, many successful directed evolution experiments show that mutations occur in regions that would be hard or impossible to predict (and difficult to explain that they do), even when a high-resolution structure and much information about the enzyme is available [14, 98-100]. One possibility is to make use of knowledge gained from early rounds of random point
mutagenesis that targeted the whole enzyme. The content of the mutant library can be improved by only mutating sites that do not severely disrupt stability. A structurally tolerant protein allows more mutations, and therefore more potentially beneficial ones, making it more likely that there is a connected path in sequence space of single mutations that leads to regions of higher fitness. By reducing the evolutionary search to regions of sequence space that retain structure, functional space can be explored more thoroughly. This concept can also be inverted: if the goal is to improve stability while retaining functionality, then eliminating the sequence space inconsistent with the function improves the search.

Several groups have proposed targeting mutagenesis to residues where natural diversity is observed. Fersht and coworkers reengineered the tumor suppressor p53 by creating a small library of mutants where the hot-spots were determined from a sequence alignment of 23 homologous proteins. The mutations were made in the wild-type sequence background, and several were found that improved stability. Using a similar methodology, Lehmann et al. constructed a thermostable phytase from the consensus sequence of 13 homologous proteins. The mutant phytase exhibited a 15–22 °C increase in melting temperature.

Alanine scanning has been widely used to identify the residues which are contributing to various protein properties. Alanine substitutions are made at various positions and the perturbation in the property of interest is measured. This has several potential applications to directed evolution. For instance, it can be used to predetermine which positions are essential to the structure (or function) of the protein and therefore should be avoided. Conversely, positions that tolerate the alanine substitutions may be good candidates for saturation mutagenesis. Unfortunately, this procedure is tedious. To surmount this difficulty, Kollman and coworkers proposed a method to determine the effects of alanine substitutions computationally. Kollman's method could be used to scan the protein structure for positions to mutagenize in directed evolution.

The observation that some sequence positions are more tolerant to mutation initiated the application of information theory to studying the importance of these residues to structure and function. The sequence entropy can be calculated from the probability distribution of allowed amino acids substitutions at each residue. Using simulations of evolution on fitness landscapes, Voigt et al. predicted that beneficial mutations are found by directed evolution at amino acids that are largely uncoupled to other sites (Figure 4.9). To test this prediction, they compared the calculated site entropies with mutations found from previous evolution experiments on subtilisin E and T4 lysozyme. The sequence space considered in the subtilisin E computation was enormous: 10^43 amino acid combinations (274 residues). Seven out of the nine mutations that improved the thermostability of subtilisin E occur at positions computed to be highly tolerant. Mutations that improved activity in organic solvent similarly occurred at high-entropy positions. This calculation may be used to determine the positions where improvement will likely be found in an evolution experiment.
4.3 Creating a Library of Diverse Solutions

4.3.2 Recombination

Another approach to creating genetic diversity is based on DNA recombination. Multiple positive variants are used to parent the next generation, which allows for recombination of beneficial mutations, elimination of deleterious mutations as well as creation of new diversity. Using recombination also has a practical advantage. More than one mutant may show improved fitness during a single screening effort. Allowing only the fittest mutant to continue to the next generation can be wasteful (Fig. 4-3). For highly non-additive (rugged) fitness landscapes, recombining positive mutations is less certain to be beneficial because combining individually good mutations can have deleterious effects. In this discussion, we focus on the experimental techniques for recombination and describe the theoretical basis for the optimal parameters.

4.3.2.1 In Vitro Recombination

*In vitro* recombination of DNA, often referred to as DNA shuffling, was introduced by Stemmer for evolutionary protein design [36, 37]. The method is based on recursive PCR, which allows for whole gene synthesis from several DNA fragments [93]. As outlined in Fig. 4-4, onc or several parental genes are cut by enzymatic digestion using the endonuclease DNase I in the presence of Mg$^{2+}$. This generates overlapping DNA fragments that are randomly distributed over the gene. The isolated DNA fragments are then reassembled in a PCR-like reaction with denaturation, annealing, and extension steps, during which recombination occurs through the reannealing of DNA fragments from different parents.

![Diagram of In Vitro Recombination](attachment:image.png)

Figure 4-3. Comparison of the progress of evolution for a random mutagenesis approach (A) where the best mutant is used as parent for the next cycle of mutagenesis and screening and a DNA-recombination approach (B) where several improved mutants are used as parents for the next generations. Not shown here is any additional screening cost associated with finding several improved variants in each generation.
Diversity is created by combining parental mutations and random point mutations which are introduced at a rate of about 0.7% because of the intrinsic error rate of Taq DNA polymerase.\(^{37}\) A high error rate, however, can mask the relationship between evolved phenotype and combined parental mutations. Using different DNA polymerases and substituting Mg\(^{2+}\) with Mn\(^{2+}\) during DNase I digestion reduces the mutation rate to as little as 0.05%\(^{61}\). A high-fidelity protocol is also important if recombination is used to distinguish between functional and non-functional mutations or for structure-function studies of evolved sequences\(^{111}\).

In normal PCR, recombination can also occur at a low rate. This is caused by incomplete extension of primer during the extension cycle and annealing to a different template\(^{112-114}\). Increasing the recombination efficiency of incomplete primer extension motivated development of the staggered extension process (StEP)\(^{115}\). The basis of StEP is repeated switching of the template caused by fast annealing and extension cycles (Fig. 4-5). During each cycle, the growing oligonucleotide can randomly anneal to different templates and thereby combine information from different parents. A further method for \textit{in vitro} DNA recombination is the random-priming method (RPR)\(^{38}\), which involves production of gene fragments by annealing and extension of random-sequence primers. The fragments are reassembled as in the Stemmer procedure.

StEP, RPR and Stemmer's method were compared based on their recombination efficiency of truncated GFP genes\(^{116}\). The Stemmer method using small fragments (<100 bp) and StEP yielded the highest recombination efficiencies. However, the efficiencies may differ from gene to gene and are highly dependent on the experimental conditions chosen. Detailed protocols for RPR, StEP and the Stemmer method are given in\(^{115}\).

![Diagram of recombination process](image)

\textbf{Figure 4-4.} \textit{In vitro} recombination by DNA shuffling as described by Stemmer\(^{117}\). Parent genes carrying mutations (indicated by X) are digested with DNase I and randomly reassembled in a cyclic PCR-like reaction to yield a library of recombined genes. New point mutations are introduced (indicated by underlined X) during the reassembly reaction.
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Figure 4-5. *In vitro* recombination by the staggered extension process (SteP)\(^{119}\). Only one primer and single strands from two parents are shown. Fast annealing and extension cycles during PCR of the parent genes cause template switching of the extending strand. After full-sized recombined genes are synthesized, parent genes are removed by treatment with DpnI.

It is possible to recombine any number of parent genes with the available methods, which raises the question of what is the optimal number. Similar to determining the optimal mutation rate for random mutagenesis, the answer will depend on the number of screened mutants and the additivity of the combined mutations. It could be advantageous to screen all the permutations of mutations from the parents. Assuming independent and additive recombination, the probability \( P_d \) that an offspring has \( d \) mutations is given by

\[
P_d = \frac{T!}{(T-d)!d!} \left( \frac{1}{M} \right)^d \left( \frac{M-1}{M} \right)^{T-d}
\]

where \( M \) is the number of sequences and \( T \) is the total number of mutations\(^{117}\). Unfortunately, only 25% of the sequences in the recombination library have novel combinations of mutations because there is a statistical disadvantage for the presence of new combinations of mutations. The probability of creating a mutant that contains all mutations is only \((1/M)^T\). If all mutations from four parents (each having two mutations) are recombined, the probability of creating an offspring gene with all the parental mutations is only \(1.5 \times 10^{-5}\). This probability decreases rapidly as the number of mutations and parent genes increase. The probability is further reduced if recombination is accompanied by a high error rate. It is clear that recombination of large pools of sequences quickly reaches the throughput limitation for available screening or selection methods if the mutant containing all mutations needs to be sampled.

One strategy for reducing the screening requirements is to divide the recombination experiment into multiple generations\(^{117}\). The following example demonstrates the advantage of this procedure when the goal is to combine eight mutations onto a single gene from four double-mutant parents. The experiment is divided such that
two libraries are created using two parents each. In each library a mutant is created that contains all four mutations of its parents. The probability of creating this quadruple mutant is \( p = (1/2)^4 = 1/16 \). All recombinant mutants at this step can be sampled by screening only 32 clones (some oversampling will be required). If the quadruple mutants from each library are then recombined to create a mutant with all eight mutations, the probability of creating it is \((1/2)^8\). In sum, screening fewer than 300 clones \((16 + 16 + 256)\) would be sufficient, whereas the simultaneous recombination of all four mutants to create the same mutant in one recombination experiment would require screening about 65 000 clones. The success of this procedure also relies on the additivity of the mutations. If some of the mutations are non-additive, then combining all mutations is not guaranteed to be optimal. In addition, not all combinations of mutations are screened. If a particular double or triple mutant is the fittest, it may not be found (see pooling strategies, Sect. 4.2.3). Note that if the mutations to be combined were discovered in previous generations, then it is likely that they are relatively additive (or uncoupled, see Mutagenesis Hot-Spots, Sect. 3.1.3).

All of the recombination methods described above require considerable sequence identity among the parents for crossovers to occur. For example, in the Stemmer method, the fragments require a minimum nucleotide sequence identity to reanneal and form an offspring gene. Non-homologous recombination methods seek to remove the sequence identity restriction from the recombination process. Ostermeier et al.\(^ {118, 119} \) describe a method based on generation of N- or C-terminal fragment libraries of two genes by progressive truncation of the coding sequences with exonuclease III followed by ligation of the products to make a single-crossover hybrid library. An intrinsic problem of this approach is that random ligation of two DNA fragments results in two-thirds out-of-frame sequences, yielding mostly non-functional products. In addition, recombination of more than two parents is hard to realize. Another approach to create a mutant enzyme library is based on the permutation of modules or secondary structure units\(^ {120, 121} \). It is not yet known to what extent non-homologous recombination generates useful genetic diversity, rich in improved functions.

4.3.2.2

*In vivo* Recombination

*In vivo* homologous DNA recombination mechanisms are known for various host cells such as *E. coli*\(^ {139} \) or *Saccharomyces cerevisiae*\(^ {40} \) and have long been applied in protein engineering purposes, for example to shuffle mammalian P-450 substrate specificities\(^ {95} \). Because of its simplicity, it is an attractive tool for directed evolution of enzymes\(^ {122, 123} \). The most common system is based on the ability of *S. cerevisiae* to rescue plasmids with a double-bond break by intermolecular homology-dependent recombination\(^ {124} \). A plasmid is cut by restriction enzymes and transformed into yeast together with different genes or fragments thereof. Recombination at homologous positions on both sites of the gap and within homologous regions of the fragments yields a functional, self-replicating circular plasmid. The reconstitution of
the functional plasmid allows for easy detection of recombination events based on the selection marker of the plasmid. Besides its simplicity, an important feature of in vivo recombination is that additional point mutations are extremely rare because of the high fidelity recombination mechanism in yeast. This is particularly advantageous when the aim is solely to recombine positive mutations or eliminate deleterious and neutral ones. In vivo methods are unlikely, however, to generate as many crossovers as in vitro methods.

Volkov et al. described a hybrid in vitro-in vivo recombination method involving formation of a heteroduplex between two homologous sequences in vitro and transformation into bacterial cells[125]. Mismatches present in the heteroduplex are randomly repaired by the host cell, creating recombinant sequences composed of the elements of each parent. This approach may be particularly useful in recombining large genes or entire operons.

4.3.2.3

Family Shuffling

Recombination of homologous parent genes, referred to as “family shuffling” or “molecular breeding,” introduces an additional dimension to creation of molecular diversity for directed evolution[126]. While random point mutagenesis and local recombination explores only the sequence space covered by neighboring mutants, and focused mutagenesis covers only a small fraction of the whole sequence space of the protein, family shuffling explores more distant regions of the sequence space with a lower sampling density. Since the diversity is usually created by a combination of mutations from parents that were previously selected in nature to be functional, it is suggested that family shuffling provides functional diversity that could accelerate evolution[126].

Several recent studies attest to the power of this approach[126–131]. Using a family of four genes from different species, a comparison to single-gene recombination suggested an evolutionary advantage for family shuffling[126]. Recombination of four cephalosporinase genes (57%–82% identity in amino acid sequence) and screening of 5×10⁴ clones yielded a mutant with a 540-fold increased moxalactam resistance compared to wild-type. The mutant that was generated by family shuffling was an offspring of three out of the four parent sequences and contained 33 new amino acid substitutions. The large number of new point mutations raises the question of how so many could be tolerated. It is unclear whether these mutations contribute to the improved β-lactamase function.

An important characteristic of the family shuffling approach is that the diversity changes after each generation. During early generations, many different combinations of mutations are tested, and the best combination becomes fixed during subsequent generations. After fixation, new diversity is created only by additional random point mutations inherent to the recombination method. Thus, the most promising distant regions in sequence space are explored by recombination, followed by step-wise mutagenesis towards the fitness optimum. This hybrid methodology might take more than the maximum of four generations of recombinat—
tion that have been used so far in family shuffling experiments\textsuperscript{[129]}. Based on studies of genetic algorithms it was also proposed that the recombination process with additional mutations provides a powerful method for finding higher fitnesses\textsuperscript{[132]}.

Family shuffling should gain increasing importance with the greater availability of homologous genes as a result of the rapid accumulation of new sequences in public databases. The restrictive licensing policies for the known recombination technologies might be limiting the commercial use of recombination methods at the moment. However, this situation and technical limitations of the existing methods are stimulating the development of new and improved recombination methods.

4.4

Finding Improved Enzymes: Screening and Selection

Given a thoughtful strategy to generate a mutant library, developing a method to detect positive mutants is probably the single most important step determining success (or failure) of directed evolution. Screening refers to a qualitative or quantitative assay of each single clone or few pooled clones of a mutant library, whereas selection refers to methods that enrich positives in a pool of all members of a mutant library or, even better, allow growth only of desired variants (extensively reviewed in\textsuperscript{[133–135]}). In directed evolution, diversity is created on the genotype (DNA) level, whereas screening and selection acts on the phenotype (protein) level. A physical link between the genotype and phenotype is required because direct amino acid sequencing of positive mutants is not feasible and because DNA or RNA is required for replication of the desired mutants as well as for introduction of diversity in subsequent cycles of evolution. For the evolution of enzymes, the most simple, versatile and therefore most commonly used approach to couple genotype and phenotype employs recombinant cells. Genes are introduced (transformed) into cells and translated into proteins by the cell’s natural transcription-translation machinery. For organisms such as \textit{E. coli}, \textit{Bacillus subtilis}, and \textit{S. cerevisiae}, efficient transformation protocols are available that allow for the production of reasonably sized mutant libraries of $10^5$–$10^9$ different members. In addition, recombinant protein expression is well established for those organisms, making them the current preferred choices for directed evolution experiments. In this section, we focus on general principles for searching mutant libraries and discuss important characteristics of available systems such as throughput, error level and versatility. We also discuss theoretical approaches to determining the required screening effort, analyzing the immense amount of data that are generated during the screening step and the theoretical advantages of different screening strategies.
4.4 Finding Improved Enzymes: Screening and Selection

4.4.1 You Get What You Screen For

The first rule of directed evolution is “you get what you screen for.” The importance of establishing appropriate screening or selection methods cannot be overestimated. Conditions used for screening or selection should be as close as possible to the conditions where the enzyme is going to be applied. This includes the actual substrate, its concentration, pH, buffer, salt, temperature, co-solvents, and any other conditions that affect the enzyme behavior. For example, the screen can be established conveniently using an artificial substrate, the enzymatic products of which produce color or fluorescence. However, optimizing an enzyme on an artificial substrate bears the risk that gains in performance will disappear on the desired substrate. This is true for virtually all enzyme behaviors. If compromises in reaction conditions or substrates cannot be avoided, the risk of obtaining undesired solutions during several rounds of directed evolution can be reduced by using a secondary screen under more “real” reaction conditions or by testing the chosen mutant(s) with the actual substrate after each cycle of directed evolution\textsuperscript{[99]}. For \textit{in vivo} selection, which uses the indirect measurement of cell growth as an indicator of enzyme performance, one has to be aware of the immense flexibility (and creativity) of living organisms to circumvent selection pressures by inventing new solutions unrelated to the enzyme and its property we wish to optimize. Many such examples are well known from studies of metabolic acquisitions through laboratory selection, where strong selective pressure uncovered solutions to biochemical blocks\textsuperscript{[32, 33, 130]}. Directed evolution experiments using combinatorial mutant libraries have also found complementation that was caused by activating a novel gene locus rather than by the mutated enzyme\textsuperscript{[137]}. Starvation under selection conditions may induce low-fidelity polymerases and speed up the evolution of new solutions\textsuperscript{[138, 139]}.

4.4.2 Screening Strategies

Screening methods allow enzyme behavior to be measured independently of biological function. Novel enzyme properties can be explored, such as activity or stability in unnatural environments (e.g., extreme pH, temperatures, or organic solvents) or activity on unnatural substrates. These properties are impossible to target using \textit{in vivo} selection methods, because of the cell’s inability to survive under such biologically harsh conditions. The advantage of higher versatility and better control of the applied selection pressure comes at the cost of lower throughput (defined as the number of clones that can be tested in a given time period). Depending on the screening methods, libraries of about $10^4$–$10^6$ clones can be screened within a few days, which is several orders of magnitude less than the $10^7$–$10^{11}$ clones that can be tested with selection methods. High throughput is usually accompanied by a high error level in the measurement, which dictates the minimum change that can be detected. On one hand, the consequence of low throughput is missing rare beneficial mutants because they are not sampled. On the
other hand, mutants will also be missed if the method cannot resolve the small fitness difference between a mutant and the parent (Fig. 4-6). Part of this difficulty stems from the fact that only a few mutations are made at each generation, and it is often only over multiple generations that large fitness improvements are observed.

It is important to take both the throughput and the error level of the assay into account when setting up a screen. The intrinsic error level of a screen can be tested by screening a number of wild-type clones, which allows an estimate to be taken of the minimal change that can be resolved. Fig. 4-7 shows typical statistics of a screen using a 96-well microtiter plate pH-indicator assay, specifically applied for the evolution of a hydantoinase. From the standard deviation (in this case, 5%) and maximal deviation (20%), one can estimate that mutants differing in more than 50% activity can be detected with high confidence. Thus, this screening method is suitable for detecting small improvements in activity in a mutant library.

4.4.2.1 Low-Throughput Screening

Screening in 96- or 384-well plate formats allows precise fitness measurements. The accuracy of the detection system, kinetic assays (in contrast to end point assays), the possibility to normalize activity values (e.g., using measured cell concentrations), and better control over cell growth and protein expression contribute to the improved

![Diagram A](image1)

**Figure 4-6.** Throughput (sampling size) versus error level of a screen. Low error level and small sampling size (A) might miss the best mutant because it is too rare to be sampled. Large sampling size and high error level (B) might miss the mutant because it cannot be distinguished from wild-type background even though it was sampled.
Figure 4-7. Statistics of a typical screen using a 96-well microtiter plate pH-indicator assay which was applied for the evolution of a hydantoinase.^{[65]} Total # clones = 192. The experimental data roughly follow a Gaussian distribution.

The accuracy of microtiter plate-based screening systems compared to colony-based screens. Semi-quantitative visual screens are usually based on fluorescence^{[140–142]}, color formation^{[16, 143]}, or formation of clear zones (halos)\(^{[144, 145]}\) of colonies grown on agar plates (often filter membranes). The time-consuming process of gridding transformed colonies into microtiter plates is not required. Further, sample preparation steps are straightforward (and sometimes not necessary at all), which simplifies and accelerates the screening process. If coupled to digital imaging analysis, visual screens can be used for quantification^{[146, 147]}. However, the high throughput and simplicity of these methods are often balanced by a larger error. Standard analytic systems such as HPLC, GC or capillary electrophoresis allow very sensitive and precise measurement and are highly versatile. However, throughput is very limited to fewer than \(10^3\) samples per week. This is too low for efficient screening of most mutant enzyme libraries. Further developments, such as integration of HPLC, GC, or capillary electrophoresis into automatic liquid handling systems, coupling of mass detection systems, and parallel separations will increase the throughput.

4.4.2.2

High-Throughput Screening

Complete automatic systems are now available that can screen up to a thousand 96-well plates per day (about \(10^6\) samples per week). Systems have been developed to increase the density of the plates (number of samples per area) by reducing the required sample volume\(^{[148, 149]}\). This reduces the cost of screening each mutant and increases the throughput of the assays. In screening enzyme libraries, the throughput seems to be more limited by the required step to transfer single clones from agar plates into the arrays of microtiter plates or silicon wafers rather than by the assay step. Robotic systems are sometimes used to speed up this transfer. An alternative to this step might be a dilution that adjusts cell density to one cell within a certain liquid volume, which could be transferred into adequate plates (or onto silicon wafers) much faster. Although the theoretical throughput would be increased, the statistical problem arises that a huge fraction of the transferred volume would be empty or contain more than a single cell. Other ultra-high-throughput systems that
can directly analyze single cells or single proteins do not require a transfer step and thus have a potentially higher throughput. Confocal fluorescence coincidence analysis (CFCS) can analyze up to $10^7$ single molecules or cells per week\textsuperscript{[159]. Although the reported sensitivity ($<10^{-15}$ mM can be detected by regular FCS\textsuperscript{[151]}) and throughput are impressive, applications for directed evolution of enzymes have not yet been reported. A fluorescence-activated cell sorter (FACS) can be used to analyze as many as $10^9$ mutants per week. It is currently the only available screening tool with a sufficiently high throughput to exhaustively screen mutant libraries for all possible double mutants in less than a week. So far, most reported applications of evolutionary protein design using FACS to screen and sort mutant libraries have been for binding molecules\textsuperscript{[68, 92]. Joo et al.\textsuperscript{[146] describe a plate-based fluorescence digital imaging screen with a throughput of $\sim10^5$ clones per day.}

4.4.2.3

Choosing Low versus High Throughput

In this section, we will discuss the critical issues in deciding the balance between throughput and accuracy in the screen. A minimal screening requirement can be roughly estimated from the frequency of positive mutants found either in earlier rounds of directed evolution or from results reported in literature. The frequency of positive mutants for different enzymes and different properties varies, but is usually found in the range of about one out of $10^2$ to $10^5$ mutants (Table 4-2). However, it should be noted that the frequency of positive mutants will strongly depend on the fitness of the parent the property, and the strategy chosen to create the mutant library.

A theoretical approach to determine the required number of screened mutants is based on the landscape paradigm. Following this paradigm, several studies have shown that, when the landscape is additive, the number of mutants that need to be screened in order to find fitness improvements increases linearly as the wild-type sequence increases in fitness\textsuperscript{[29, 158]. However, as the landscape ruggedness increases, the number of fitter neighbors decreases more rapidly as the sequence becomes optimized\textsuperscript{[30, 159, 160]. Thus, in order to discover improved mutants, the number of mutants screened has to increase more rapidly on rugged landscapes. This implies that a protein that is tolerant (corresponding to a smooth landscape) can undergo more rounds of mutation and improvement.

There is a tradeoff between generating large libraries for a few generations and generating small libraries for many generations. In other words, if the total number of mutants that can be screened is fixed, what is the optimal number of generations? While the improvement in fitness increases with the size of the screening library, the benefit of accumulating stepwise positive mutations over multiple generations is compromised. Both experimental and theoretical studies have suggested that the best method may be short, adaptive walks utilizing small libraries\textsuperscript{[14, 161]. Husimi and Aita further studied the effect of the screening cost on the optimal search strategy\textsuperscript{[78]. They found that screening multiple generations of small libraries has the advantage of evolving more rapidly; however, it has a greater potential of being
Table 4-2. Frequencies of positive mutants found during directed evolution studies. Calculation is based on reported mutants and might not represent all positive mutants. Actual frequencies may be higher.

<table>
<thead>
<tr>
<th>Frequency of positive mutants</th>
<th>Evolved property / mutagenesis procedure / test</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3x10⁻³ (1500/5)</td>
<td>Thermostability / PCR / screen</td>
<td>[152]</td>
</tr>
<tr>
<td>5x10⁻⁴ (2000/1)</td>
<td>Thermostability / family shuffling / screen</td>
<td>[128]</td>
</tr>
<tr>
<td>2x10⁻⁵ (1.5x10⁶/34)</td>
<td>Thermostability / chemical / selection</td>
<td>[153]</td>
</tr>
<tr>
<td>3x10⁻⁵ (6.4x10⁴/2)</td>
<td>Thermal and oxidative stability / PCR / screen</td>
<td>[123]</td>
</tr>
<tr>
<td>3x10⁻³ (10³/3)</td>
<td>Activity in organic solvent / PCR / screen</td>
<td>[99]</td>
</tr>
<tr>
<td>2x10⁻⁵ (2x10⁹/35)</td>
<td>Activity at elevated temperature / PCR / screen</td>
<td>[154]</td>
</tr>
<tr>
<td>4x10⁻⁵ (2x10⁹/7)</td>
<td>Activity / cassette mutagenesis / selection</td>
<td>[88]</td>
</tr>
<tr>
<td>1x10⁻² (10⁴/124)</td>
<td>Activity / family shuffling / screen</td>
<td>[129]</td>
</tr>
<tr>
<td>3x10⁻³ (300/1)</td>
<td>Activity in organic solvent / PCR</td>
<td>[155]</td>
</tr>
<tr>
<td>2x10⁻³ (1.7x10³/4)</td>
<td>Activity / family shuffling</td>
<td>[130]</td>
</tr>
<tr>
<td>8x10⁻⁵ (1.2x10⁷/1)</td>
<td>Functional expression / PCR / screen</td>
<td>[156]</td>
</tr>
<tr>
<td>1x10⁻² (10⁵/12)</td>
<td>Enantioselectivity / PCR /screen</td>
<td>[157]</td>
</tr>
<tr>
<td>1x10⁻³ (750/1)</td>
<td>Enantioselectivity / mutator strain / screen</td>
<td>[49]</td>
</tr>
<tr>
<td>4x10⁻² (150/6)</td>
<td>Substrate specificity / focused mutagenesis / screen</td>
<td>[87]</td>
</tr>
</tbody>
</table>

trapped in a local optimum. If the cost of screening a mutant is high, then the walk should tend towards many generations of small libraries, whereas, if it is low, it should tend towards fewer generations of large libraries. A powerful strategy for balancing the limitations of throughput and sensitivity is tiered screening (Fig. 4-8). In this method, a series of screening or selection methods with decreasing throughput and increasing accuracy are combined. This strategy has been used successfully for the evolution of subtilisin towards a combination of properties[127] and for the evolution of an esterase towards increased enantioselectivity.[49].

A computational method, such as the entropy calculation presented in Sect. 4.3.1.3, can be used to eliminate the portions of sequence space where improvement is unlikely (Figure 4.9).[82] Pre-screening drastically reduces the number of mutants that have to be experimentally screened.

Another approach to increasing throughput is to use a pooling strategy.[162-164]. This methodology is conceptually equivalent to the recombination strategy presented in Section 4.3.2, in which the recombination load is subdivided into multiple generations, thus reducing the required screening effort. Most screens, however, are not sufficiently sensitive to use a pooling strategy to find small improvements.

4.4.2.4
Analyzing the Mutant Fitness Distribution

During the screening, a large amount of fitness data is generated, but only the fitness information of the improved mutants is used to continue to the next round of evolution. The large ensemble of less fit mutants provides a view of the local fitness landscape. By analyzing these data, certain statistical landscape parameters can be
deduced, such as the fitness landscape ruggedness, which can then be used to guide in setting evolutionary parameters. In this analysis, sequencing is time-consuming and expensive, so a sequence cannot be assigned to each measured fitness. The lack of sequencing data means that only the probability distribution of mutant fitnesses can be analyzed. In this section, we discuss some methods that have been developed to extract useful information from the mutant fitness distribution.

Several theoretical approaches based on the additivity of mutations have been proposed to analyze the screening data. Urabe and coworkers developed a model that captures additive and non-additive mutational effects in directed evolution and fit their theoretical model to the experimental fitness distribution of catalase I. By investigating the degree of non-additivity of specific properties, they tuned the parameters of the experiment to suit the fitness landscape\cite{165}. In a similar approach, Aita and Husimi proposed that the additive model can be applied to give a rough estimate for the Hamming distance from the wild-type to the optimum, the fitness slope near the wild-type, and the height of the optimum\cite{158}. They calculated the expected fitness distribution and compared this to experimental data produced by the mutagenesis of a region of \textit{E. coli} lac promoter. Based on a fit between the theoretical and experimental distributions, they estimated that the Hamming distance between the wild-type lac promoter and the optimum is 7–10 nucleotide substitutions and the activity could be improved 100- to 1000-fold.

Mean-field theory can be used to predict the effects of mutation rate, landscape ruggedness, and parental fitness on the moments of the mutant fitness distribution\cite{79}. In this analysis, only the portion of the mutant distribution that is not dead (zero fitness) or parent (unmutated) is considered. The mutant effects are averaged over the transition probabilities. In order to obtain the fitness distribution, two sets of probabilities are required: (1) the probabilities $P_i(a)$ that a particular amino acid identity $a$ exists at a residue $i$, and (2) the transition probabilities that one amino acid
will mutate into another, \( q_a \rightarrow b \). The probabilities \( P_i(a) \) can be determined through a mean-field approach and the probabilities \( q_a \rightarrow b \) are calculated based on the genetic code\(^{[110, 166-168]} \). Using the mean-field solution, the change in the mutant fitness distribution is captured as the sequence ascends the fitness landscape. By increasing the coupling interactions between residues, the effect of the landscape ruggedness on the moments is calculated. As the fitness of the wild-type increases, the first and second moments increase (Fig. 4-9). In other words, as the sequence ascends the fitness landscape, the mutant distribution spreads out (diffuses) and becomes skewed towards less-fit mutants (drifts). In addition, the dependence of the moments on mutation rate can be predicted. As the mutation rate increases, both the drift and the diffusion of mutants from the parent increases. Because rugged landscapes have less correlation between parent and offspring fitnesses, the drift-diffusion effect becomes exaggerated as the coupling between residues increases. Through this approach, it may be possible to model the mutant fitness distribution to experimentally obtain statistical parameters that describe the fitness landscape.

### 4.4.3

**Selection and Methods to Link Genotype with Phenotype**

The advantage of high throughput, together with minimal experimental and technical effort, makes selection an attractive tool for \textit{in vitro} evolution. The most commonly used \textit{in vivo} selection approach links a targeted enzyme property to cell growth through their contribution to resistance\(^{[36, 37, 88, 126, 169]} \) or complementation of auxotrophy or genetic defects that block the metabolism of a host strain\(^{[62, 89, 90, 100]} \). Most of these systems allow cells with improved enzymatic properties to grow, while cells with wild-type properties do not. Such systems are especially powerful if the selection pressure can be adjusted as the evolution progresses, for example, by increasing antibiotic concentrations\(^{[36, 37]} \), decreasing substrate concentrations or changing the enzyme expression level\(^{[62]} \).

If the growth of cells with wild-type activity cannot be prevented and positive mutants contribute only to an increased growth rate, cells with improved variants are usually enriched by continuous culture techniques, dilution series, or detection of the size of tested colonies. A problem for selection arises if enzymes are secreted into the culture medium. This problem was avoided in one case by growing cells in hollow fibers that limit cross-feeding between neighboring colonies\(^{[170]} \). Another limitation of \textit{in vivo} selection methods is that selection conditions must be compatible with the requirements of the host organisms, which are often very different from conditions under which the enzyme is going to be applied. This is particularly true if the enzyme is to be used in an industrial reactor, where conditions often involve extreme temperatures and the substrates are suspended in organic solvents. It might be advantageous to use other expression hosts that grow under different reaction conditions than the commonly used organisms (\textit{E. coli}, \textit{S. cerevisiae} or \textit{B. subtilis}). Thermophilic hosts with reasonable transformation efficiencies, such as \textit{Bacillus stearothermophilus} or \textit{Tetrahymena thermophilus}, have been used for selection for increased thermostability by growth at elevated temperatures\(^{[171-173]} \). Other \textit{in vivo}
Figure 4-9. A histogram of the residue entropies for the structure of subtilisin E\textsuperscript{[82]}. The entropy is a measure of the number of amino acid mutations that can be made at a residue without disturbing the structure, as determined using a model of stabilizing interactions (van der Waals, electrostatics, hydrogen-bonding, and the hydrophobic effect). When all amino acids are equally allowed at a residues, then $s_i = \ln 20 \approx 3.0$. When a single amino acid is allowed at a residue, then $s_i = 0.0$ (marked by the arrow). The connected bar in the center of the graph marks the mean and standard deviation of the histogram. The lines above this bar indicate where beneficial mutations that improved stability (top row) and activity in organic solvents (bottom row) occurred in directed evolution experiments. Most of these beneficial mutations occurred at residues that are predicted to have high entropies.

Selection approaches are based on infectivity of phages\textsuperscript{[174, 175]} rather than on cell growth. This method has been applied to select for proteins with improved thermostability\textsuperscript{[153]} and stability against proteolysis\textsuperscript{[176]}. Unfortunately, many of the targeted enzyme properties and catalyzed reactions cannot be linked to cell growth or infectivity of phages. Even if selectable traits exist, it remains a tedious task to guarantee that the targeted enzyme property and not other factors such as substrate uptake or other metabolic steps is limiting for growth.

Selections also face difficulties in controlling biological complexity. New in vitro selection methods might reduce some of the limitations imposed by biological complexity of living cells\textsuperscript{[5, 19, 24]}. In addition, much larger mutant libraries can be searched by in vitro selection methods if they are used together with in vitro genotype-phenotype coupling systems. However, such methods often select enzymes based on single turnover events\textsuperscript{[177, 178]}, binding of transition state analogs\textsuperscript{[150, 179, 180]} or suicide inhibitors\textsuperscript{[181]} and therefore do not necessarily reflect enzyme properties of highly active catalysts.

Another in vivo approach to couple the genetic information with a screenable or selectable phenotype is phage display, which has been extensively reviewed elsewhere\textsuperscript{[19, 21, 23, 182, 183]}. The most commonly used approach is to fuse the mutage-
nized target genes to a coat protein gene of filamentous bacteriophages. After transformation of bacteria with the recombinant DNA, bacteriophages are assembled that display the protein of interest on their surface fused to the coat protein and carry the genetic information for the displayed enzyme in the DNA. The typical diversity of a library produced by phage display is high: $10^7$–$10^{11}$ different sequences. However, it is difficult to screen for properties other than binding$^{[184]}$. Furthermore, the folding of displayed proteins occurs in the periplasm of the bacteria, which is often problematic for the functional display of cytoplasmic enzymes. In addition, large and multimeric enzymes are difficult to display. However, a few examples exist where some of these limitations have been overcome, such as alkaline phosphatase$^{[185]}$, β-lactamase$^{[186]}$, glutathione transferase$^{[181]}$ and penicillin G acylase, which is a 86 kDa heterodimeric enzyme$^{[187]}$. Several theoretical models have been proposed to capture the dynamics of phage display$^{[188$–$191]}$.

All in vivo approaches require the transformation of the mutated genes into cells, which limits the library size that can be produced to a maximum of about $10^6$–$10^{10}$ members$^{[192]}$. In vitro approaches circumvent the transformation step by using cell-free transcription/translation systems that can produce the mutant proteins directly from the mutated genes$^{[193]}$. The required link between the gene and the protein produced can be achieved with ribosome display$^{[194]}$ or mRNA-protein fusions$^{[195, 196]}$. These elegant systems allow the generation of protein libraries that contain up to $10^{13}$ different members, which is a 10 000-fold expansion of accessible sequence space. Theoretically, such methods can cover all possible triple mutants of an enzyme with 300 amino acid residues if coupled to a suitable selection system. Another approach to coupling a phenotype with its genotype is based on In vitro transcription / translation reactions compartmentalized in water-in-oil emulsions$^{[126, 139]}$. In principle, each aqueous compartment contains one single gene that is transcribed and translated into protein. In practice, however, many compartments will be empty or contain more than one gene. All these approaches require suitable screening or selection technology to make use of the larger libraries.

### 4.5 Applications of Directed Evolution

With directed evolution we can engineer enzyme properties rapidly and with a high probability of success. Many enzymes that have been improved by directed evolution are listed in Tab. 4-3. This powerful biocatalyst engineering strategy creates new opportunities in organic synthesis: new and improved bioconversion processes can be developed and novel compounds that are otherwise inaccessible by classical chemistry can be synthesized. In addition, the molecules created by directed evolution offer an excellent opportunity for improving our still poor understanding of sequence-structure-function relationships.

The specific applications of directed evolution that are described below focus on properties that are important for efficient enzyme production as well as on those that are of special interest for applications in organic synthesis, including enzyme
specificity, activity towards non-natural substrates, and function in non-natural environments.

4.5.1 Improving Functional Enzyme Expression and Secretion

Pharmaceutical or industrial applications of enzymes require their production at a large scale. This is usually done by overexpressing the enzyme in *E. coli*, *Bacillus* sp., yeasts or fungi. Many enzymes, however, fail to fold properly in heterologous hosts. This is a problem particularly for membrane-bound, highly glycosylated or disulfide bond-containing eukaryotic proteins. Eukaryotic enzymes such as glycosylases, peroxidases or cytochrome P-450s will require significant improvements in functional expression to make them available in large quantities and at low cost.

Enzyme expression can be affected by mutations in the structural gene that may or may not change amino acid sequence or by mutations in regulatory elements that control expression. It was reported that horseradish peroxidase (HRP), a glycosylated heme enzyme, cannot be expressed in functional form in *E. coli* or yeast. We found, however, that we could obtain significant levels of HRP activity in the supernatant of yeast cultures by accumulating point mutations in the structural gene\(^{[156]}\). Furthermore, mutants expressed at high levels in *S. cerevisiae* were also better expressed in *Pichia pastoris*. Although far from sufficient for commercial enzyme production, the activity level that was achieved enabled us to carry out further generations of directed evolution to tailor the enzyme for specific applications.

Other reports show that intracellular expression of misfolded or unstable proteins can be dramatically improved by directed evolution. For example, directed evolution increased the expression of disulfide-containing antibody fragments in *E. coli* 50-fold, to reach a level of more than 0.5 g/L\(^{[212]}\). The expression of a wide spectrum amidase of *B. stearothermophilus* in *E. coli* improved 23-fold by two mutations\(^{[210]}\). And, *in vivo* fluorescence of the green fluorescent protein was improved 45-fold by increasing its solubility and native folding in *E. coli*\(^{[140]}\).

Secretion into the culture medium is preferred for some industrial enzymes because it can facilitate purification. This is especially true if the protein reaches high concentrations. Schellenberger’s group at Genencor devised a method to select for improved subtilisin-secreting mutants in *Bacillus*\(^{[170]}\). Mutants secreting up to five times as much enzyme were found after one round of error-prone PCR and selection.

Directed evolution can improve functional enzyme expression and even allow expression of enzymes that are otherwise difficult to produce in recombinant systems. Results obtained by directed evolution, however, will depend on the particular system, and high expression will undoubtedly be best achieved by a combined approach of molecular biology, fermentation optimization and directed evolution.
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4.5 Applications of Directed Evolution
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4.5 Applications of Directed Evolution

4.5.2

Engineering Enzymes for Non-natural Environments

Bioconversion processes performed in organic solvents or at elevated temperatures impart such benefits as increased substrate solubility, decreased viscosity of the reaction medium, altered reaction selectivity and equilibria, higher rates, and reduced risk of microbial contamination. High thermostability also tends to translate to resistance to other denaturants and better long-term stability at lower temperatures. Most natural enzymes are poorly suited for function in organic solvents or at high temperatures, however, and their limited stability and activity in these environments can be a limiting factor for applications in organic synthesis. These properties are good targets for engineering by evolution.

Directed evolution has generated a large number of thermostabilized enzymes (see Table 4-3 for examples); there are too many reports for a comprehensive review here. We will discuss only the general picture that arises from those studies; the interested reader is referred to a recent review that deals with evolution of enzyme stability in greater detail.[213]

The increase in thermostability imparted by single amino acid substitutions is usually small and is typically in the range of a 1–2 °C increase in melting temperature or optimal reaction temperature.[152, 171, 173, 214–216] Larger changes are possible, but rare. Significant changes in thermostability therefore require the accumulation of multiple substitutions, e.g., by sequential rounds of mutagenesis or recombination. This strategy has generated 20 °C and higher increases in thermostability.[15, 171]. The stabilization mechanisms are consistent with those found in naturally thermophilic enzymes and include reduction of surface loop flexibility, new hydrogen bonds, altered core packing, helix stabilization and acquisition of surface salt bridges.[217] Although the mechanisms are familiar, most of the changes would have been difficult or impossible to predict.

In nature, thermophilic enzymes tend to be less active at low temperatures than their mesophilic counterparts, which in turn are less thermostable (Fig. 4-10). One popular explanation for this observation is that activity and stability make mutually exclusive demands on enzyme flexibility. Two properties coupled in this way cannot evolve independently. However, directed evolution experiments have shown that these properties can evolve independently.[15, 152, 202] A 17 °C increase in the melting temperature of a mesophilic esterase was achieved at the same time as catalytic efficiency was increased several fold by random mutagenesis and screening over several generations.[152, 213] A similar approach taken with mesophilic subtilisin E generated a 17 °C increase in the temperature optimum and a >200-fold increase in half-life at 65 °C.[202] The thermostable subtilisin was also more active than wild-type over the whole temperature range. Most recently, directed evolution of a psychrophilic subtilisin[15] led to a 500-fold increase in half-life at 60 °C at no cost to its activity at low temperature. The evolved enzyme is more stable than homologous mesophilic subtilisins. The stabilized enzymes contained between 7 and 13 amino acid substitutions. In the studies described above, mutants were screened simultaneously for activity and thermostability, and mutations were accepted only when...
Figure 4-10. Enzymes isolated from organisms growing at different temperatures often exhibit a tradeoff between thermostability and catalytic activity measured at low temperature. Enzymes that are both highly thermostable and highly active at low temperatures are rare in nature but highly desired for various applications and can be obtained by directed evolution with relatively few mutations\textsuperscript{[184]}.

enhanced thermostability came at little or no cost to activity. If the selection pressure is not maintained, thermostability can easily be lost\textsuperscript{[218, 219]}.

Creating enzymes that are both more thermostable and more active is particularly exciting for industrial applications. In addition, these studies nicely demonstrate that behaviors of natural enzymes may not necessarily be due to physical limitations intrinsic to proteins themselves, as is often assumed. Instead they reflect what is both relevant to the organism and accessible to natural evolution\textsuperscript{[184]}.

Directed evolution has also been very effective for increasing enzyme activity in organic solvents\textsuperscript{[14, 99]}. For example, the serine protease subtilisin can catalyze specific peptide syntheses and transesterification reactions, but organic solvents are required to drive the reaction towards synthesis. Sequential rounds of error-prone PCR and visual screening yielded a subtilisin variant with twelve amino acid substitutions that was 471 times more active than wild-type in 60\% dimethylformamide (DMF)\textsuperscript{[145, 220]}; this enzyme is much more effective for peptide and polymer synthesis.

The production of cephalosporin-derived antibiotics requires a deprotection step usually catalyzed by zinc in organic solvents. Since this step produces large amounts of solvent- and zinc-containing waste material, scientists at Eli Lilly were interested in using an enzyme. Classic screening identified an esterase that catalyzed the desired reaction but performed poorly in the solvents required to solubilize the substrate. Directed evolution was therefore used to try to improve the performance of the enzyme for efficient hydrolysis of an antibiotic $p$-nitrobenzyl ester intermediate in aqueous-organic solvent mixtures\textsuperscript{[99]}. Four rounds of random mutagenesis by error-prone PCR and screening followed by one recombination step improved the esterase activity 50- to 60-fold in 25\% DMF and yielded mutants that performed
as well in 30% DMF as the wild-type enzyme in water. None of the six mutations found in the best mutant were in direct contact with the substrate and some were as far away as 20 Å. Thus, focused mutagenesis in the substrate binding site may have overlooked important beneficial mutations.

High product concentrations are important in organic synthesis but often detrimental to enzymes. Scientists at Celgene reduced product inhibition in transaminases[221] which are valuable for the production of chiral amines or amino acids. A single round of error-prone PCR and screening of 10,000 clones revealed mutants with better product tolerance that translated to a four-fold increase in volumetric productivity for a substituted amphetamine.

4.5.3

Engineering Enzyme Specificity

Enzymes are particularly valuable for the production of enantiomerically pure compounds, as shown in examples throughout this book. However, the narrow range of substrates that some enzymes accept and the less than impressive enantioselectivities exhibited by others often frustrate attempts to develop new synthetic applications and to commercialize existing ones. Directed evolution can efficiently tune substrate specificity and catalytic efficiency towards non-natural substrates; it can also tailor enantioselectivity, as illustrated in the examples below.

4.5.3.1

Substrate Specificity

Zhang et al. evolved a fucosidase from a galactosidase[16]. Seven rounds of DNA shuffling and screening using a chromogenic fucose substrate yielded a mutant with 66-fold increase in fucosidase activity. Kinetic analysis of the purified enzyme revealed a 10- to 20-fold increase in $k_{cat}/K_m$ for the fucose substrate and a 50-fold decrease for galactose (a total of 1000-fold increased substrate specificity for fucose).

Kumar et al. recombined two biphenyl dioxygenases (96% identical) and visually screened for mutants whose substrate range differed from the parents'. These mutants degraded various biphenyl compounds more efficiently and also exhibited oxygenation activity for single-ring aromatic compounds on which neither parent was active[131].

Lanio et al. reported the tailoring of restriction endonucleases EcoRV specificity[87]. Focused combinatorial mutagenesis was used to make variants that cleave specific DNA sequences of eight or ten base pairs rather than the six recognized by the natural enzyme. Twenty-two amino acid residues were targeted by oligonucleotide-directed mutagenesis within three different regions of the enzyme. Screening a total of only 500 colonies over three cycles of mutagenesis was sufficient to find several mutants with high activity and high specificity for AT- or GC-flanked GATATC cleavage sites.

Aspartate aminotransferase catalyzes amino group transfer between acidic amino
acids, aspartate and glutamate, and their corresponding 2-oxo acids. The wild-type activity for β-branched amino acids is barely detectable, but was dramatically increased by directed evolution\textsuperscript{[62, 100].} The aspartate aminotransferase gene derived from \textit{E. coli} was subjected to DNA shuffling and introduced into an \textit{E. coli} host lacking the branched-chain amino acid aminotransferase gene and therefore allowing selection by complementation with mutant aspartate aminotransferases. The stringency of the selection was increased during the progression of evolution by omitting the substrate (2-oxovaline) from the medium, shortening the incubation time and decreasing the expression level of the mutant enzymes by manipulating the construction of the plasmid. A mutant with 10\textsuperscript{5}-fold increased catalytic efficiency (\(k_{\text{cat}}/K_m\)) for β-branched amino acids and 30-fold decrease for the natural substrate was created after five cycles of shuffling and selection\textsuperscript{[62].} This mutant was further improved to yield a mutant with a remarkable 2.1×10\textsuperscript{6}-fold improved catalytic efficiency compared to wild-type\textsuperscript{[100].} Analysis of the structure of the mutant enzyme complexed with a valine analog provided detailed insight into how the mutations affected substrate binding and demonstrated the importance of cumulative effects of residues far from the active site.

The P-450 monoxygenase from \textit{Pseudomonas putida} was evolved for efficient utilization of hydrogen peroxide in lieu of \(\text{O}_2\) and \(\text{NADH}\) and for improved activity towards the non-natural substrate naphthalene\textsuperscript{[142].} One round of error-prone PCR and screening of about 200 000 clones by high-throughput digital image analysis\textsuperscript{[146]} revealed several mutants with increased activity. Subsequent recombination of five improved mutants yielded several variants with about 20-fold improvements in naphthalene hydroxylation activity over wild-type using hydrogen peroxide as sole cofactor.

Fructose 1,6 bisphosphate (FBP) is an allosteric activator of the thermostable 1,2-hydroxyacid dehydrogenase from \textit{B. stearothermophilus}, which might be useful for the asymmetric synthesis of chiral compounds. Since FBP is quite expensive, Allen and Holbrook wished to create an FBP-independent variant\textsuperscript{[208].} Three rounds of shuffling and screening produced a mutant 1,2-hydroxyacid dehydrogenase with three amino acid substitutions that is almost as active in the absence of FBP as the wild-type is in its presence.

Recently, Schmidt-Dannert et al. reported the molecular breeding of carotenoid biosynthetic pathways in \textit{E. coli}\textsuperscript{[209].} Two different phytoene desaturases were shuffled and expressed in the context of a carotenoid biosynthetic pathway assembled from different bacterial species. Clones containing mutant desaturases were visually screened to identify new carotenoid products. One out of approximately 10 000 colonies turned pink and produced shuffled tetrahydrolycopene instead of lycopene. The new pathway was extended with a second library of shuffled lycopene cyclases. Visual screening identified a cyclase with altered substrate specificity that produced the cyclic carotenoid torulene for the first time in \textit{E. coli}. Complementary to the strategy of creating new polyketides by mixing and matching subunits in a multi-enzyme complex\textsuperscript{[222, 223],} the combination of a rational pathway assembly and directed evolution is an exciting opportunity to create libraries of otherwise inaccessible biologically active compounds.
4.5.3.2
Enantioselectivity

Matcham and Bowen were among the first to apply an evolutionary approach to improve the enantioselectivity of an enzyme for use in chiral synthesis[221]. The wild-type enzyme (an S-selective transaminase) converts a particular β-tetralone to the corresponding amine at only 65% ee. By screening a mutant library of 10,000 variants in a microtiter plate-based assay, they identified 10 mutants that produced the (S)-aminotetraline with 80–94% ee. Sequencing the mutants revealed positions important for enantioselectivity and, interestingly, the existence of synergistic combinations of mutations.

The lipase from *Pseudomonas aeruginosa* (PAL) catalyzes the hydrolysis of 2-methyldecanoic acid p-nitrophenyl ester with only 2% ee in favor of the (S)-acid. Reetz and Jaeger used four rounds of error-prone PCR and screening on enantiomerically pure R and S substrates to generate a more enantioselective variant that produced the desired (S)-acid with 81% ee[157]. Additional cycles of error-prone PCR in combination with saturation mutagenesis further improved the enantioselectivity of this enzyme, which hydrolyzes the 2-methyldecanoic acid p-nitrophenyl ester with 91% ee (E = 25.8) in favor of the (S)-acid[12].

Bornscheuer et al. improved the enantioselectivity of an esterase from *Pseudomonas fluorescens*[49, 207]. In their first report, the enzyme was evolved for hydrolysis of a 3-hydroxy ester serving as a building block in epithilone synthesis[109]. Isolated plasmids obtained from a mutator strain were transferred into *E. coli* and plated onto two different kinds of agar plates. One plate contained a pH indicator which shows active clones by a color change. The other plate contained a minimal medium and a glycerol ester as the sole carbon source. Cleavage of the gycero-ester releases glycerol, which leads to growth of active cells. One clone that produced the desired enantiomer with 25% ee was identified, compared to no enantioselectivity for wild-type. The screen allowed for detection of active clones, but is not sensitive to enantioselectivity; this might explain why further improvements in enantioselectivity were not reported.

A subsequent report describes the evolution of the same enzyme for the hydrolysis of 3-phenylbutyric acid resorufin ester using both a mutator strain and error-prone PCR[207]. Mutants were screened for improved enantioselectivity based on a microtiter plate assay using the optically pure R- or S-esters. Both mutagenesis methods generated first-generation mutants with higher enantioselectivity (E=6.6 and 5.8 compared to wild-type E=3.5).

Recent results show that directed evolution can also invert enzyme enantioselectivity[65]. The hydantoinase derived from *Arthrobacter sp.* shows a substrate-dependent inversion of enantioselectivity which limits its use for the production of certain L-amino acids such as L-methionine (for applications of hydantoinases in organic syntheses see Chapter 12). By accumulation of mutations through sequential rounds of error-prone PCR and saturation mutagenesis, the enantioselectivity of the hydantoinase was inverted from ee = 40% for the D-enantiomer to ee = 20% for the L-isomer at 30% conversion. Only one amino acid substitution was required for the
inversion of enantioselectivity. Furthermore, mutant hydantoinases exhibiting high
D-selectivity (ee = 90% at 30% conversion) were also found. The L-selective mutant,
whose overall activity was improved 5-fold over wild-type, was co-expressed with a
racemase and L-specific carbamoylase in E. coli. This yielded a recombinant whole-
cell catalyst with an improved hydantoin converting pathway. Application of this
whole-cell catalyst for the production of L-methionine resulted in >5-fold improved
productivity for >90% conversion of the racemic substrate into the optically pure
product.

The optimization of whole pathways by directed evolution and their introduction
into recombinant whole-cell catalysts may offer the possibility of substituting
complicated multi-step processes with straightforward single-pot processes. This, of
course, is highly desired for industrial applications and a major advantage of
biocatalysis over other competing technologies used in organic synthesis.

4.6
Conclusions

The power of directed evolution is now well documented. These methods are robust
and are able to improve industrial enzymes in reasonably short times. The first
laboratory-evolved enzymes are now used commercially in laundry detergents; other
commercial applications are on the horizon. Directed evolution may well help
move biocatalysis from an “enabling tool” to a “lowest cost approach”. It also offers
new opportunities to engineer multi-enzyme pathways and even whole
microbes, which will lead to straightforward single-pot, multi-enzyme
bioconversions and new fermentation processes based on “green” resources such as
glucose or inexpensive waste materials.

Sixteen years after Manfred Eigen and William Gardiner presented the basic
algorithm for evolutionary molecular engineering; it is worth commenting on the
conclusion of their paper:

"... The clones have to be addressable; the analytical methods must combine parallel
processing and automatic sampling with sensitivity and speed. With such elaboration
and scale, experimental biology might well become ‘Big science’.”

Today’s tools of evolutionary engineering certainly fulfill these requirements, and
directed evolution has in fact emerged as the method of choice for biocatalyst
improvement. However, we are only beginning to explore the power of evolutionary
design.

The most obvious limitations of these methods are still related to the tools.
Screening or selection methods require significant development time. This might be
reduced by the development of versatile enzyme assays that can be adapted rapidly to
specific conditions. The problem will also be reduced by integrating versatile
standard analytic systems such as mass spectroscopy, HPLC or capillary electro-
phoresis into automatic high-throughput systems.

The finite sampling capacity of most screening methods and the low versatility of
selection methods will probably remain significant limitations. This makes it
difficult, if not impossible, to generate surprising new functions that require
multiple simultaneous amino acid substitutions. It is clear that more "rational"
approaches, based on structure/sequence comparisons or computation, will be
necessary to target key amino acid positions. Other limitations of directed evolution
are inherent in the current mutagenesis and recombination methods, which
strongly bias the combinatorial libraries. It is not yet clear how best to create
molecular diversity for evolution. What is clear is that many of these questions and
limitations can and will be addressed in the near future.

The field of molecular evolution used to focus on the past and aimed to explain the
existence of today's fantastic array of biological molecules. Applied molecular
evolution is changing this focus to the future, by creating molecules for a bio-
technology industry of unlimited opportunities.

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