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MINI-REVIEW

K.-E. Jaeger · T. Eggert · A. Eipper · M.T. Reetz

Directed evolution and the creation of enantioselective biocatalysts

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Abstract Directed evolution has emerged as a key technology to generate enzymes with new or improved properties that are of major importance to the biotechnology industry. A directed evolution approach starts with the identification of a target enzyme to be optimized and the cloning of the corresponding gene. An efficient expression system is needed before the target gene is subjected to random mutagenesis and/or in vitro recombination, thereby creating molecular diversity. Subsequently, improved enzyme variants are identified, preferably after being secreted into culture medium, by screening or selection for the desired property. The genes encoding the improved enzymes are then used to parent the next round of directed evolution. Enantioselectivity is a biocatalyst property of major biotechnological importance that is, however, difficult to deal with. We discuss recent examples of creating enantioselective biocatalysts by directed evolution.

Introduction

The production of enantiomerically pure compounds is of steadily increasing importance to the chemical and pharmaceutical industries and, therefore, the world market for chiral fine chemicals, pharmaceuticals, agrochemicals, and flavor compounds rapidly expands (Table 1). In the year 2000, the worldwide sales volume for chiral drugs exceeded the US $ 100 billion barrier for the first time (Stinson 2000). The demand for chiral drugs is caused by the fact that cell surface receptors are biological molecules that are chiral by themselves, and efficient drug molecules must match the receptor's asymmetry. Furthermore, the US Food and Drug Administration (FDA) asks companies to rigidly evaluate whether or not a novel drug molecule can be produced as a single isomer.

How can the growing requirements for the synthesis of optically active compounds be met? Basically, two options are available: asymmetric chemical catalysis using transition metal catalysts (Brunner and Zettlmeier 1993; Ojima 1993; Noyori 1994; Jacobsen et al. 1999) or biocatalysis using enzymes (Davies et al. 1989; Wong and Whitesides 1994; Drauz and Waldmann 1995; Faber 1997). In the latter case, large screening programs have revealed a number of enzymes with the ability to catalyze enantioselective reactions (Bornscheuer and Kazlauskas 1999). However, in the majority of cases the enantioselectivity of a given enzyme is not high enough for a desired reaction. Therefore, it is necessary to develop novel methods allowing creation of enantioselective enzymes.

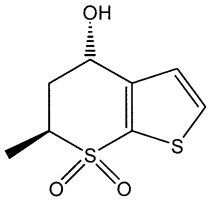
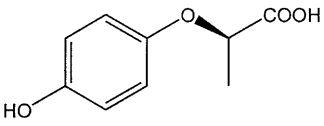
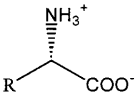
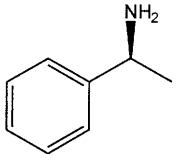
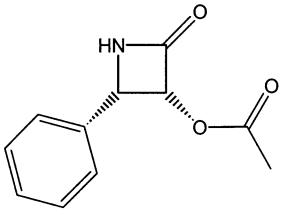
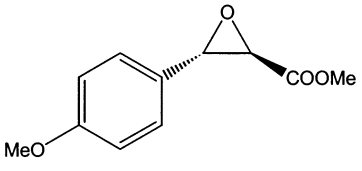
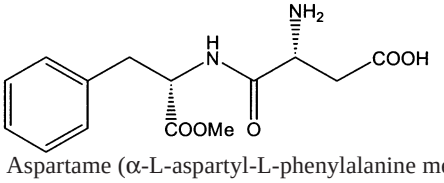
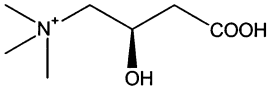
Today's enzymes are the product of biological evolution which has taken several millions of years. They usually catalyze a given reaction with high specificity and enantioselectivity. However, since they are adjusted perfectly to their physiological role, their activity and stability are often far away from what organic chemists need. This is true for the stability of enzymes in organic solvents and particularly for enantioselectivity of reactions yielding industrially important compounds.

Nature itself appears to provide a solution for this apparent dilemma: natural evolution produces a large number of variants by mutation and subsequently selects the 'fittest' variant. This process can be mimicked in the test tube by using modern molecular biology methods of mutation and recombination. This collection of methods has been termed 'directed' or '*in vitro*' evolution and provides a powerful tool for the development of biocatalysts with novel properties, without requiring knowledge on enzyme structures or catalytic mechanisms (Jaeger and Reetz 2000; Petrounia and Arnold 2000). It has been demonstrated that directed evolution can produce en-

K.-E. Jaeger (✉) · T. Eggert
Ruhr-Universität Bochum,
Lehrstuhl Biologie der Mikroorganismen,
Universitätsstrasse 150, 44780 Bochum, Germany
e-mail: karl-erich.jaeger@ruhr-uni-bochum.de
Tel.: +49-234-3223101, Fax: +49-234-3214425

A. Eipper · M.T. Reetz
Max-Planck-Institut für Kohlenforschung,
Kaiser-Wilhelm-Platz 1, 45470 Mülheim/Ruhr, Germany

Table 1 Biotechnologically important chiral products produced by enantioselective biocatalysis (data from Liese et al. 2000)

Product	Applications	Enzyme (EC no.), Source	ee value (%)	Process capacity	Company
 (4*S*,6*S*)-5,6-Dihydro-4-hydroxy-6-methyl-4H-thieno[2,3b]thiopyran-7,7-dioxide	Intermediate in the synthesis of Trusopt for treatment of glaucoma	Alcohol dehydrogenase (1.1.1.1), *Neurospora crassa*	>98	Multi t	Zeneca Life Science Molecules, UK
 (*R*)-2-(4´-Hydroxyphenoxy)propionic acid	Intermediate in the synthesis of herbicides	Oxidase (1.X.X.X), *Beauveria bassiana*	>98	120,000 1 reactor volume	BASF AG, Germany
 D-amino acid	Food additive	D-Amino acid transaminase (2.6.1.21), *Bacillus* sp.	100	Multi t	NSC Technologies, Monsanto, USA
 (*S*)-1-Phenylethylamine	Intermediate for pharma-ceuticals and pesticides	Lipase (3.1.1.3), *Burkholderia plantarii*	>99	>100 t×a^{-1}	BASF AG, Germany
 (3*R*,4*S*) *cis*-Azetidinone acetate	Intermediate for the synthesis of paclitaxel (taxol) used for cancer treatment	Lipase (3.1.1.3), *Burkholderia cepacia*	>99.5	Multi kg	Bristol-Myers Squibb, USA
 (2*R*,3*S*)-3-(4-Methoxyphenyl) glycidic acid methyl ester	Intermediate in the synthesis of diltiazem	Lipase (3.1.1.3), *Serratia marcescens*	99.9		Tanabe Seiyaku Co. Ltd., Japan DSM, The Netherlands
 Aspartame (α-L-aspartyl-L-phenylalanine methyl ester)	Low-calorie sweetener for food	Aminopeptidase (3.4.11.1), *Pseudomonas putida*	>99.9	ton scale	DSM, The Netherlands
		Thermolysin (3.4.24.27), *Bacillus proteolicus*	>99.9	>2000 t·a^{-1}	Holland Sweetener Company, The Netherlands
 (*R*)-L-Carnitine (3-hydroxy-4-(trimethylamino)butanoate)	Additive in infant, health sport, and geriatric nutrition	Carnitine dehydratase (4.2.1.89), *Escherichia coli*	99.9	300 t·a^{-1}	Lonza AG, Switzerland

zymes with altered substrate specificity (Stemmer 1994a; b; Zhang et al. 1997; Takato et al. 1998; Altamirano et al. 2000), thermal stability (Giver et al. 1998; Zhao and Arnold 1999), and organic solvent resistance (You and Arnold 1994; Moore and Arnold 1996). However, it is unclear at present which strategy is the most efficient for evolution of a desired property or for a given protein. This mini-review summarizes current methods for directed evolution and also briefly discusses some recent examples for evolution of enantioselective biocatalysts.

General strategy for directed enzyme evolution

The general strategy to isolate enzymes with novel properties by directed evolution is outlined in Fig. 1. Molecular diversity is created by random mutagenesis and/or recombination of a target gene or a set of related genes. A powerful (over)expression system is needed to express the variant proteins at a level high enough to allow for screening and/or selection of better variants. In many cases, secretion of the protein of interest into the bacterial culture supernatant greatly facilitates screening, in particular when microtiter plates are used. As soon as enzyme variants with improved properties are identified the cognate genes are used to parent the next round of evolution.

Several excellent recent reviews describe various aspects of directed evolution approaches to improve key properties of biocatalysts (Petrounia and Arnold 2000; Tobin et al. 2000). In the following paragraph we briefly discuss the different steps of a directed evolution experiment.

Overexpression of genes and secretion of enzymes

The first step needed to set up a successful directed evolution protocol is frequently considered as trivial. Fortunately, some proteins can easily be overexpressed and secreted by using commercially available systems (Wong 1995; de Vos et al. 1997; Baneyx 1999), a prominent example being subtilisin of *Bacillus subtilis* (Rao et al. 1998). However, many enzymes of interest are not amenable to those systems; examples include a variety of different lipases. In such cases, the development of a

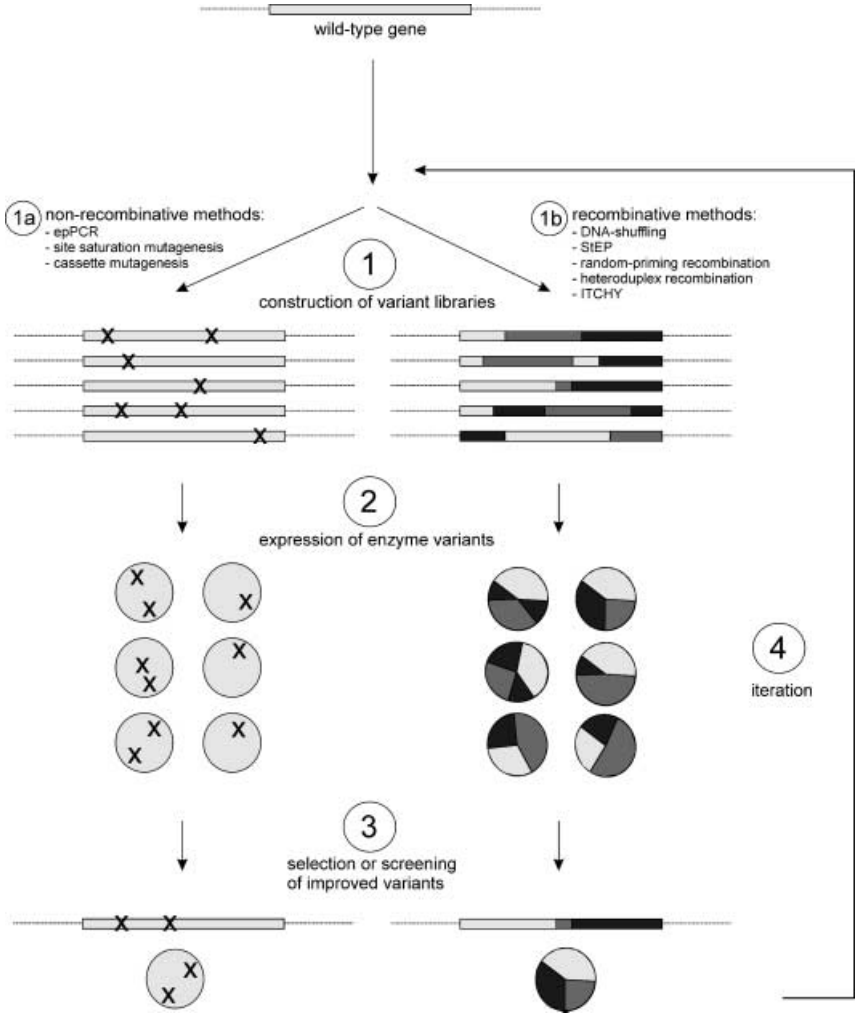


Fig. 1 Experimental strategy for directed evolution of a biocatalyst. Variant libraries are produced by random mutagenesis (*1*) using non-recombinative (*1a*) or recombinative methods (*1b*). The mutant genes are expressed in a suitable bacterial host (*2*) and the enzyme variants are selected or screened for an improved property (*3*). The genes encoding these enzyme variants are used as templates for the next round of directed evolution (*4*) (*epPCR* error-prone polymerase chain reaction, *StEP* staggered extension process, *ITCHY* incremental truncation for the creation of hybrid enzymes)

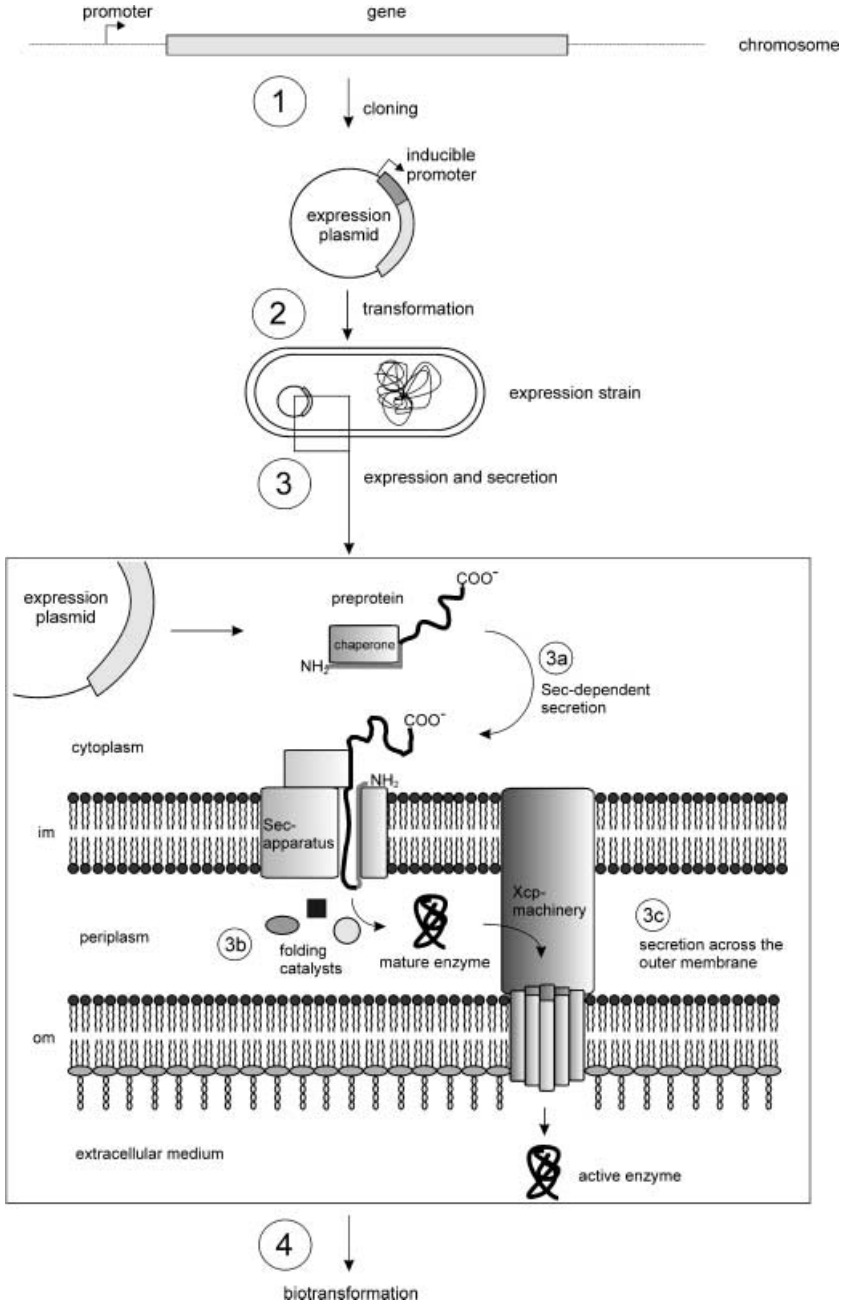


Fig. 2 Schematic presentation of a suitable system for gene overexpression and enzyme secretion. The expression and secretion system for lipase of *Pseudomonas aeruginosa* is shown which allows for high-throughput screening in microtiter plates (Liebeton et al. 2000). The gene of interest is cloned into an expression vector (*1*) fused to a signal sequence. The plasmid is then transferred into a bacterial cell by transformation (*2*). Gene expression (*3*) results in the synthesis of a preprotein that is secreted across the cytoplasmic membrane in a Sec-dependent manner (*3a*). Several different catalysts assist folding of the mature enzyme in the periplasm (*3b*). The mature enzyme is transported across the outer membrane via the main terminal branch of the type II secretion pathway, which is formed by the Xcp-machinery in *P. aeruginosa* (*3c*). The culture supernatant containing the active enzyme can finally be used to catalyze the desired biotransformation reaction (*4*)

cloning and overexpression system is needed which preferably also ensures the secretion of the enzyme to be evolved. Figure 2 shows that the construction of such systems may require sophisticated molecular cloning strategies as well as a thorough understanding of the cellular processes underlying protein folding and secretion.

Examples include lipases from *Pseudomonas* species which are frequently used for a variety of biotransformations (Jaeger and Reetz 1998). These enzymes require the functional assistance of about 30 different cellular proteins before they can be recovered from the culture supernatant in an enzymatically active state (Rosenau and Jaeger 2000). Further complications may arise from the fact that many enzymes cannot be overexpressed in their homologous hosts, making expression in heterologous host organisms essential. For *Pseudomonas* enzymes, it has been demonstrated that folding and secretion are highly specific processes, which normally do not function properly in heterologous hosts.

Methods to identify enantioselective biocatalysts

The application of random mutagenesis and /or recombinative methods yields large libraries of mutant genes which may comprise 10^6–10^{10} members, with each encoding a different enzyme variant. An enzyme consisting

of 300 amino acid residues can theoretically exist in 20^{300} possible linear combinations of all 20 amino acids at each position.

Table 2 illustrates the dimensions of the libraries that should in principle be sampled if a protein consisting of 300 amino acid residues is mutated in a way that each variant contains only a single amino acid residue exchange (Arnold 1996). Given these library sizes, the goal to identify enzymes with improved enantioselectivity is not easy to attain. In general, the selection of a better or even the best variant would be the method of choice. During the last few years, many publications have appeared describing novel selection systems, including phage display and ribosome display systems (Schultz and Lerner 1995; Vanwetswinkel et al. 1996; Avalle et al. 1997; Hanes and Plückthun 1997; Hanes et al. 1998; Sieber et al. 1998). Although compounds have been synthesized which may be applicable to identify enantioselective lipases by phage display (Deussen et al. 2000a, b), no methods have been described to date that allow for the direct selection of enantioselective enzymes. Therefore, screening for enantioselectivity is currently the method of choice. We have developed a number of efficient screening systems, one of which is described in detail in the accompanying paper (Reetz 2001a). The methods for high-throughput screening include a spectrophotometric assay (Reetz et al. 1997), IR-thermography (Reetz et al. 1998), electrospray ionization mass spectrometry (ESI-MS) (Reetz et al. 1999), and capillary array electrophoresis on chiral columns (Reetz et al. 2000). A detailed discussion of these and other screening methods for identification of enantioselective biocatalysts can be found in a comprehensive review article (Reetz 2001b).

Table 2 Theoretical number of variants to be generated from a model enzyme composed of 300 amino acids (Arnold 1996). The values were calculated by using the formulae $N = \frac{\lg^M X!}{(X-M)!M!}$ with
N=number of variants
M=number of amino acids exchanged per enzyme molecule
X=number of amino acids per enzyme

Number of amino acids exchanged (*M*)	Number of variants (*N*)
1	5,700
2	16,190,850
3	30,557,530,900

Generation of libraries by non-recombinative methods

Error-prone polymerase chain reaction

The development of the polymerase chain reaction (PCR) (Saiki et al. 1985; Mullis and Faloona 1987) and its improvement by using thermostable DNA polymerases (Saiki et al. 1988) paved the way for the development of efficient methods for directed evolution. A large number of thermostable DNA polymerases are commercially available, examples include *Taq*- (Roche Diagnostics, Gibco-BRL, Fermentas), *Tth*- (Roche Diagnostics, Perkin-Elmer), *Pwo*- (Roche Diagnostics), and *Pfu*-polymerase (Stratagene). The *Taq*-polymerase isolated from the thermophilic bacterium *Thermus aquaticus* (Chien et al. 1976) lacks the so-called proof-reading activity *in vitro* and therefore incorporates wrong nucleotides at a frequency of $0.1–2\times10^{-4}$ (Tindall and Kunkel 1988; Eckert and Kunkel 1990). In nature, the incorporation of "wrong" nucleotides into DNA during replication is an important driving force to produce novel protein variants that can cope with altered environmental situations. Accordingly, several protocols have been developed with the aim of increasing the error rate of *Taq* polymerase, which can infinitely be varied by: (1) increasing the concentration of $MgCl_2$, (2) addition of $MnCl_2$, (3) using unbalanced concentrations of nucleotides, or (4) using a mixture of triphosphate nucleoside analogues (Zhou et al. 1991; Cadwell and Joyce 1992, 1995; Zaccolo et al. 1996). Furthermore, these modifications of standard PCR protocols can also be combined to achieve even higher error rates (Table 3).

Further variations of error rates, which are usually given as base substitutions per 1,000 base pairs (bp) of

Table 3 Experimental protocols for error-prone polymerase chain reactions (ep-PCR) yielding different error rates. The gene *lipA* of *Bacillus subtilis* encoding extracellular lipase (181 amino acids) was used as the target DNA. The error rates were determined by sequencing of five mutant genes generated by each protocol

	ep-PCR protocol 1	ep-PCR protocol 2	ep-PCR protocol 3
Primer 1	5 pmol	5 pmol	5 pmol
Primer 2	5 pmol	5 pmol	5 pmol
Buffer	67 mM TRIS/HCl pH 8.8	75 mM TRIS/HCl pH 8.8	75 mM TRIS/HCl pH 8.8
$(NH_4)_2SO_4$	16,6 mM	20 mM	20 mM
$MgCl_2$	6.5 mM	6.0 mM	6.0 mM
$MnCl_2$	–	0.15 mM	0.3 mM
EDTA	6.7 µM	–	–
dNTPs	0.2 mM	0.2 mM	0.2 mM
Tween 20	–	0.01%	0.01%
β-Mercaptoethanol	10 mM	–	–
Taq-polymerase (Goldstar, Statagene)	2.5 U	2.5 U	2.5 U
Template DNA	1 ng	1 ng	1 ng
Error rate (base substitutions per 1,000 bp)	1–2	2–4	5–7

the target gene, are caused by the varying base composition of the genes (e.g., GC content).

Site-specific saturation mutagenesis

Random mutagenesis by error-prone PCR (ep-PCR) generates point mutations; however, not all amino acids of a particular target protein can be exchanged at an equal rate, the major reason being the degeneracy of the genetic code, with 61 codons encoding 20 amino acids and 3 codons used as stop codons. Only 2 of 20 amino acids are specified by a single codon, namely Trp by UGG and Met by AUG, whereas the amino acids Leu, Ser, and Arg are each encoded by 6 different codons. As a consequence, approximately one-third of all base substitutions introduced by ep-PCR will not result in an amino acid substitution. Additionally, the likelihood of two or three base substitutions in a single codon is low. By altering 1 bp in a triplet, 9 different codons can be created, i.e., only a small number of amino acids can be introduced at a given position in the enzyme. An estimation of amino acid exchanges that could be introduced into the *Pseudomonas aeruginosa* lipase (a 285-amino acid protein) revealed that the actual number of variants produced by a single base substitution was only 34% of the theoretical number (Liebeton et al. 2000). This problem can partly be overcome by application of site-specific saturation mutagenesis, which can be used to introduce all possible amino acids at any predetermined position in a gene. In a first round of random mutagenesis, screening, and DNA sequence analysis, positions can be identified that are important for improving the property of interest. Such "hot spots" can then be subjected to site-specific saturation mutagenesis to investigate whether the optimal amino acid has been introduced by the preceding round of random mutagenesis.

Several techniques are available for site-specific saturation mutagenesis. Cassette mutagenesis can be performed by synthesis of the entire target sequence and ligation into two unique restriction sites of the target gene. During synthesis, all nucleotides (dATP, dCTP, dGTP, and dTTP) are incorporated at equal proportions in a given position, simply by using 25% of each nucleotide during the process. If appropriate restriction sites are missing, the site-specific saturation mutagenesis can also be performed by PCR methods used for site-directed mutagenesis (Kammann et al. 1989; Landt et al. 1990; Barettino et al. 1993). For this purpose we have developed a simple and efficient PCR technique named the one-step overlap extension PCR (Urban et al. 1997), which is outlined in Fig. 3.

Site-specific saturation mutagenesis is performed by synthesizing the mutagenic primers a and b using equimolar concentrations of the nucleoside phosphoramidites dA, dC, dG, and dT at a particular codon position. However, such mutant populations have been shown to be biased towards the original nucleotides. This bias can be eliminated by modifying the concentrations of nucleoside phosphoramidites during oligonucleotide synthesis (Airaksinen and Hovi 1998).

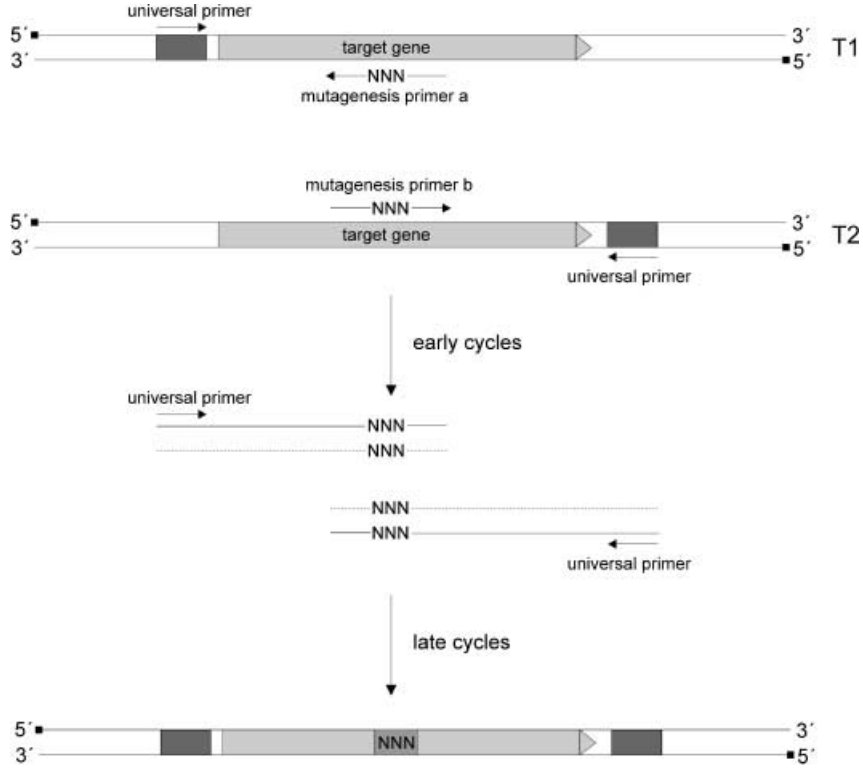


Fig. 3 One-step overlap extension PCR (Urban et al. 1997) for site-specific saturation mutagenesis. The target gene is cloned in two orientations with respect to the location of the universal primer (*grey box*) into a standard cloning vector (e.g., pUC18/pUC19 or pBluescript KS/pBluescript SK). The PCR reaction is performed with the universal primer and a mixture of two mutagenesis primers a and b that contain at a predefined position the complete set of 64 codons. Intermediate products are amplified during the early PCR cycles. Due to their terminal complementarity, these products will overlap and will subsequently be extended. During the late PCR cycles, full-length products are formed that encode variants containing all 20 amino acids at the predefined position

Cassette mutagenesis

This method is used when mutagenesis of limited and defined gene segments is required. Such segments may encode functionally important domains of an enzyme, which may have previously been identified by a rational design approach using three-dimensional structural data or by defining hot spot regions upon screening of ep-PCR libraries. Those gene segments can be replaced by randomly mutated DNA cassettes, which can be generated by different methods depending on the size of the DNA fragment. Synthetic oligonucleotides can be created that carry random point mutations when doped nucleotide phosphoramidites (one nucleotide contaminated with small amounts of the other three nucleotides) are used during the synthesis process. For larger size cassettes (>50 bp) conventional ep-PCR methods can be used.

Cassette mutagenesis methods use high error rate mutagenesis and therefore create libraries with a high diversity at a predefined position. Furthermore, the size of the variant libraries to be screened is minimized. However, this method is not entirely random and also does not allow complete sampling of the sequence space of a given enzyme.

Generation of libraries by recombinative methods

DNA shuffling was the first method described for *in vitro* recombination (Stemmer 1994a, b) and immediately proved to be a valuable tool for directed evolution of biocatalysts. More recently, other efficient recombinative methods for generating variant libraries have been developed, including the PCR-based staggered extension process (StEP) (Zhao et al. 1998), random-priming recombination (Shao et al. 1998), heteroduplex recombination (Volkov et al. 1999), and the incremental truncation for the creation of hybrid enzymes (ITCHY) (Ostermeier et al. 1999a). In the following paragraphs, we will briefly discuss these techniques.

DNA shuffling

Homologous DNA sequences carrying mutations are recombined *in vitro* in a process consisting of gene fragmentation and subsequent reassembly in a self-priming chain extension method catalyzed by DNA polymerase. Related genes are digested with DNase I to yield double-stranded DNA fragments of 10- to 50-bp lengths. These fragments are reassembled into a full-length gene by a PCR-like reaction consisting of repeated cycles of annealing to a parent template strand and polymerization by DNA polymerase. Recombination occurs by template switching: a fragment originating from one gene anneals to a fragment from another gene. During this process additional point mutations are randomly introduced into the DNA at a rate of 0.7%, which is similar to the rate obtained by ep-PCR (Stemmer 1994b). Modified shuffling protocols with higher fidelity of DNA replication (i.e., lower rate of wrong nucleotide incorporation) are also available if only recombinative events are strived for (Zhao and Arnold 1997).

A valuable extension of the original DNA shuffling method allows recombination of variants of the same gene or homologous genes from different species. This method, termed "molecular breeding," was captured as Family Shuffling™ technology by the company Maxygen (Redwood City, Calif., USA). It is now extensively used to generate or improve enzymes of biotechnological importance (Crameri et al. 1998; Christians et al. 1999; Ness et al. 1999).

Staggered extension process

The StEP (Zhao et al. 1998) is based on a modified PCR reaction. Two or more DNA template sequences are annealed with a specific oligonucleotide. Priming of the template sequences is followed by repeated cycles of denaturation, extremely abbreviated annealing periods, and DNA polymerase-catalyzed extensions of the product. This process is repeated until full-length genes are formed. Template switching, which occurs during the denaturation and annealing phases, finally yields a full-length gene carrying randomly recombined parental sequences.

Random-priming recombination

Another PCR-based recombinative method uses random oligonucleotide primers to create a large number of short DNA fragments spread randomly over the template sequences (Shao et al. 1998). These fragments are reassembled to a library of mutant full-length products by cycles of denaturation, annealing, and enzyme catalyzed DNA polymerization. During this process additional point mutations are introduced due to mispriming of the random-sequence primers. The individual error rate as well as the frequency of recombination can be varied by manipulating the length and concentration of the random primers and by the choice of the annealing temperature and reaction time.

Heteroduplex recombination

The *in vitro* heteroduplex formation and *in vivo* repair method (Volkov et al. 1999) provides a tool for efficient recombination of large genes or even entire operons. A heteroduplex consisting of two closely related DNA sequences is formed *in vitro* and subsequently transformed into bacterial cells where the mismatches are recognized by the natural DNA repair machinery. The recombinative process does not involve physical exchange between double-stranded DNA molecules, but nevertheless gives

rise to a set of sequences with different combinations of mutations.

Incremental truncation for the creation of hybrid enzymes

All *in vitro* recombination methods described to date require relatively high levels of DNA homology in the target sequences, otherwise the recombination events will occur only in regions of homology or they will not occur at all. A novel approach for creating libraries of fused gene fragments independent of sequence homology has been developed (Ostermeier et al. 1999a, b, c), and was termed incremental truncation for the creation of hybrid enzymes (ITCHY). Two parental genes are digested with exonuclease III in a tightly controlled manner to generate truncated gene libraries with progressive 1-bp deletions. The truncated 5´-fragments of the one gene and the truncated 3´-fragments of the other gene are fused to yield a library of chimeric sequences, which are expressed and screened or selected for improved enzyme activity. This method allows creation of functional fusions of genes from overlapping amino- or carboxy-terminal gene fragments independent of DNA sequence homology. Current limitations include the fusion of only two genes per experiment and the creation of just a single crossover between two fragments, thereby restricting diversity of the libraries (Lutz and Benkovic 2000). Therefore, a combination of ITCHY with DNA shuffling may result in an optimized sampling of protein sequence space (Ostermeier et al. 1999c).

Directed evolution of enantioselective enzymes

Despite the wealth of molecular biological techniques available to create libraries of enzyme variants by random mutagenesis and/or recombination, significant studies on the directed evolution of enantioselective enzymes are still scarce (Jaeger and Reetz 2000). This can best be explained by looking at lipases, which constitute the most-important class of enzymes used in organic chemistry (Jaeger et al. 1994; Jaeger and Reetz 1998):

1. Many lipases are known to convert interesting substrates with at least moderate enantioselectivity; however, the corresponding genes are not available at all, or they have not been cloned yet and no overexpression system is available (Jaeger et al. 1999).
2. *Pseudomonas* lipases, which are known to be versatile enzymes with a broad substrate specificity and a high enantioselectivity (Jaeger et al. 1996a), are not readily amenable to cloning and overexpression by using commercially available systems, and sophisticated expression systems have been constructed (Jaeger et al. 1996b; Rosenau et al. 1998).
3. Experimental systems allowing for selection of enantioselective enzymes are not yet available.
4. Generally applicable high-throughput screening methods have been developed only very recently (Reetz 2001b).

Attempts have been made to improve the enantioselectivity of an esterase from *P. fluorescens* by generating variant libraries using the commercially available mutator strain *Epicurian coli* XL1-Red (Bornscheuer et al. 1998). Mutants were assayed for their ability to hydrolyze a sterically hindered 3-hydroxy ester, which resembled a building block for epothilones that exhibit a taxol-like biological activity. Screening was performed on agar plates containing a pH indicator, and one variant was identified with an enantioselectivity of *ee*=25%. This variant, which contained two mutations L181V and A209D, was subjected to further rounds of mutagenesis and screening, but variants with a further improved enantioselectivity were not found (Bornscheuer et al. 1999). To date (December 2000), significant success in evolving enantioselective biocatalysts has been reported for three enzymes which will be discussed below.

Hydantoinase

In an elegant approach the Arnold group has inverted the enantioselectivity of a hydantoinase from *Arthrobacter* species for the production of *L*-methionine in *Escherichia coli* (May et al. 2000). All known hydantoinases are selective for *D*-5-(2-methylthioethyl)hydantoin, which leads to the accumulation of *N*-carbamoyl-*D*-methionine. The wild-type hydantoinase gene was subjected to two successive rounds of random mutagenesis by ep-PCR followed by saturation mutagenesis, and the resulting libraries were screened for altered enantioselectivity by a pH indicator assay. One variant was identified carrying three amino acid exchanges which was 1.5-fold more active than the wild-type enzyme and produced *N*-carbamoyl-*L*-methionine with an *ee*=20% at 30% conversion. When the corresponding gene was co-expressed with a hydantoin racemase and the *L*-*N*-carbamoylase in *E. coli*, this led to the conversion of 90% of the precursor compound D,L-5-(2-methylthioethyl)hydantoin in less than 2 h, compared with 10 h for the wild-type enzyme. This result clearly demonstrated the possibility of optimizing multi-enzyme pathways by directed evolution of single enzyme genes, thereby improving whole-cell catalysts for the production of chiral compounds.

Aldolase

The stereo-controlled formation of carbon-carbon-bonds can be performed biocatalytically using aldolases (Fessner 1998). The 2-keto-3-deoxy-6-phosphogluconate aldolase from *E. coli* catalyzes the reversible addition of pyruvate to a number of aldehydes to form 4-substituted-4-hydroxy-2-ketobutyrates. This enzyme is highly specific for aldehydes with the *D*-configuration at the C2

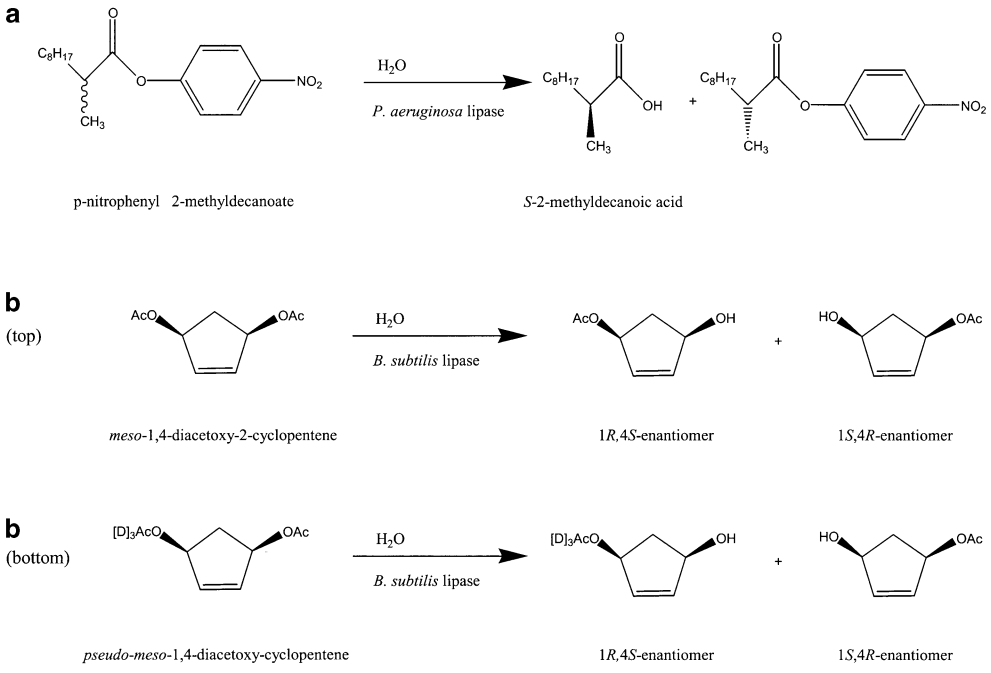


Fig. 4 **a** Lipase-catalyzed hydrolytic kinetic resolution of *p*-nitrophenyl 2-methyldecanoate. **b** Lipase-catalyzed hydrolysis of *meso*-1, 4-diacetoxy-2-cyclopentene (*top*), and of *pseudo-meso*-(1*S*,4*R*)-1-trideutero-acetoxy-4-acetoxy-2-cyclopentene (*bottom*)

position. Recently, Wong's group has evolved novel aldolases capable of accepting both *D*- and *L*-glyceraldehydes as substrates (Fong et. al. 2000). A library was generated by ep-PCR with a low mutation rate (one amino acid exchange per enzyme and generation) and screened for variants that accepted the non-phosphorylated *D*-2-keto-3-deoxygluconate substrates or the *L*-glyceraldehyde instead of the *D*-enantiomer. The screening method consisted of a coupled enzyme assay in microtiter plates with spectrophotometric determination of the time-dependent decrease of NADH absorbance at 340 nm. Four first-generation variants with improved properties were subjected to DNA shuffling and the best variant was again randomly mutated by ep-PCR, yielding several third-generation variants that showed a tenfold improved activity towards the non-phosphorylated substrate *D*-2-keto-3-deoxygluconate and a more than fivefold improved rate for the addition of the (non-natural) *L*-gyceraldehyde to pyruvate. This study demonstrated that novel aldolases can successfully be created by directed evolution and screening of a relatively small number of variants. A total of 4–5 amino acid exchanges introduced into the wild-type enzyme were sufficient here to evolve aldolases that, in contrast to the wild-type enzymes, catalyzed the efficient synthesis of both *D*- and *L*-sugars from non-phosphorylated aldehydes and pyruvate.

Lipases

The first and most-comprehensive study with respect to directed evolution of an enantioselective enzyme was performed with a lipase from *P. aeruginosa* by the groups of Reetz and Jaeger (Reetz et al. 1997; Jaeger and Reetz 1998, 2000; Reetz and Jaeger 1999, 2000; Liebeton et al. 2000; Jaeger et al. 2001). The model reaction studied here was the asymmetric transformation shown in Fig. 4A, namely the hydrolytic kinetic resolution of the chiral 2-methyldecanoic acid *p*-nitrophenyl ester by this lipase, which catalyzed the reaction with an enantioselectivity of only *ee* 2% in favor of the *S*-ester at about 50% conversion corresponding to a selectivity factor *E* of 1.1.

Screening was performed spectrophotometrically in microtiter plates by separate determination of the hydrolysis rate for the *S*- and *R*-enantiomers, respectively. After four rounds of mutagenesis using ep-PCR at a low mutagenesis rate corresponding to an average of one amino acid exchange per enzyme molecule, enantioselectivity was increased to *ee*=81 % (*E*=11). A significant further increase was obtained by combining ep-PCR with saturation and site-directed mutagenesis at those positions at which amino acid substitution had occurred ("hot spots"). Thus, several variants were obtained which displayed *ee*-values of 90–94% (*E*=20–25) (Liebeton et al. 2000). In other studies, the direction of enantioselectivity was reversed, i.e., *R*-selective variants were created. Upon repeating the first round of ep-PCR at a higher mutagenesis rate corresponding to an average of two amino acid exchange events, even better results were obtained (M.T. Reetz, D. Zha, S. Wilensek, K. Liebeton, K.-E. Jaeger, unpublished results), inverting the sense of enantioselectivity of a lipase-catalyzed reaction by directed evolution. At present, it seems that ep-PCR with low or high mutagenesis rates in combination with saturation mutagenesis at "hot spot" positions identified previously is a viable approach to evolve an enantioselective enzyme.

Recently, the three-dimensional structure of *P.aeruginosa* lipase was solved at 2.54-Å resolution in complex

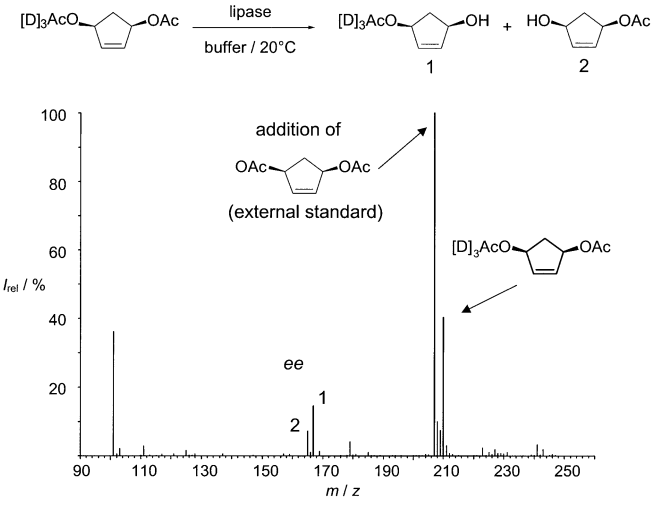


Fig. 5 Typical electrospray ionization mass spectrum of the hydrolysis product of the reaction of the *pseudo-meso* substrate (Fig. 4B) catalyzed by the wild-type *Bacillus subtilis* lipase (LipA)

with a covalently bound inhibitor (Nardini et al. 2000). The availability of the three-dimensional structure allowed to locate the positions of the amino acid substitutions and to rationalize the effects of the mutations (Liebeton et al. 2000). The lipase variant that showed the highest enantioselectivity towards the *S*-enantiomer of the substrate contained five amino acid substitutions, namely, V47G, V55G, S149G, S155F, and S164G. It is interesting to note that four of these substitutions lead to the introduction of a glycine residue that may increase the overall conformational flexibility of this lipase. Surprisingly, none of the substitutions was in direct contact with the stereocenter of the substrate, thus excluding a direct spatial effect on the enantioselectivity of the reaction. Instead, the substitutions are located directly or in close vicinity to loops that are involved in the enzyme's transition from the closed to the open conformation.

More recently, we have started to use a *Bacillus subtilis* lipase as the catalyst in the asymmetric hydrolysis of *meso*-1,4-diacetoxy-2-cyclopentene, with formation of chiral alcohols as shown in Fig. 4B (top). This conversion does not constitute a kinetic resolution and can thus be carried out to 100 % conversion. In order to be able to screen for enantioselectivity, the ESI-MS system developed earlier is being used (Reetz et al. 1999). In the present case the synthesis of a *pseudo-meso*-compound [Fig. 4B (bottom)] was necessary. Hydrolysis leads to the two chiral products shown, which are *pseudo*-enantiomers. The deuterium labeling allows for a distinction in the mass spectrum and integration of the two peaks gives the *ee* value directly. Addition of the non-labeled starting *meso*-diacetate to the product mixture as an external standard allows for the determination of conversion. A typical example of an ESI-MS spectrum in which the *ee* value amounts to 38% is shown in Fig. 5. The sample was taken from the reaction catalyzed by the wild-type *B. subtilis* lipase LipA. An excellent agreement was observed with the value determined by conventional gas chromatography using a chiral phase.

Conclusions

Directed evolution has previously been used to generate mutant enzymes displaying improved properties such as higher activity and stability. It is now well established that the extremely difficult goal of improving enantioselectivity can also be achieved by directed evolution. The major challenges to be met included the evaluation of efficient strategies for exploring sequence space with respect to enantioselectivity and the development of high-throughput screening systems for assaying enantioselectivity. Future efforts in this fascinating area of protein design are necessary, including the further optimization of enantioselectivity of the model reactions, as well as the use of novel substrates with industrial relevance and other enzymes such as oxidases, reductases, or epoxide hydrolases. The development of more-efficient methods to explore protein sequence space will be an ongoing endeavor. Finally, it should be emphasized that directed evolution of enantioselective enzymes offers a unique opportunity to increase our knowledge about the way enzymes function. Thus, the determination by protein crystallography of the three-dimensional structures of enantioselective enzyme variants in combination with molecular modeling and molecular dynamic calculations will help to uncover the structural basis of enantioselectivity. In the near future we expect that directed evolution will successfully be used to create novel biocatalysts that are not only enantioselective *and* active, but also stable enough to allow for a variety of biotechnological applications.

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