Discovery of Superior Enzymes by Directed Molecular Evolution

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1. Introduction

Natural selection has created optimal catalysts that exhibit their convincing performance even with a number of sometimes counteracting constraints. Optimal performance of enzyme catalysis does not refer necessarily to maximum reaction rate. Rather, it may involve a compromise between specificity, rate, stability, and other chemical constraints; in some cases, it may involve "intelligent" control of rate and specificity.[1] Because enzymes are capable of catalyzing reactions under mild conditions and with high substrate specificity that often is accompanied by high regio- and enantioselectivity, it is not surprising that a continually increasing number of industrial and academic reports concern the use of enzyme catalysts in chemical synthesis as well as in biochemical and biomedical applications.

However, the demands of modern synthesis and their commercial application were obviously not targeted during the natural evolution of enzymes. Considering a specific, non-natural application, any property (or combination of properties) of an enzyme may therefore need to be improved. Of course, scientists desired to mimick nature's powerful concepts for tailoring specific enzymatic properties:[2] Following pioneering experiments for evolving molecules in the test tube,[3–6] evolutionary engineering of biomolecules was successfully realized with first selections of functional nucleic acids (ribozymes) by using the SELEX (*s*ystematic *e*volution of *li*gands by *ex*ponential enrichment with integrated optimization by non-linear analysis) procedure,[7, 8] and with the development of high-affinity ligands (aptamers) by using similar techniques. Meanwhile, evolutionary engineering, also termed "directed evolution", has emerged as a key technology for biomolecular engineering and generated impressive results in the functional adaptation of enzymes to artificial environments.[9–11] Certainly, evolution in the laboratory does not come to a halt at the optimization of single genes and proteins. Recent results excitingly demonstrate the success of "molecular breeding" of metabolic pathways, and even of complete genomes,[12] and the end is not in sight yet.

Directed evolution in the laboratory is highly attractive because its principles are simple and do not require detailed knowledge of structure, function, or mechanism. Essentially like natural evolution, directed evolution comprises the iterative implementation of (1) the generation of a "library" of mutated genes, (2) its functional expression, and (3) a sensitive assay to identify individuals showing the desired properties, either by selection or by screening (Figure 1). After each round, the genes of improved variants are deciphered and subsequently serve as parents for another round of optimization. This review covers the most important aspects of directed evolution and summarizes key solutions to problems of optimizing and understanding enzyme function.

2. Creating diversity

The route of evolutionary optimization of a certain enzyme can be described as an adaptive walk in a "fitness landscape" that consists of peaks (sequences with high fitness) which are connected by ridges and separated by saddles, valleys (sequences with low fitness), or planes. The fitness landscape is associated to a "sequence space"—a network-like arrangement of all possible amino acid sequences of a given length.[13, 14] Under the influence of mutation and selection, the enzyme may walk along the ridges toward peaks, that is, sequences of higher fitness.[15] However, exhaustive searching of all possible protein sequences for the individual variant with maximum fitness seems like a daunting undertaking because the sequence space even for a protein of moderate sequence length of 100 amino acids is extraordinarily large (about 10^{130} sequences). Taking again nature as an example, optimal solutions can be found by exploring only small fractions of the sequence space: A series of experimental strategies have been developed for generating mutant libraries in the laboratory which differ in diversity, that is, in the extent of covered sequence space, and in approaching intelligent solutions for dealing with complexity.

2.1. Random mutagenesis

Random mutagenesis at the nucleotide level is a widespread strategy which targets whole genes. This may be achieved by passing cloned genes through mutator strains,[16, 17] by treating

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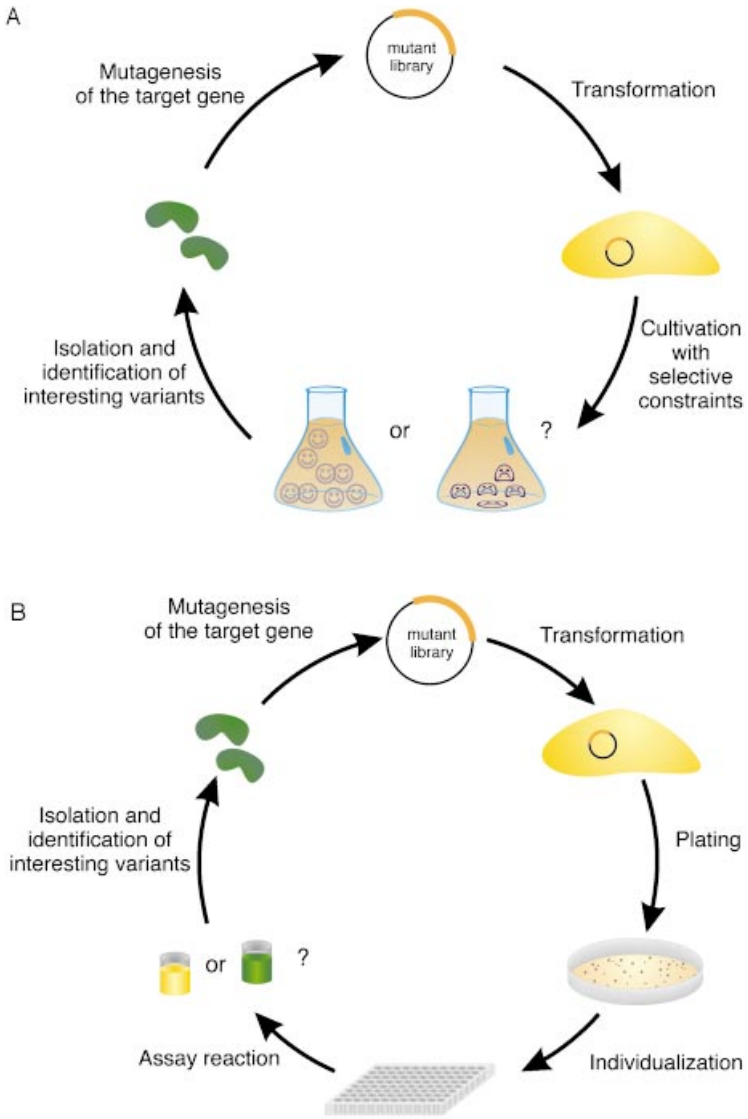

Figure 1. *A) Schematic representation of directed enzyme evolution by in vivo selection. Target genes are mutated and inserted into a plasmid vector to yield a mutant gene library. After transformation of a suitable host (which is, for example, auxotrophic with respect to the target function), selective conditions are applied to the culture. Those cells which express active target gene variants that perform their activity under the applied constraints can survive, whereas other cells harboring inactive gene products die out. B) Schematic representation of directed evolution by screening. Bacteria harboring mutant target genes are plated and subsequently individualized, for example, in a microwell array. The target reaction is started with the addition of such substrates that facilitate the detection of successfully performed reactions, for example, chromogenic substrates. Desired variants are then isolated and analyzed.*

single-stranded DNA with various chemical mutagens,[18–20] or by error-prone PCR.[21, 22] Due to its simplicity and versatility, random PCR mutagenesis emerged as the most common technique which can result in mutation frequencies as high as 2% per nucleotide position. With alterations of some PCR conditions, the mutation rate may also be adjusted to lower values. However, the number of amino acid substitutions accessible by using error-prone PCR is limited because this reaction biases the distribution of mutation type in favor of transitions (A $\rightleftharpoons$ G, T $\rightleftharpoons$ C) and because multiple substitutions within a single codon are extremely rare. Codon-level random mutagenesis of complete genes therefore would be desirable, but has not been realized yet.

Much effort, however, has been devoted to targeting single amino acids or selected regions of a protein which have been identified in previous experiments to be important for a certain function. By focusing only on the positions of interest and/or their close environment, also the size of a mutant library can be drastically reduced (so-called "doped libraries").[23] Methods for randomizing small gene fragments are among the earliest techniques applied to in vitro evolution. Typically, they employ the substitution of wild-type gene fragments by synthetic oligonucleotides which contain random positions or stretches (random cassettes),[5, 24] or semi-random ranges (spiked oligonucleotides).[25] Randomization of defined positions or regions is achieved with automatic solid-phase DNA synthesis by programming the desired International Union of Biochemistry (IUB) mix codes. The introduction of stop codons can be excluded by allowing only G and C (mix code: S) at the third position of each codon. Complete permutation of a single amino acid position (saturation mutagenesis) may thus enable the finding of nonconservative replacements which are inaccessible by random point mutagenesis.[26] Meanwhile, automatic solid-phase DNA synthesis also allows for the selective introduction of codon mixtures by using trinucleotide β-cyanoethyl phosphoramidites,[27] even by combining conventional dimethoxytrityl (DMT) protection with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry.[28]

2.2. Recombination

Recombination of DNA represents an alternative or additional approach for generating genetic diversity that is based on the mixing and concatenation of genetic material from a number of parent sequences. As compared to random mutagenesis, recombination may be advantageous in concentrating beneficial mutations which have arisen independently and may be additive, and likewise, in concentrating deleterious mutations which subsequently might be more efficiently purged from the population by selection.[29, 30] DNA shuffling was the first technique introduced for random in vitro recombination of gene variants created by random mutagenesis.[31] This method employs the PCR reassembly of whole genes from a pool of short overlapping DNA sequences (typical length: 100 – 300 bp) which are generated by random enzymatic fragmentation of different parental genes. Alternative protocols include StEP (staggered extension process),[32] and random-priming recombination.[33] In vitro recombination by StEP is forced in a PCR-like reaction with very short annealing and extension steps that promote the formation of premature extension products. In following cycles, the truncated strands may anneal randomly to a parent strand, thus

combining the information of different parent strands. As an alternative to DNA shuffling, random-priming recombination produces random fragments for reassembly by annealing of short, random primers to a certain template gene and extension by a polymerase.

There is no reason that the concept of in vitro recombination should be limited to pools of gene variants generated by random mutagenesis. In an extension of the idea, naturally occuring genes showing high similarity in sequence and function can serve as an enormous pool of "information" for the creation of new, chimeric enzymes. Recombination of homologous parent genes, also called "family shuffling",[34] could access yet unexplored regions of the sequence space because it combines genetic variability that has already been selected in nature to be functional.

Homologous recombination may also be achieved in vivo. Most common are methods based on the transformation of *Saccharomyces cerevisiae* with a linearized plasmid and target gene variants. Intermolecular homology-dependent recombination may occur, which yields a circular plasmid that can be detected by using a selection marker.[35]

Another concept for exploiting natural sources is called modular protein design. This idea emphasizes the predominance of a limited number of elementary secondary structure units which could be adopted by protein sequences having a low degree of similarity. The permutation of protein modules requires nonhomologous recombination for generating functional diversity rather than sequence diversity.[36]

3. Strategies for searching improved biocatalysts

3.1. Linking genotype and phenotype

The classic evolution experiments with RNA and DNA were successful in vitro because nucleic acids represent both function (phenotype) and genetic information (genotype). The directed evolution of enzymes, however, differs insomuch as diversity is created on the DNA level, but selection or screening act on the level of encoded protein. Therefore, functional expression of the information-carrying DNA libraries, whether generated by random mutagenesis or by recombination, is a necessary prerequisite for the detection of improved enzyme variants. The most common approaches for recombinant protein expression employ the cellular trancription and translation machineries of well-established organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, or *Bacillus subtilis*. These cellular expression systems also guarantee the association of a specific protein variant and its encoding gene. This is essential for the identification and amplification of desired mutants after selection or screening, as well as for further cycles of evolution. Alternatively, a physical link between genotype and phenotype may be established by generating fusions between the protein of interest and a bacteriophage coat protein. Following intracellular assembly, recombinant phages express the protein variants on their surface while enclosing the appertaining genetic information within their genome.[37, 38]

Irrespective of whether a protein library is expressed in a recombinant host or displayed on bacteriophage, the available protein diversity is limited by the transformation or transfection efficiencies of bacterial, or eukaryotic cells. Furthermore, the expression of nonhomologous or even toxic proteins may severely interfere with some host environments. Thus, selection may enrich only those cells which survive by reducing or circumventing the expression of the specific protein, or by preventing the correct protein folding. Cell-free transcription–translation systems that were recently developed may provide the basis for protein evolution in the absence of cells: They establish a physical genotype–phenotype linkage in vitro either by stabilizing the mutual attachment of correctly folded complete protein and its encoding mRNA to the ribosome (called ribosome display),[39] by generating covalent fusions between a peptide or protein and its mRNA,[40] or by distribution of a library-based transcription–translation system within aqueous droplets in a water-in-oil emulsion.[41]

3.2. Selection

Functional protein libraries can be created rather easily by using one of the strategies described above. Therefore, the most challenging step in directed evolution experiments is to develop a screening or selection scheme that is sensitive to the properties of interest. Selection can be used in vivo if a substantial growth advantage is conferred to those clones that harbor a protein variant with the desired improvement. Most often, this is achieved by genetic complementation of hosts that are deficient in a certain pathway or activity. In other cases, the positive feedback coupling between a property of interest and cell survival may be achieved with alteration of genetic contexts, for example, employing enzyme-specific control elements like transcriptional promoters.[42] Selective enrichment of only those clones that express the particular enzyme function can be very efficient. It should be noticed, however, that in vivo selection systems usually represent highly specific solutions and often are difficult to implement because microbial hosts are extremely flexible in circumventing the applied constraints and in inventing solutions that are not necessarily related to the targeted activity.

In vitro enrichment procedures that are detached from cell survival may also be termed selection. Originally, these techniques have been developed for the "biopanning" of phage-displayed peptide libraries by binding to a ligand that is immobilized on an appropriate column matrix. Recently, the approach has also been applied to the selective enrichment of phage-displayed functional enzyme libraries. Therefore, the idea of panning optimal binding partners needs to be extended to a panning of optimal catalysts by using either transition state analogues,[43] immobilized suicide substrates,[44] or reaction substrates that are covalently linked to the same or another phage via a second fusion.[45–47] However, the enrichment of improved enzymes by biopanning remains challenging because the assessment of phage-displayed enzymes on the basis of their catalytic activity, that is, on the basis of their kinetic parameters, has not yet been possible.[48]

3.3. Screening

Screening is an important alternative to selection which requires that the specific property is directly observable by using physical or biochemical analysis. As compared with in vivo selection, the screening approach enables a better control of the applied constraints, and also is more versatile, predominantly in unnatural environments, or with unnatural substrates. The number of individual mutants that can be tested in a certain period of time (throughput), however, may be lower—depending on the enzymatic reaction and the sensitivity of the applied detection principle.

The comparative assessment of individual mutants usually requires that the mutant libraries are diluted and distributed. This can either be achieved by conventional plating of transformants on agar plates or filter membranes, or by distribution of the mutant pool in a microtiter format (usually 96- or 384-well plates, but also formats with higher sample density, including silicon wafers). This time-consuming step is sometimes accelerated by using robotic systems. Common assays are based on visual or spectroscopic detection, for example formation, alteration, or destruction of colors or fluorescence characteristics. The determination of the optical parameters can also be accomplished by using automatic plate-reading systems, which enable a normalization of measured intensity values to the respective cell densities and, furthermore, may be used for monitoring reaction kinetics. There is an increasing tendency toward automation and parallel processing by using decreasing sample volumes and concentrations, for example by applying the highly sensitive fluorescence correlation spectroscopy (FCS) technique, which requires concentrations in the femtomolar range.[49] Alternative approaches like confocal fluorescence coincidence analysis (CFCS)[50] or a fluorescence-activated cell sorter (FACS) can directly analyze single cells or proteins and, thus, gain (ultra)high throughput by avoiding the transfer of individuals.

Disregarding whether a specific directed-evolution experiment employs a selection or screening approach (for a comparison, see Table 1), it should finally be emphasized that it is important to choose selective constraints that precisely reflect the desired property. Otherwise, "you get what you screen for".[51]

4. Successful application of directed evolution

During the past few years, many enzymes have been improved by directed evolution (Table 2). Some of these new biocatalysts are tuned for use in organic synthesis, and commercial applications of some other enzymes are already in sight. Enzymes that exhibit increased activity in aqueous-organic solvents were among the first products of directed-evolution experiments.[52, 53] These biocatalysts enable the performance of reactions at increased substrate solubility and stability, and thus effect altered reaction equilibria, higher reaction rates, and higher product yields. The directed evolution of a large number of enzymes that exhibit increased performance at elevated temperatures has been driven by a similar motivation.[26, 32, 54–58, 60–62] Furthermore, increased thermostability may be beneficial regarding the long-term stability of proteins at lower temperatures.

Directed evolution has also been employed to improve the expression and folding of recombinant eukaryotic enzymes which fail to adopt their active conformation in a heterologous host, or which are expressed in an artificial context, for example, in the form of a fusion protein.[63–66] Likewise, the secretion of correctly folded enzymes has been facilitated by using evolutionary optimization.[67] In most of these cases, the increased levels of expression and native folding have been attributed to few point mutations within the structural genes.

The narrow range of substrates that are accepted by natural enzymes often retards or prevents their use in new synthetic and commercial applications. By far most results therefore reflect the efficient tuning of catalytic efficiency toward nonnatural substrates.[34, 42, 43, 65, 68–88] Similarly, the enantioselectivity of specific bioconversions has been significantly improved by using evolutionary approaches.[89–91] Enzymes with altered substrate specificity that yield yet inavailable products have also recently been generated by using the molecular breeding of new biosynthetic pathways.[92] Together with similar attempts at mixing subunits of multi-enzyme complexes,[93] this approach opens up the horizon toward new biologically active compounds.

The conversion of a specific enzymatic activity into another has very recently been achieved by using the methods of directed evolution, and by using a combination of rational and

Table 1. *Comparison of strategies for searching improved biocatalysts.*

Method	Requirements	Advantages	Limitations
selection	linkage between desired activity and cell survival	enrichment of positives	false positives (viable but undesired mutants)
		elimination of undesired variants	indirect measurement (enzyme performance $\rightleftharpoons$ cell growth)
		simultaneous assessment of large populations (ca. 10^{10} individual mutants)	complex, nontrivial assay
screening	individualization of mutant clones; in some cases, isolation of mutant proteins from competitive cellular activities	direct testing of each single clone for the desired activity	multiple pipetting/washing/transfer steps
	often: need for fluorogenic or chromogenic substrates	assays in nonnatural environments (artificial substrates, organic solvents, etc.)	low throughput (ca. 10^5 individual mutants in a few days if automation is employed)
		qualitative and quantitative assay of one or more parameters	detectable minimum change and details of analysis determine throughput

Table 2. *Examples of enzymes that were successfully optimized by using directed evolution.*[a]

Target enzyme	Evolved property	Change	Ref.
amidase (*B. stearothermophilus*)	increased expression in *E. coli*	23-fold	[63]
aminoacyl-tRNA synthetase	increased aminoacylation of orthogonal suppressor tRNAs with diverse ribosomally accepted molecules	55-fold	[68]
arsenate detoxification pathway	increased activity	40-fold	[97]
aspartate aminotransferase	(1) increased activity toward β-branched amino and 2-oxo acids;	10^5-fold	[69]
	(2) increased activity toward valine	2.1×10^6-fold	[70]
biphenyl dioxygenase	activity toward polychlorinated biphenyls (PCBs)	degradation of various PCBs, polychlorinated benzene, and toluene	[71]
β-glucosidase A	thermostability at 65 °C without decrease in catalytic activity	$t_{1/2} = 12$ min	[54]
β-glucosidase CelB (*Pyrococcus furiosus*)	increased catalytic activity at 20 °C	3-fold	[55]
β-glucoronidase	retention of function after glutaraldehyde treatment	increased stability toward glutaraldehyde and formaldehyde	[98]
β-lactamase	increased activity toward cefotaxime	32 000-fold	[72]
	increased activity toward cefotaxime	inactivation of 20 000-fold higher concentration of cefotaxime, 2383-fold increase in activity	[73]
$(\beta\alpha)_8$-enzyme IGPS[b]	activity switch to PRAI[b]	sixfold higher PRAI activity than wild-type enzyme	[94]
$(\beta\alpha)_8$-enzyme ProFARI[b]	PRAI activity while retaining ProFARI activity	PRAI activity $3 - 11 \times 10^4$ lower than wild-type; ProFARI activity 26-fold lower than wild-type	[95]
carboxymethyl cellulase	increased activity	2.2 – 5-fold	[99]
catalase I	increase in intrinsic peroxidase activity	increase from 2% to 58%	[100]
catechol-2,3-dioxygenases	increased thermostability	13 – 26-fold activity at 50 °C	[56]
cephalosporinase	increased activity toward moxalactam	270 – 540-fold	[34]
chorismate mutase (dimer)	conversion to soluble monomeric form	activity of monomeric and hexameric enzyme	[64]
cytochrome P450 BM-3	altered substrate specificity	hydroxylation of indole	[74]
cytochrome P450 monooxygenase	increased activity toward naphthalene in the absence of cofactors	5 – 20-fold	[75]
cytochrome *c* peroxidase	increased activity toward guaiacol	300-fold	[76]
D-selective hydantoinase (*Arthrobacter* sp.)	enantioselectivity and increased activity	conversion into L-hydantoinase, 5-fold activity	[89]
DNA polymerase β	increased spontaneous mutation frequency	10 – 30-fold	[77]
deoxyribonucleoside kinase (*Drosophila melanogaster*)	sensitivity toward nucleoside analogues	316-fold decrease in LD_{100} of transformed *E. coli* in the presence of AZT,[b] 11-fold decrease with ddC[b]	[78]
esterase (*Pseudomonas fluorescens*)	increased enantioselectivity	twofold	[90]
	hydrolysis of sterically hindered 3-hydroxy esters	activity with an increase in enantiomeric excess from 0% to 25%	[79]
Flp recombinase	increased thermostability	improved recombination efficiency	[57]
fungal peroxidase	increased thermal and oxidative stability	174-fold thermostability, 100-fold oxidative stability	[58]
galactosidase	conversion to fucosidase	1000-fold increase in specificity towards *p*-nitrophenyl furanoside, 66-fold increase in specific activity	[80]
glutathione transferase	substrate specificity	activity toward a range of new substrates	[43]
HIV reverse transcriptase	resistance toward nucleoside analogues	DNA-dependent DNA polymerase activity and resistance to AZT	[81]
horse heart myoglobin	increased intrinsic peroxidase activity	25-fold	[101]
horseradish peroxidase	increased activity toward ABTS[b] and guaiacol	total, 40-fold; ABTS, 5.4-fold; guaiacol, 2.3-fold	[65]
3-isopropylmalate dehydrogenase (*B. subtilis*)	increased thermostability	3.4-fold activity at 70 °C	[59]
kanamycin nucleotidyl transferase (*B. stearothermophilus*)	increased thermostability	(1) 200-fold increase in $t_{1/2}$ at 60 – 65 °C	[60]
		(2) 20 °C increase	[102]
lactate dehydrogenase (*B. stearothermophilus*)	increased activity in the absence of cofactor fructose-1,6-biphosphate	70-fold	[103]
lipase (*Pseudomonas aeruginosa*)	increased enantioselectivity	increase in enantiomeric excess from 2% to 81%	[91]
lipases (*Staphylococcus hyicus*, *S. aureus*)	substrate specificity (phospholipids vs. short-chain fatty esters)	3-fold increase in activity toward long-chain pNB[b] esters	[82]
lipase (*S. aureus*)	substrate specificity (activity on phospholipids)	11.6-fold increase in abolute phopholipase activity, 11.5-fold increase in phospholipase/lipase ratio	[88]

Table 2. (Continued)

Target enzyme	Evolved property	Change	Ref.
N-carbamylase – D-hydantoinase fusion protein	stabilization of fusion protein	6-fold increase in yield of D-amino acids	[66]
O^6-alkylguanine alkyltransferase	resistance toward inhibitor BG[b] and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine	reduction of BG inhibitory concentration to 50%	[104]
O^6-methylguanine methyltransferase	resistance toward *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in vivo	2.7 – 5.5-fold decrease in mutation frequency	[105]
phytoene desaturase, lycopene cyclase	new carotenoid pathway	synthesis of 3,4,3′,4′-tetradehydrolycopene and torulene	[92]
pNB esterase	increased thermostability	14 °C increase in T_m without any decrease in activity at all temperatures	[61]
	increased activity in aqueous/organic solvents	50 – 150-fold activity toward different pNB esters in 25 – 30% DMF	[52]
protease SSII (*B. sphaericus*)	increased activity at 10 °C	6-fold activity, while retaining 3.3-fold lower activity at 70 °C	[106]
restriction endonuclease *Eco*RV	extended recognition site	recognition of 8-bp and 10-bp sites	[83]
serine protease (*Lysobacter enzymogenes*)	altered specificity and increased activity	20 – 45-fold increase in rate and a greater selectivity	[84]
subtilisin (*B. lentus*)	expression level of secreted enzyme	50% increase	[67]
subtilisins	various properties	increase in activity, stability	[107]
subtilisin BPN′	increased activity at decreased temperature	2-fold increase in activity at 10 °C	[108]
subtilisin E	activity in aqueous/organic solvents	170-fold activity in 60% DMF	[53]
	increased thermostability	17 °C increase in T_{opt}, increased activity at all temperatures	[62]
	increased thermostability	50-fold increase in $t_{1/2}$ at 65 °C	[32]
subtilisin S41 (psychrophilic)	increased thermostability	100-fold increase in $t_{1/2}$	[26]
Taq DNA polymerase I	functional complementation of *E. coli* DNA polymerase I in vivo	retention of activity of active-site mutants	[109]
	increased mutation frequency	7 – 25-fold	[85]
T7 RNA polymerase	increased mutation frequency	$\geq$ 20-fold	[42]
thymidine kinase (HSV)[b]	increased sensitivity toward ganciclovir and/or aciclovir	43-fold toward ganciclovir, 20-fold toward aciclovir	[86]
thymidine kinase-1 or -2 (HSV)	decreased sensitivity toward AZT	TK-1, 32-fold; TK-2, 16 000-fold	[87]

[a] See also ref. [96]. [b] Abbreviations: ABTS = 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid; AZT = 3′-azido-3′-deoxythymidine; BG = O^6-benzylguanine; ddC = dideoxycytidine; IGPS = indoleglycerol phosphate synthase; pNB = *para*-nitrobenzoate; PRAI = phosphoribosyl anthranilate isomerase; ProFARI = *N*′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide isomerase.

evolutionary design principles.[94, 95] These approaches essentially copy natural evolution by recruiting existing functional protein scaffolds and refitting them to new enzymes. Experiments of this type may also unravel the evolutionary relations between enzymes that share some common properties.

In conclusion, many impressive examples have demonstrated that directed evolution represents a powerful and reliable tool for improving biocatalysts in reasonably short periods of time. The technique itself will continue to evolve and address many of the present questions and limitations. Certainly, the future of evolutionary biotechnology will be exciting!

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