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Principles and Methods of Evolutionary Biotechnology

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Abstract

Evolutionary biotechnology applies the principles of molecular evolution to biotechnology, leading to novel techniques for the creation of biomolecules with a great variety of functions for technical and medical purposes. Several basic principles for the application of evolutionary strategies can be derived from a comprehensive theory of molecular evolution. Prerequisites for evolutionary biotechnology are summarized with respect to the different classes of biomolecules and a few, selected applications are described in detail. Concepts for the technical implementation of evolutionary strategies are presented which allow automatized, high throughput processes. © 1997 Published by Elsevier Science B.V.

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1. Introduction

Nature is itself a demonstration of the versatile capabilities of biomolecules. Even in cells that are seemingly primitive, biomolecules simultaneously control hundreds of different and complex chemical reactions under mild conditions. These molecules play a key role in the life sciences and are a nearly infinite source for biotechnological and medical purposes. Unfortunately, nature does not provide molecules for every desired feature, and the activities of natural molecules are often reduced when they are used for technological or medical purposes.

In the past 20 years, the utilization of biomolecular functions has grown at a rapid pace due to innovations in biotechnology, particularly in genetic engineering. Nucleic acids, peptides, proteins and a wide variety of secondary metabolites are exploited for a tremendous number of applications, such as drug design, diagnostics, chemical and biotechnological synthesis. Most of these molecules were discovered in nature and their utilization for technical

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purposes often leads to non-optimal functions. Evolutionary molecular engineering which was proposed by Eigen and Gardiner more than a decade ago [1], overcomes these drawbacks. Today there is an increasing need in the life sciences to create new functions, as well as to alter the functions of biomolecules such that they are more efficient and better suited for particular purposes. These demands lead to two technologies which have the potential to create biomolecules with desired features, namely *rational design* and *evolutionary biotechnology*. Rational design assumes precise knowledge of quantitative structure-function relationships for designing a molecule. This approach must struggle with insufficient insights into the biophysics of such complex systems which do not allow the precise prediction of structures or structure-function relationships. It has been shown to be successful for the case of minor changes in biomolecules of known structures, as well as in combination with evolutionary strategies in small-molecule design based on peptides.

In contrast, evolutionary biotechnology creates molecules with complex functions without any knowledge of its structures. The prerequisites for the successful implementation of evolutionary biotechnology are the amplification of genotypes by an *in vitro* or *in vivo* reproduction mechanism, the variation of genotypes, either by replication errors or synthetic randomization, and a selection strategy to select phenotypes with the desired features from a large pool of related phenotypes. Nucleic acids are the only molecules that possess all the prerequisites of a genotype, whereas phenotypes can be realized by all biomolecules, such as nucleic acids, peptides, proteins, enzymes, antibodies, as well as secondary metabolites. Due to the great variety of applications that have utilized the principles and methods of evolutionary biotechnology, this article puts his focus on only a few applications that demonstrate the basic techniques and underlying general concepts for the design of biomolecules from different substance classes.

2. Implications of the theory

With the aim of basing Darwinian evolution on solid physical principles, Manfred Eigen, together with others, developed a theory of evolution at the molecular level [2-4]. By analyzing the kinetics of competing replicators, natural selection was found to be a direct physical consequence of self-reproduction under conditions far from thermodynamic equilibrium and *not* an *a priori* property of states of matter. The theory was influenced by the increasing knowledge concerning nucleic acid structure and function, and by the pioneering experiments of Spiegelman and coworkers who made use of the Qβ replicase system for studying for the first time *in vitro* evolution [5-7]. The inh*erent auto-catalytic* replication mechanism of nucleic acids via template copying is a particularly important prerequisite for evolution: Every replicator unit replicates solely its own genotype, unless there are mistakes which result in closely related replicator units. Thus, a model for populations of self-amplifying and competing nucleic acid molecules was derived [2]. This theory, including the concepts of the quasispecies and the hypercyclic coupling has been described in detail and reviewed several times [4,8-11]. Rather than delving into the details, some of the major implications of the theory for the technological utilization of evolutionary principles are pointed out below.

First of all, the theory has indeed redefined the view of the object of evolution. According to the quasispecies concept, the target of selection is not a single sequence, but rather a distribution of related sequences occupying a region in sequence space. The expansion of this so-called quasispecies is determined by the error rate of the self-replication mechanism. Its appearance is defined by the underlying fitness landscape: The population density at every point in sequence space is determined by its fitness value and by the densities of the neighboring positions. In contrast to earlier conceptions that mutants appear completely spontaneously, a stable quasispecies already includes a large spectrum of more and less adapted individuals. Therefore, when selection pressures alter, better adapted variants are likely to be present already, and hence need not be created by mutation. Thus, selection is by no means a random

drift, but a shift of the quasispecies distribution towards regions of higher fitness, guided by the underlying fitness landscape. The rapidity of adaptation depends largely on the mutation rate. Mathematical analysis, however, demonstrated that beyond a certain error threshold - which is inversely related to the sequence length - the information content of the quasispecies is lost [4,8,12]. Consequently, the highest rate of evolutionary adaptation is found at an error rate slightly below the error threshold. Another important point refers to the relationship between sequence space and function. Schuster and coworkers have shown that (i) a particular function is coded by several sequences distributed equally over the sequence space and (ii) within a relatively small radius around a particular sequence all common functions are coded [13,14]. These facts have been obtained by modeling studies with short RNA sequences concerning RNA based evolutionary optimization [15]. However, examples for relatively unrelated sequences realizing a function by the same structural motif, such as the catalytic triad of chymotrypsin, subtilisin [3] and similar structures of catalytic antibodies [16] indicate that this may be a common feature of biomolecules.

Evolution means the generation of information - which refers to a set of functions - by self-organization. Evolutionary biotechnology makes use of this information-generating principle to create biomolecules with desired functions. In order to set up an experimental system for evolutionary biotechnology, first it must be decided which particular class of molecules should be chosen. A second decision concerns the question how the basic features of molecular evolution - variation, amplification and selection - are realized and linked together. There are two extremes conceivable: A completely natural selection system with selection linked to amplification and variation introduced by erroneous copying, and, in contrast, a completely artificial system, where all these features are substituted by *in vitro* techniques and where all steps are controlled separately. For both extremes, and for almost all intermediate stages, applications already exist. Manipulations of the three basic features - (i) variation, (ii) amplification, and (iii) selection - shall be discussed separately in more detail.

Variation can be introduced either during amplification or independently. Since every copying process occurs with limited accuracy, all amplification techniques introduce mutations. If error-prone amplification is achieved through cellular or viral reproduction, it is called *in vivo mutagenesis*; if it is based on nucleic acid *in vitro* amplification, it is called *random-point mutagenesis*. In both cases the level of variation can be enhanced artificially over the background mutation rate through the use of mutator strains, error-prone polymerases, base analogs or base-modifying reagents [17,18].

Alternatively, mutations may be introduced independently from the amplification step. The technique to produce a synthetic random RNA library for selection of functional RNA molecules was invented by Biebricher and Orgel 25 years ago [19]. Today, the technical possibilities of synthesizing completely or partially randomized oligonucleotides make it possible to achieve almost every level of variation. A second advantage of this method refers to the particular target of variation. Depending on our knowledge of the genetic organization it is possible to introduce mutations at specific sites of interest, whereas amplification-coupled mutagenesis usually introduces mutations that are randomly distributed over the whole replicating unit. Target sites can be distinct genes coded on a plasmid, or certain sequences within a gene as small as a particular set of amino acid positions. The latter applications need detailed information and they are usually called *random-cassette mutagenesis* techniques [20-22]. Whereas the condition of error threshold usually strictly limits the level of variation, the selective manipulation of only part of a replicator allows much higher levels of variation.

Methods for *amplification* are of great importance in the case of peptides, proteins and several secondary metabolites. They do not fulfill the requirements for inherent self-replication. Therefore, information must be stored, varied and amplified by another molecule, i.e. by RNA or DNA. In all these cases, a tight linkage of any genotype to its particular phenotype must be realized by technical means.

Whereas nature achieves *selection* by coupling fitness to amplification, evolutionary biotechnology allows to uncouple these two processes. In principle,

there are three different categories for introducing selective pressure: (i) natural selection by coupling fitness to amplification as nature does, (ii) selection by physical separation, usually for the search of binders, and (iii) selection by screening, i.e. spatially separating a population, analysing individual clones by using some detection device, and selecting those that match the predefined condition.

Combinations of different variation, amplification and selection strategies are possible, resulting in a variety of different applications individually suited for a particular class of biomolecule and a particular desired function.

3. Biomolecules for evolutionary biotechnology

The suitability of different classes of biomolecules in evolutionary biotechnology depends largely on the nature of desired functions. If the aim is to find novel ligands which are specific binders, activators or inhibitors of a given target, promising candidates are peptides, oligonucleotides, as well as secondary metabolites. On the other hand, enzymes, antibodies, as well as single-stranded oligonucleotides can serve as molecules with novel catalytical functions.

3.1 Oligonucleotides

Nucleic acids are unique among all known classes of biopolymers due to their ability to act as both, information carriers and functional molecules. Their potential for folding into complex structures and their intrinsic base-pairing properties enable them to carry out certain functions as well as to store information and to be copied by self-replication [23,24]. The capability of replication allows cell-free evolutionary systems to be designed; whereas the transformation of cells usually reduces the number of molecules that can be worked with, the completely *in vitro* techniques increase the theoretical complexity of the population by at least five orders of magnitude. Although double-stranded molecules were also used for *in vitro* selection procedures, only single-stranded oligonucleotides have the potential for building structures that are sufficiently complex, e.g. to bind specifically to a given target. Furthermore, because RNA is more chemically reactive, it is the more versatile oligonucleotide in finding novel functions.

Oligonucleotide-based evolutionary biotechnology provides a tool useful for two separate tasks: (i) finding a binding partner or a substrate for a given target, and (ii) finding a catalyst for a given reaction [25-28]. The techniques for both tasks are very similar. As a starting point, completely randomized RNA, as well as existing RNA motifs that are randomized either at certain positions or all over the molecule may be used. Even the combinatorial assembly of known RNA motifs, combined with partial randomization, may provide a good starting point. Using a standard DNA oligonucleotide synthesizer a library consisting of up to 10^{15} molecules is generated. By applying a certain mixture of the four monomers at defined positions almost every kind of variation may be introduced at this level. With the aim of finding DNA binders or catalysts, the resulting single-stranded DNA molecules may be used directly after amplification via PCR and subsequent strand separation. For RNA studies, usually a T7-promotor is introduced into one of the amplification primer binding sites in order to produce single-stranded RNA by transcription of one of the two DNA strands. Details of the selection protocols depend mainly on the intended feature and are given in some examples below. In general, they enrich the fraction of those molecules in the population that show a higher fitness under applied conditions compared to the average. Selected fractions are amplified by any *in vitro* nucleic acid amplification protocol [29-32]. Additionally, variation by mutagenic PCR or recombination may be introduced at this level. The resulting populations, with enhanced fitness under the applied conditions, are used as the starting point for additional rounds of selection trying to approach the highest possible level of fitness.

There are many more or less different protocols for directed evolution of oligonucleotides described in the literature, especially with respect to the introduction of genetic variation. Most interest has been concerned with the diversity of the starting library. Compared with other biopolymers, oligonucleotide libraries can reach enormous complexities: Chemical

synthesis of DNA oligonucleotides leads to population sizes up to 10^{15}. Nevertheless, only for a maximum of 25 completely randomized positions a library of this size includes all possible variants. However, the smallest known natural ribozyme, the hammerhead ribozyme, requires a minimum of about 40 nucleotides in order to be functional [33]. To find a single sequence in a library of random 40mers would mean that one individual must be selected from a pool of 10^{24} sequences. Therefore, despite their large complexity, oligonucleotide libraries cover only a vanishing portion of this sequence space. It is this inherent relation between the information content (given by the number of monomers in one molecule) and the complexity of the intended function that provides the challenge facing all approaches to this problem. Therefore, it seems to be impossible (or at least very unprobable) to find oligonucleotides that fulfill complex functions - such as specific and efficient catalysis - only by selecting from a pool of the highest achievable complexity. Several approaches exist for dealing with this limitation; these include starting with a natural function and changing it by evolutionary means, or rationally dissecting the targeted function into parts and generating them separately via selection; both methods are discussed in examples below.

3.1.1 Oligonucleotides as binders

Selecting oligonucleotides from a random pool that bind to a given target was originally an extension of techniques that were used to determine promotor sites and other protein-recognizing sequences on genomic DNA or RNA. The technique is now called SELEX (Systematic Evolution of Ligands by EXponential enrichment) [34]; the selected oligonucleotide molecules that are capable of cognate binding to a target substance are usually called aptamers [35]. In recent years, a large repertoire of these aptamers was developed which bind specifically to small-molecule targets, such as organic dyes, nucleotides and cofactors, amino acids and derivatives, biotin, cyanocobalamin, theophyllin and antibiotics, as well as to peptides, nucleic-acid-binding proteins, e.g. translation inhibitors, transcription termination factors, ribosomal proteins, tRNA synthetases, retroviral proteins, and other proteins as, for instance, proteases, phospholipases, growth factors and antibodies. Selection of aptamers to these targets has been reviewed in detail [28,36-38]. Selection of binders can be achieved by any method that enables the separation of specific binders from non-binding and unspecifically binding molecules. For this purpose, well-known separating techniques, such as band-shift assays, filter-binding methods or affinity chromatography, have been used [39]. In every case where the target molecule is immobilized on a solid support, *specific elution* steps with a soluble target and additional *counterselection* steps with unmodified resin have been proposed in order to omit unspecific binders [36,40].

Small molecules were one of the first targets for *in vitro* selection of aptamers. Szostak and coworkers reported several basic studies with the goal of selecting binders to organic dyes, nucleotides, and several other small biomolecules [35,41-44]. In an interesting comparison they selected for RNA as well as for DNA aptamers binding to ATP. In the case of RNA, the completely randomized region had a length of 120 bases, in the case of DNA of 72 bases, each flanked by constant primer binding sites. The initial complexities of the library (around 10^{14}) and the number of selection rounds (six and eight for RNA and DNA respectively) were comparable. The resulting aptamers showed no sequence homologies and they folded into completely different secondary and tertiary structures: the RNA aptamer formed a stem-bulge-stem structure, whereas the DNA aptamer was composed of two stacked G-quartets. As shown already for dye-binding aptamers, the DNA version of the RNA aptamer was nonfunctional, and vice versa. Despite these differences the dissociation constants of both aptamers (1-10 μM) and the sizes of minimal functional structures (32 and 25 nt) were similar. Therefore, the potential for RNA and DNA to form complex, ligand-binding structures does not seem to differ much, although the ways of achieving the functional role differ considerably. Furthermore, the information content required to build a certain function, as measured by the minimal length, also seems to be similar for DNA and RNA. Accordingly, the additional 2'-OH group in each monomer of an RNA strand seems neither to improve nor to impair the polymer's general functional potential.

Gold and coworkers have focused their SELEX studies to more complex targets, such as polymerases, transcription factors and viral proteins [36]. For instance, HIV-1 reverse transcriptase was used to find specific inhibitors of cDNA synthesis [45-47]. IN SEveral approaches RNA as well as DNA aptamers were obtained that bound HIV-1 RT with high affinity (K_d of 1 nM) and inhibited cDNA synthesis specifically as tested by cross-reactivity assays with reverse transcriptases. These oligonucleotides, as well as others that are targeted to different viral proteins, may be useful directly as therapeutics or as lead structures for the design of small-molecule drugs.

Among the proteins that do not carry a natural recognition site for nucleic acids, the first and probably the most intensively studied target was the serine protease thrombin. Bock and coworkers reported the selection of a single-stranded DNA aptamer that binds to thrombin [48]. A library of 10^{13} ssDNA molecules comprising a thoroughly randomized 60 nt region was used to select for thrombin binding aptamers. Clones isolated after five SELEX cycles, including counterselection, exhibited a 15 nt consensus sequence that was shown to be responsible for thrombin binding and to block specifically fibrinogen cleavage *in vitro*. NMR and X-ray crystallography studies revealed a novel structural motif of the thrombin DNA aptamer, consisting of a G-quartet, and provided detailed information of the binding site [49]. An RNA thrombin aptamer with anticoagulating activity was described by Kubik and coworkers [50]; it shares no homology with the DNA aptamer, neither the sequence nor the structure. This result agrees with the DNA/RNA differences in small-molecule aptamers as discussed above.

3.1.2 Oligonucleotides as catalysts

Since the discovery of self-splicing introns [51,52] there has been a substantial interest in finding new or altered catalytic activities of RNA molecules. Inspired by the hypothesis of an RNA-world [53] prior to the present protein-based world much effort has been expending on finding catalytic RNAs (termed ribozymes) working as kinases, ligases, polymerases, isomerases, etc. In general, two separate approaches can be distinguished: (i) the alteration of existing ribozymes, and (ii) the selection of RNA molecules with catalytic activities from completely random pools.

Existing ribozymes can be used and modified with respect to their tolerance for environmental conditions, as well as to their substrate specificity. One of the most successful keys for selecting for catalytical activity is to allow the ribozyme to change its structure by tagging itself in a way that can be used to apply selective pressure. For example, Joyce and coworkers used the well-known Tetrahymena ribozyme, altered its substrate specificity from RNA to DNA, thereby inventing a ribozyme able to cleave ssDNA site-specifically [54]. They used the reverse exon-ligation step of the self-splicing mechanism resulting in an additional tag at the 3' end of the ribozyme that was then used as a primer binding site for subsequent PCR amplification steps. Therefore, the desired feature (substrate cleavage) was linked directly to amplification, allowing several cycles of selection to be performed with no need for physically separating or screening for fitter variants. In later studies, Joyce and his group have selected DNA-cleaving ribozymes with 10^5-fold better activity by applying successively stronger selection pressures [55,56]. Although linking fitness directly to amplification is the most powerful method of selection [2], this linkage is very hard to find and until now has been limited only to special applications.

Alternatively, different groups have tried to find ribozymes *de novo* from a library of random RNAs. In contrast to the selection of simple binders, the functional complexity for catalyzing specifically a distinct reaction is rather high. The probability for finding such a catalyst directly in a pool with an average size of 10^{15} molecules is accordingly low. Therefore, Szostak and coworkers have proposed a step-wise strategy to evolve a ribozyme able to act as a kinase [57]. The catalyst's functions were dissected into several parts and selected sequentially for these simpler features. Because one indispensable feature of polynucleotide kinases is to recognize specifically ATP as one of the substrates, they divided the selection procedure into two main parts: (i) selection for ATP binding, and (ii) selection for kinase activity. A minimal RNA aptamer with a length of 40 nt, which binds to ATP with a K_d of 0.7 μM, was designed

using the consensus sequence obtained after six SELEX rounds [58]. This 40 nt sequence was used as the core structure in the initial library for the second selection [59]. Partial mutagenesis of the core structure, together with completely randomized surrounding regions, led to a new pool of RNAs with a strong bias for ATP-binding capability. Selection was achieved by self-phosphorylation of the ribozyme with a modified ATP that allowed the separation of tagged from untagged molecules via covalent binding to a solid support. During the subsequent cycles of selection and amplification, mutagenic PCR was used to introduce further variation, and selective pressure was successively increased (by shortening the incubation time and decreasing the substrate concentration). The experiment resulted in several classes of novel kinase ribozymes with k_{cat} ranging between 0.03 and 0.37 min^{-1} and K_m values for the oligonucleotide substrate between 41 and 456 μM [59]. At least one of the selected ribozymes was able to catalyze the intermolecular reaction as well, thereby acting as a true catalyst.

The principle of creating catalytic antibodies by using transition-state analogs (TSAs) as immunogens [60,61] has also been applied to random RNA libraries. Schultz and coworkers [62] have found a ribozyme with isomerase activity by using the corresponding TSA as a target. The isolated ribozyme accelerated the isomerisation reaction by about two orders of magnitude. In contrast to this, Gold and coworkers [63] found RNA aptamers binding to a Diels-Alder TSA with affinities in the submillimolar range, but failed to create a ribozyme catalyzing the corresponding Diels-Alder reaction. There is no doubt that RNA molecules have catalytic activities; but it remains to be shown, whether the transition-state methodology is generally applicable for the *de novo* creation of ribozymes.

3.2 Peptides

Peptides are short oligomers of amino acids, that are distinguished from proteins by their lack of higher order structures. In contrast to oligonucleotides with four monomers, peptides are usually synthesized from a set of 20 different amino acids. The greater number of building blocks, together with the wider range of physicochemical properties of amino acids compared to nucleotides, allows a much greater chemical diversity for the same molecule length. This diversity corresponds to the great number of biologically active peptides that have been discovered, acting as hormones, cytokines, inhibitors, antibiotics, etc. Therefore, it is quite reasonable to use peptides as the chemical basis in the search for novel drugs with various targets. Identified peptides with biological activities may act directly as pharmaceuticals or as lead structures for the rational design of synthetic compounds. Not surprisingly, drug development on the basis of peptides has grown tremendously during the last decade [64], including rational design, as well as the application of evolutionary biotechnology.

Randomized pools of peptide molecules are usually called *combinatorial peptide libraries* and then constitute just a special case of *combinatorial chemical libraries*. Several recent reviews describe principles and applications of combinatorial synthesis of peptides as well as of a variety of other polymers in detail [64,65]. This article focuses only on biological expression systems for peptides which inherently include amplification and mutation possibilities. Nevertheless, there are certainly several analogies between chemical and biological peptide libraries, and evolutionary strategies for optimization can be applied to both.

Up to now, there is no known biological peptide amplification system, that works without the instruction of nucleic acids. This limitation may be overcome someday because there are reports of self-replicating peptides [66]. However, at present, a nucleic acid expression system is required and the expressed peptide must be coupled to the coding nucleic acid in order to link function to information. Expression of peptides can be achieved *in vivo* by employing cells and phages, or *in vitro* with cell-free translation systems; both methods allow different coupling strategies:

A *physical linkage* between a peptide (or a protein; see section 3.3) and the coding nucleic acid sequence is usually obtained during expression by fusing it to a host protein. Such expression systems are called *display* systems. *Phage display systems*

that use fusions to coat proteins of filamentous phages are now the most popular display systems [67-69]. Phage particles displaying different peptides on their surfaces can easily be separated by their affinity to a given target, a technique termed *biopanning*. Since the DNA coding for the peptide is contained in the phage particle, the corresponding information is accessible. The selected phages can be applied directly to further rounds of amplification and selection. This technique was invented by Smith [70] and since then it has been applied to peptides, protein fragments, and proteins. A variety of phage vectors based on filamentous phages, as well as on other phages, have been described for different purposes. Particularly, the expression of antibody fragments gave rise to a novel antibody production method without the need for immunization [71,72] (see also section 3.4).

Bacterial display systems use proteins on the surface of cells as fusion partners, such as receptors, pili, or engineered translocation systems, e.g. the IgA protease [73-75]. Applications have been reviewed in detail [76-78]. Selection can be achieved by affinity separation as is done with phage display systems; in addition, techniques, such as fluorescence-activated cell sorting (FACS), may be employed to detect and isolate cells that express the desired peptide.

Cull and coworkers used an intracellular approach which they called *peptide-on-plasmids*. In this method a peptide is fused to a DNA-binding protein in order to link it to the coding gene [79]. A similar effect can be achieved through the use of cell-free translation systems and the display of nascent peptides on polysome complexes [80].

A *biological linkage* between a phenotype and a genotype is realized by any correlation between the expressed peptide and the amplification of the corresponding gene. For instance, yeast-based transactivation systems with growth coupling have been engineered, where only those cells which are stimulated through activation of their native G-protein coupled receptor can grow. The receptor-activating peptide agonist can be secreted either by the same cell (autocrine system) [81,82], or by another cell (endocrine system) [83]. With such a strong growth coupling, peptide agonists can easily be selected from large libraries. Furthermore, selection through receptor activation goes beyond simple methods for selecting binders, providing a method for directly detecting molecules with biological activities. A second approach with a biological linkage uses the phage display principle for coupling peptide function to amplification [84-86]. Instead of selecting binders by affinity separation on a solid support, the expression of a cognate binding partner allows the restoration of the phage infection protein, leading to the restored infectivity of otherwise non-infectious phage particles. Since in both approaches, the yeast and the phage system, selection is linked to amplification, no physical separation is required. Therefore, large populations may be screened, and the integration into a closed system based on natural selection in the Darwinian sense becomes possible.

Applications of peptide displays have recently been reviewed for bacterial [78] and for phage display systems [68,87]. Peptide libraries are mostly used to identify antibody epitopes, but the possible range of target molecules is much larger. One of the most intensively studied targets is streptavidin. Devlin and coworkers [88] were the first to publish peptide sequences with an affinity for streptavidin. The sequences were selected from a library of 15 random residues, and beared the consensus motif HPQ (Histidine-Proline-Glutamine). This study was followed by others resulting in the same motif and a bias for an odd number of cystein residues in the displayed peptide [89]. This led to the development of conformationally constrained peptide libraries with varying numbers of random positions flanked by two cystein residues. The disulfide bridge formed between the two cysteins seems to lead to a more rigid structure of the peptide, resulting in binding affinities at least two orders of magnitude higher than the linear peptides [90]. The crystal structure of cyclic peptides containing the HPQ motif binding to streptavidin have recently been determined [91].

3.3 Enzymes and other proteins

Due to their catalytical and regulatory functions, proteins are one of the most interesting targets for optimization by evolutionary biotechnology. Here, strategies only for the creation of novel or altered enzymes are discussed, but most of the techniques

are transferable to the design of other proteins as well. All mutagenesis techniques described below have already been applied to improve other proteins, such as the antifreeze protein or the green fluorescence protein (GFP) [92-94].

Enzymes are nature's choice for the execution of almost every biological function. They are polypeptide chains, typically with hundreds of amino acids, and exhibit a multitude of catalytic functions, such as oxydoreductases, transferases, hydrolases, lyases, isomerases and synthetases. Enzymes are very efficient catalysts (enhancing reactions by factors of 10^6 to 10^{12}) with catalytic specificity and highly sensitive regulation mechanisms. These unique properties allow controlled and simultaneous processing of hundreds of different reactions within living cells. The ability of enzymes to work under mild reaction conditions and to catalyze very complex chemical reactions with stereospecificity and without undesired by-products makes them ideal tools for a variety of medical and technical applications. Unfortunately, the use of natural enzymes under conditions useful for biotechnological applications or with unnatural substrates often leads to a dramatic reduction of catalytic efficiency or no activity at all. To overcome these limitations there are basically two approaches: (i) rational design and (ii) application of evolutionary strategies for the creation of enzymes with desired properties.

Rational design requires extensive knowledge of the structure and the function of the enzyme as well as detailed information about the catalytic mechanism. If such data are available, directed modifications by exchanging one or a few amino acids at specific positions may result in an enzyme with desired properties. A recent successful example was the conversion of papain into a peptide nitrile hydratase by the exchange of a single amino acid residue [95]. Rational design is a highly sophisticated and time-consuming process providing a great challenge for structure prediction by protein crystallography, construction of transition-state analogs and other tools of molecular structural biology. Progress in this discipline will certainly make more and more classes of enzymes accessible to rational design, such as proteases and their inhibitors [96].

Evolutionary strategies do not require structural information. Even in cases without knowledge of the structure and the reaction mechanism of an enzyme, evolutionary biotechnology can create molecules with desired properties by means of directed molecular evolution. Prerequisites are suitable systems for carrying out mutation, reproduction and amplification, as well as selection (screening) on the molecular level. Theoretically, *in vitro* as well as *in vivo* strategies can be designed for these purposes. The expression of polypeptides by *in vitro* translation is limited. *In vivo* expression systems have been commonly used on bacteriophages [97], as well as on procaryotic and eucaryotic organisms (see section 3.2).

3.3.1 In vivo mutagenesis

All techniques based on *in vivo* mutagenesis necessarily require coupling between enzyme function and cell growth [98,99]. Examples for directed evolution *in vivo* are the modification of the acid phosphatase pH optimum in *Saccharomyces cerevisiae* [100] and the spontaneous single-point mutations in the evolved β-galactosidase A (ebgA gene) of *E. coli* K12 (lacZ deletion mutant) to permit growth by metabolized lactose [101-103]. Many directed evolution strategies were reported, for example, the utilization of cellobiose in *E. coli* K12 [104,105] and a second xylitol catabolic pathway in *Klebsiella pneumoniae* [106] by activation of a cryptic gene [107] in order to obtain new catalytic activities under selective pressure. Recently, a mutant of *Rhodobacter sphaeroides* Si4 was selected by continuous cultivation under selective pressure utilizing galactitol [108]. The galactitol dehydrogenase that evolved has not been identified in the wild-type strain and it is believed to result from an activation of the cryptic gene. Although *in vivo* selection shows the potential for discovering new activities, it is inherently limited to only a few functions, such as metabolic activities or resistance. In addition, the rather low mutation rates when compared to *in vitro* systems leads to only very slow variation. Acceleration may be achieved, for example by using a mutator strain. For instance thermostable kanamycin nucleotidyltransferases have been created by several rounds of passaging a shuttle plasmid from an *E. coli* mutator strain to a *Bacillus stearothermophilus*

strain, which can grow at 63°C [109]. However, every *in vivo* mutagenesis technique which requires the whole genome is limited to rather moderate mutation rates by the error threshold condition.

3.3.2 *Applying in vitro mutagenesis*

In order to overcome the limitations of *in vivo* mutation it is replaced by *in vitro* mutation. The selective manipulation of only one gene or a sequence of interest is not restricted to the *in vivo* mutation rate. If oligonucleotides are used, the error rate corresponds to that of total randomization because the oligonucleotides can be synthetically produced. If gene fragments or genes were used, the randomization depends on the error rate introduced by mutagenic PCR or chemical mutagenesis. Basically, there exist three different strategies for *in vitro* mutagenesis which were discussed by means of recent examples:

(i) Random-cassette mutagenesis

Alteration in substrate specificity of an enzyme can be achieved by the introduction of a synthetic randomized oligonucleotide cassette into the active site of a structural gene or by its replacement. The first examples were reported by Dube and Loeb on the β-lactamase gene; they replaced five base pairs near the active site of serine-70 by random oligonucleotides. This led to approximately 10^6 different amino acid substitutions in $Phe_{66}XXXSer_{70}XXLys_{73}$ of the β-lactamase enzyme. By transforming an *E. coli* population with plasmids containing the random inserts, several active-site mutants were isolated by screening, rendering *E. coli* that were resistant to carbenicillin and a series of related analogs [110]. Oliphant and Struhl reported the insertion of a semi-randomized (doped) cassette with 80 % of wild-type nucleotides and a mixture of the three (mutagenesis rate 20 % per base pair) which code a 17 amino acid portion in the active site of the β-lactamase gene. From a collection of approximately 10^5 altered β-lactamases with amino acid substitutions from Arg_{61} to Cys_{77}, 2000 colonies of *E. coli* were able to confer ampicillin resistance. Several isolated clones were examined in detail; clones with altered specificity against different antibiotics were found, and specific suicide inhibitors, as well as different temperature-dependent activities, were obtained [111].

The potential of random-cassette mutagenesis was impressively demonstrated by Loeb and co-workers who recently reported several developments, such as the creation of drug-specific *Herpes simplex* virus type 1 thymidine kinase mutants [112-119], novel human DNA alkyltransferases [120], functional mutants of *human immunodeficiency virus type 1* reverse transcriptase (HIV-1 RT) [121] and functional mutants of *Thermus aquaticus* (Taq) DNA polymerase I [122]. Furthermore, a novel polymerase screening system was introduced by functional polymerase activity complementation in *E. coli* strain recA718 polA12. This strain contains a temperature-sensitive DNA polymerase I that is unable to replicate its genome at 37°C in rich media at low density [123-126]. Functional complementation by transformation with a variety of different polymerase genes, such as DNA polymerase β [124], HIV-1 reverse transcriptase [123] and Taq DNA polymerase I [122], demonstrated that this system is a suitable tool for polymerase manipulations based on evolutionary biotechnological principles. Recently, these results have been summarized [127-129]. They demonstrate the potential of random-cassette mutagenesis, and it should be noted that this method can also be applied to other classes of substances, for example to ribozymes [22].

(ii) Random-point mutagenesis

Whenever knowledge is lacking concerning an active site or other coding regions, random-point mutagenesis can be applied. Random-point mutations are introduced a gene of interest by mutagenic PCR or chemical modifications. The library found thereby can be transformed into cells, yielding in a population of clones that express individual variations of an enzyme. Selection can be carried out either by coupling to growth or by screening. Several sequential rounds of *in vitro* mutagenesis, amplification of transformed clones, and selection will lead to an enzyme with desired features.

Among the first enzymes subjected to random-point mutagenesis were subtilisin and related peptidases. Selection was applied in order to obtain higher catalytic activity [130], tolerance to organic

solvents [131-134] and extreme pH [135], as well as thermostability [136]. In the presence of organic solvents subtilisin can catalyze acylations regioselectively [137,138] and stereoselectively [139]. Subtilisin also catalyzes peptide synthesis in organic solvents [140]. There is an increasing need to obtain such enzymes with high catalytic activity in organic solvents for synthetic applications [141]. Arnold and her group has made rapid progress in developing an evolved subtilisin adapted to high concentrations of dimethylformamide (DMF) [131-133,142]. Random-point mutations were introduced into the subtlilisin gene via mutagenic PCR, and Bacillus subtilis was transformed by means of the randomized subtilisin library. A large number of different clones was isolated and individually selected by screening for enhanced activity in the presence of organic solvents. Clones showing higher proteolytic activity were tested in detail and transferred to the next evolution round. After several rounds, Arnold and coworkers [131,142] achieved an adapted subtilisin which exhibited in the presence of 60 % DMF a catalytic efficiency (k_{cat}/K_m) 471-fold higher than the wild-type subtilisin, whereas the stability was not affected (mean life-time, $\tau_{1/2}$ around 460 h, both in adapted and in wild-type subtilisin).

Recently, by applying the same technique, Moore and Arnold [143] reported randomization and rapid screening of an esterase for deprotection of an antibiotic p-nitrobenzyl ester in aqueous-organic solvents. The use of a chromogenic substrate analog to mimic the original substrate of the desired reaction allows fast and efficient screening for the desired features. Applying this assay, mutants with higher resistance to the organic solvent can be prescreened, selected and finally tested with the desired substrate. After four generations with population numbers of around 10^4 to 10^5 per round, an enzyme that was 24 times more efficient than the wild-type was evolved. The recombination of different forward mutations of the fourth generation by cleaving the different genes at specific restriction sites, shuffling the fragments and ligating them again, resulted in a further enhancement of catalytic activity by a factor of 50 - 60 (compared to the wild-type) [143]. Both examples, subtilisin and esterase optimization demonstrated the requirement for sensitive detection of enzyme activities and for well-designed screening strategies.

(iii) DNA shuffling

DNA shuffling or *sexual* PCR refers to the *in vitro* homologous recombination of a pool of genes with different mutations. Even when the mutation frequency within amplified genes is only slightly, homologous *in vitro* recombination allows combinatorial accumulation of mutations.

DNA shuffling was first applied by Stemmer [144,145]. The method involves the digestion of mutagenic copies of a gene with DNase I, resulting in a pool of DNA fragments with different sizes. These fragments can be reassembled into full-length genes by repeated cycles of annealing in the presence of DNA polymerase. The fragments prime each other based on their complementarity; recombination occurs when two fragments from different copies prime each other. The results are combinations of mutations from different copies.

The first approach was carried out with *E. coli* that contains a plasmid with a gene that encodes β-lactamase. Amplification via PCR with a point mutation rate of 0.7 % leads to a pool of different genes, each containing few mutations. Genes were digested and reassembled into full-length genes using a process similar to PCR. After transformation, several hundred colonies from the highest level of antibiotic exposition (cefotaxime) were chosen for the next round. After three rounds, increasing the exposition of the concentration of the antibiotic, some colonies achieved an antibiotic resistance 16,000 times higher than the wild-type. A β-lactamase gene of a selected colony exhibits nine base substitutions, including four silent mutations. Finally, an enhancement of antibiotic resistance up to 32,000 times higher than the wild-type resistance was achieved through removing all non-essential mutations by backcrossing, i. e. shuffling the evolved β-lactamase gene with 40-fold excess of the wild-type β-lactamase gene for two rounds [144,145].

3.4 Catalytic antibodies

Antibody catalysis provides a novel tool to mimic enzyme catalysis and exhibits the potential to create

de novo catalytic activity. Early ideas that enzymes lower the activation barrier of a reaction by stabilizing the transition states were suggested by Pauling [146]. These ideas led to the theoretical consideration concerning the possibility to design new enzymes by creating transition-state binders by Jencks [147]. Because the transition-state conformation of a reaction is too instable to be used as an effective vaccine, Lerner and his group used chemical analogs of this state to make antibodies [60]. The creation of catalytic antibodies for a given reaction requires the correct anticipation of the reaction mechanism and the chemical synthesis of a stable analog of the predicted transition conformation. Lerner and coworkers immunized animals with a transition-state analog (TSA) and produced monoclonal antibodies using the hybridoma technology. After a first screening for transition-state analog binding, positive antibodies were tested for the desired catalytic activity. There are several examples of successful catalytic antibody development, such as the hydrolysis of esters [60], stereospecific cyclization [148], hydrolysis of amide bonds [149] or stereoselective lipase activity [150]. A variety of different catalytic activities have been reported in reviews [151,152].

The time-consuming process of *in vivo* immunization for producing antibodies can be replaced by the *in vitro* generation of combinatorial antibody libraries. Expression systems for F_{ab} fragments, such as bacteria [153,154] and phages [71,155,156] have been reported (see also section 3.2). Naive combinatorial antibody libraries from non-immunized animals, as well as from preselected libraries from TSA immunized animals, were used. Further optimization of TSA antibody binding were carried out by evolutionary biotechnology or affinity maturation. After an *in vitro* selection of antibody libraries for predetermined binding specificity, an increased affinity of the selected antibody binding site can be achieved by random mutagenesis [157]. This affinity maturation utilizes the same concepts as those for evolving enhanced peptide binders (see section 3.2). Basic strategies for engineering catalytic antibodies have been discussed and reviewed in detail [158-161].

It should be emphasized that the use of TSA as targets for protein binding has the potential to create catalytic activity *de novo*. Limitations are set by the requirement for knowledge of the mechanism of the catalyzed chemical reaction and the need for a chemical synthesis of a stable TSA. Nevertheless, combining this method with evolutionary biotechnology using naive combinatorial immunoglobulin libraries derived from bacteria or phages, makes *in vitro* optimization of catalytic antibodies possible. Furthermore, principles for enhancing catalytic activities, such as those described in this section, will be suitable tools for further optimization.

3.5 Secondary metabolites

Primary metabolites of living organisms, such as carbohydrates, lipids, nucleic acids and proteins are ubiquitous and essential. In contrast, secondary metabolites are non-essential substances for growth and can be produced by a living cell during a defined growth phase or by certain specialized cells of higher organisms. Secondary metabolites are structurally very heterogeneous and can be classified by its anabolic or amphibolic synthesis, such as polyacetylenes, polyketides, isoprenoids, phenylpropane derivates, alkaloids, glycosides and cyclic oligopeptides. Functions of secondary metabolites cover a wide range including antibiotics, vitamins, carotines, exotoxines, and many others.

The pharmaceutical impact of secondary metabolites is enormous. Isolated secondary metabolites or mixtures of them, obtained from a multitude of organisms, for example plants, animals or microorganisms have been screened for drug discovery for more than 50 years. Screening programs for discovering pharmacologically active compounds for a large number of human targets are being carried out in high throughput screening systems. Setting up a library with natural compounds is a highly time-consuming and very costly process. It requires the collection of soil samples from all over the world, subsequent isolation of naturally occurring microorganisms, cultivating them under a variety of defined conditions, separation of different substances from the cultivation broth, and finally the application of a pharmaceutical test system. Genetic engineering techniques applied to manipulate molecular diversity of secondary metabolites provide an alternative. Different principles for molecular diversity by

genetically engineered microorganisms have been described [162].

Molecular diversity can be achieved when an enzyme of a secondary metabolite pathway changes or looses its substrate specificity and becomes able to catalyze the formation of different products within a given structural class. This can be done in several ways: mutagenesis strategies can be applied to a structural gene involved in a specific pathway, where each clone contains an individual randomized enzyme with altered function (see section 3.3); or an enzyme can be created which looses its substrate specificity so that a single organism can produce a broad spectrum of different products in a specific structural family, such as the formation of radical intermediates leading to a large number of different products formed from one substrate. This has been reported for Isopenicillium N synthase [163,164].

Molecular diversity will also arise if different enzymes act on a class of substances that are already synthezised by a cell, or if enzymes modify precursors in a biosynthetic pathway. Therefore, enzyme expression from an inserted heterologous structural gene that can alter the spectrum of secondary metabolites is necessary. This strategy has been reported for heterologous structural genes introduced in different Streptomyces species [165,166] and has been extended, for example to cytochrome P_{450} hydroxylases [167], O-methyltransferases [168-171] and terpene cyclases [172,173]. The molecular diversity achieved in this manner depends strongly on the number of different enzymes and their substrate specificities. If the genes that are introduced can fulfill the requirements of biotransformation, or have a rather broad substrate specificity within the desired substance class, a large diversity can be created. For instance, the molecular diversity from starting with one substrate is given by 2^m, where the base 2 stands for the fact that one enzyme can be introduced or not, and m is the number of different enzymes with a substrate specificity to the modifying substrate class.

Alternatively, molecular diversity can be achieved by shuffling different biosynthetic pathways which start by using the same substrate. This principle, which was called *combinatorial biosynthesis* by Khosla [174], includes the recombination of naturally occurring and related biosynthetic pathways by shuffling involved genes or gene modules from different species in the order corresponding to the catabolic pathway. Molecular diversity arises according to the number of different genes or modules (R) that are involved in each biosynthetic pathway and the quality of the different allelic forms (n). The theoretical value of molecular diversity is R^n. By linearly increasing n (e. g., finding a novel related biosynthetic pathway), the molecular diversity will grow exponentially. Homology searches in genes for secondary metabolism from different species by DNA hybridization and sequence analysis may facilitate the discovery of related biosynthetic pathways.

The first approach to combinatorial biosynthesis was reported by Khosla and coworkers in order to achieve molecular diversity from polyketides. Intensive research was required before an experimental set-up could be realized. First, identification, molecular cloning and characterization of genes or gene clusters were necessary. This pioneering research on the microbial genetics of the polyketide synthesis was done by Hopwood and colleagues [175-178]. Further progress was achieved in manipulating genes for polyketide synthase [179].

An early manipulation of the aromatic polyketide biosynthetic pathway through introducing a related enzyme was successfully carried out by Hopwood [180]. For the creation of large-scale, so-called *in vivo* libraries, where each clone encodes a novel biosynthetic combination of a possible polyketide, a novel host-vector strategy in *Streptomyces coelicolor* was introduced [181]. This technique can be used to express gene clusters for entire polyketide pathways. In addition to high-frequency mutagenesis technologies [181-183] these system are able to create *in vivo* libraries based on the idea of combinatorial biosynthesis for aromatic polyketides. Recently, an *in vivo* library of aromatic polyketide derived from five naturally occurring shuffled gene clusters was reported, and progress has been made in the development of combinatorial biosynthesis of macrocyclic polyketides [174].

The introduction of combinatorial biosynthesis in evolutionary biotechnology is very promising. Secondary metabolites play an important role in drug

discovery and development. It has been hardly discussed, and is still under investigation, whether the principle of combinatorial biosynthesis can be applied to other secondary metabolic pathways, too. Aminoglycoside, oligopeptide antibiotics and terpenes may serve as sources for further combinatorial biosyntheses. With increasing molecular diversity of secondary metabolites derived from combinatorial biosyntheses, evolutionary strategies for screening and optimizing very large numbers of different targets will become feasible.

4. Technological implementation

Evolutionary strategies for creating molecules with desired features may require elaborate technical equipment. For biotechnological applications, where active molecules for a variety of similar purposes are searched for, it is necessary to achieve a higher degree of automatization. Different concepts can be pursued with the help of automated, computer-controlled devices [1,11].

Technical requirements for the integration of evolutionary strategies depend on the particular principles that are applied, for example on the kind of selection method that is used (amplification-coupled selection, physical separation or screening) or on the control of the amplification conditions (continuous systems or serial transfer). These differences allow to classify the technical design of devices depending on basic concepts and purposes for evolutionary biotechnology. Highly automated machines have been described, designed and constructed for almost every concept.

4.1 The flow reactor concept

The flow reactor concept is a continuous cultivation method which allows to apply in principle every selection strategy, either *in vitro* or *in vivo*. The underlying concept is to retain constant growth conditions by a continuous flow of specific substances through a reactor. In the steady state, the amplification rate of a replicating system in the reactor is adjusted to the dilution rate. Selection can be applied in several ways: (i) amplification-coupled or natural selection favors the variants with higher amplification rates, (ii) physical separation selects those variants which have a higher retention time in the reactor, or (iii) screening by a feedback loop will accumulate variants with desired properties in the reactor.

A flow reactor system, termed *cellstat*, has been developed for the continuous propagation of phages [184-186]. In a small reactor, phages grow under controlled conditions with a continuous flow of fresh host cells that allows them to reproduce by infection. Various selection pressures can be applied and controlled by changing the environmental conditions. Furthermore, exploiting the phage display principle, such a device can be used to select for high-affinity binding of peptides or proteins that are fused to the phage particle. Selection experiments using the cellstat system have shown, that even mutants with slightly higher over-all amplification rates supersede the original population [187]. In addition, selection by physical separation can be introduced into this system, for example by an external affinity enrichment device. With its potential for continuous selection from large phage populations, the cellstat and further developments based on the cellstat technology provide a powerful means for evolutionary biotechnology.

4.2 Plug amplification reactor concept

The *plug amplification* or *capillary reactor concept* is another method to achieve continuous cultivation. This is accomplished by a traveling amplification front in a tube or capillary; ideally, the front moves in the form of a plug towards fresh substrate. In contrast to the flow reactor, where the solution is homogeneously mixed and the conditions are held constant by dilution, conditions in a plug amplification reactor are only constant in a plane perpendicular to the tube length. The velocity of the wave and the particular concentrations at the traveling front are a function of the rate of amplification. This type of reactor selects inherently for rapid amplification. Examples for the realization of a plug amplification reactor and evolution studies using the Qβ replicase in an *in vitro* amplification system have been described [188,189].

4.3 The serial transfer concept

The *serial transfer concept* is a quasi-continuous cultivation method for *in vitro* as well as *in vivo* applications. Again, all selection strategies can be applied, such as amplification-coupled selection, physical separation and screening. Often, several reactors - usually with very small dimensions - are used for serial batch amplifications. Before the termination of the exponential growth phase, a fraction of the solution is transferred from one reactor to a next reactor that contains fresh substrates. Quasi-continuous conditions are realized by keeping the population in the exponential growth phase. An automated machine has been developed for *in vitro* methods, e. g. the Qβ replicase system and other isothermal amplification systems [190,191]. This device allows the fully automated *on-line* monitoring of the nucleic acid concentration and the concentration-triggered transfer to the next reaction vessel.

4.4 The multi-channel serial transfer concept

All concepts discussed above are systems where usually one variant is selected out of a mixed pool due to its higher fitness. Only a parallel experimental design can overcome the limitations of these methods. Extending the serial transfer concept in a parallel fashion leads to the *multi-channel serial transfer concept*. A pool of variants, usually called random libraries, can either be derived from natural quasispecies, or created artificially by *in vitro* DNA manipulations. Compartmentalization of single members or parts of such pools by spatial separation leads to a distribution where fitness can be investigated on each separate channel. With this in mind, Eigen and coworkers developed a highly automated machine with large numbers of parallel sample-handling and sensitive detection units [192,193]. This machine allows the realization of evolution experiments based on the quasispecies concept where any kind of serial transfer cultivation and selection can be applied. One of the first published experiments was an *in vitro* Qβ RNA replication experiment with 960 microreactors in parallel (30 μl each) and *on-line* detection [193]. Because amplification-coupled selection was applied, this experiment was the first demonstration of *high throughput selection*, in contrast to *high throughput screening*. Further developments extended these approaches down to nano-technology. Highly automated machines based on wafer technology are able to process highly numbers of very small samples in parallel. Recently, pharmaceutical screenings showed the successful application of this technology where throughputs up to 100,000 samples per day were achieved.

5. Future Prospects

Nature is a source for a large variety of biomolecules. Pharmaceutical and biotechnological companies are finding more and more ways to explore and utilize this source. However, every natural substance is a result of the evolution on this planet during the past 4.6 billion years. Therefore, although this source is certainly very large, it is not unlimited. It is very likely that there are potential molecules with useful properties and functions which have simply not been realized on earth. For instance, the whole mass of the earth ($5.98 \cdot 10^{24}$ kg) would not suffice to produce only one molecule of each possible variant of a peptide molecule with a chain length of 37 amino acids. In addition, nature only selects for those functions that are important for the survival of the particular organism. This purpose may differ largely from current technological or medical purposes. A suitable way for overcoming these problems is to optimize biomolecules by evolutionary strategies. The examples reported in this article demonstrate the potential of evolutionary biotechnology for fulfilling these demands. In the future, this technology will probably lead to a multitude of different applications. Present limitations will be overcome by improvements in different fields:

Evolutionary strategies, such as hierarchical and genetic algorithms, as well as theoretical predictions of fitness landscapes, will lead to faster and more efficient optimization strategies.

Biological developments, especially *in vivo* assays, functional complementation assays and novel transactivation systems will focus on novel targets.

Faster and more sensitive, cell-based assays will shorten the time required to screen a large library. New library techniques, including doped positions or constrained conformations, will extend the population sizes, and progress in the development of large combinatorial biosynthesis libraries will explore new substance classes.

Technological developments, such as high degrees of automation and massive parallel processing for fast and efficient sample handling in the nanoscale range are being developed. Nano-bioreactors combined with highly sensitive detection methods, for instance the technology of fluorescence correlation spectroscopy (FCS) [194-196] will make *ultra high throughput selection* as well as *ultra high throughput screening* feasible in evolutionary biotechnology [197,198].

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