Directed evolution of biocatalysts

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Directed evolution is being used increasingly in academic and industrial laboratories to modify and improve important biocatalysts. Significant advances during this period of review include compartmentalization of genes and the *in vitro* translation apparatus in emulsions, as well as several impressive demonstrations of catalyst improvement. Shuffling of homologous genes offers a new way to utilize natural diversity in the evolution of novel catalysts.

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Abbreviations

ee	enantiomeric excess
scFv	antibody single-chain fragment
StEP	staggered extension process

Introduction

Evolution has given rise to a fantastic array of biological structures. Those who wish to redesign these molecules are now implementing evolutionary algorithms of mutation and selection to obtain new functional features, while minimizing constraints on the possible molecular solutions [1•]. This directed evolution approach contrasts with more conventional 'rational' ones involving iterative computer design and site-directed mutagenesis, processes often rendered useless by the near-complete absence of the detailed structural and mechanistic information required. Various approaches to directing protein evolution are being practiced successfully in a growing number of academic and industrial laboratories. These approaches, which pay due respect to the extreme complexity of the relationship between protein structure and function, promise to fill at least some of the gap between our primitive state of molecular level understanding of proteins and the strong and very widespread desire to engineer improved ones.

The laboratory equivalent of Darwin's survival of the fittest is mutation and recombination accompanied by selection on the basis of desired functional changes. A relatively simple algorithm of recursive random mutagenesis and recombination can deal with the many important protein engineering problems that fall in the 'tuning' category: enhancing activity or overall performance [2•,P1,P2,3], altering substrate specificity [4•,5,6••,7,8••] and enantioselectivity [9•,10], improving stability [11•,12••,13•] and a variety of other features already exhibited at some, perhaps low, level by the parent enzyme [14•,15••,16,17•]. Provided the desired enzyme is physically feasible, there appears to be a good chance that the accumulation of mutations identified in a well designed selection or screen will yield it. Once the goal has been identified and the enzyme expressed in a functional form in a suitable host, developing and validating the selection or screen becomes the most challenging part of the evolution experiment. This review covers recent advances in directed evolution related to enzymes and protein expression and stability, and includes new methods of DNA recombination, screening and selection. It does not cover evolution of ligand binding or protein–protein interactions.

Altering biocatalyst specificity

Enantioselective syntheses represent an important application of biocatalysts. The narrow range of substrates accepted by some enzymes and the less than impressive enantioselectivities exhibited by others frustrate attempts to develop new synthetic applications and to commercialize existing ones. Recent studies, however, show that improving enzyme enantioselectivity is feasible by directed evolution [9•,10]. Lipase derived from *Pseudomonas aeruginosa* hydrolyzes *p*-nitrophenyl 2-methyldecanoate with only 2% enantiomeric excess (ee) in favor of the (*S*)-acid. Four successive cycles of error-prone PCR mutagenesis and screening on enantiomerically pure *R* and *S* substrates produced a variant that catalyzes this reaction with 81% ee [9•]. An esterase derived from *Pseudomonas fluorescens* was evolved for hydrolysis of an unnatural ester having structural similarity to a key building block in the synthesis of epothilones [10]. A 25% ee was exhibited by a first-generation variant.

Because it is difficult to achieve by rational design, tuning substrate specificity is a favorite target for directed evolution. DNA shuffling (an *in vitro* DNA recombination method in which parent genes are fragmented and reassembled) and selection were used to create an *Escherichia coli* aminotransferase [4•] that accepted β-branched substrates, which were poorly accepted by the wild type enzyme. Earlier attempts to change substrate specificity by site-directed mutagenesis had resulted in limited success. This aminotransferase activity compensated for a defective host *ilvE* gene (the defect renders the cells unable to make isoleucine, leucine and valine) and allowed cells to grow in minimal media in the absence of these amino acids. Four rounds of shuffling increased the activity of aspartate aminotranferase for valine and 2-oxovaline by five orders of magnitude, while decreasing by 30-fold the activity towards the natural substrate, aspartate.

Biphenyl-dioxygenase-mediated degradation of polychlorinated biphenyls was improved by shuffling genes from two bacteria, *Pseudomonas pseudoalcaligenes* and *Burkholderia cepacia* [6••]. These biphenyl dioxygenases exhibit differences in substrate specificity, activity and the

biphenyl ring positions targeted. The *bphA1* genes responsible for substrate specificity were recombined, and the resulting bacteria were screened for their ability to degrade biphenyl compounds. The chimeric biphenyl dioxygenases showed substrate preferences differing from both parental enzymes and enhanced degradation activity toward some biphenyl-related compounds. One recombinant gained a novel activity towards the single-ring aromatic compounds toluene and benzene, an activity exhibited by neither parent. It also catalyzed the conversion of indole to indigo, as do many other aromatic dioxygenases, but not the parents.

Stability and expression

Hilvert and co-workers [18•] recently called upon combinatorial methods to assist in a difficult protein design problem, the conversion of a dimeric enzyme into a monomeric one (in order to reduce its size and obtain a minimized functional unit). A short loop was inserted into the middle of one of the helices of the thermostable chorismate mutase from *Methanococcus jannaschii* to allow the amino-terminal half of the protein to fold back and displace the second molecule of the dimer. Combinatorial mutagenesis and selection were used to identify the specific loop sequence that gives rise to the desired soluble, monomeric version. Saturation mutagenesis of all six loop residues, coupled with selection using an auxotrophic *E. coli* strain, yielded a number of positive variants, of which 26 were purified and screened by size-exclusion chromatography. One variant whose catalytic properties were very close to wild type, showed no sign of aggregation or dimerization.

Folding and stability of antibodies and antibody single-chain fragments (scFvs) require the formation of two disulfide bonds. As this is often not possible in the reducing environment of the cell cytoplasm, scFv expression in *E. coli* is painfully low. Two groups recently reported using directed evolution to improve scFv expression in bacteria. In one approach, the cysteine residues participating in disulfide bond formation were removed by site-directed mutagenesis, and compensating mutations were accumulated by directed evolution [14•]. Mutant antibodies were selected for their ability to bind antigen, overexpressed in *E. coli* and characterized by urea equilibrium denaturation, yielding four disulfide-free variants with stabilities close to that of the wild type scFv. Another approach to stabilize and express an scFv involved no explicit manipulation of the disulfide bonds [15••]. Instead, cell survival was used to test for the proper folding of an scFv, which activates inactive β-galactosidase. Only cells with active β-galactosidase are able to grow in a medium with lactose as the only carbon source. Four rounds of mutagenesis and selection produced a scFv that was soluble, correctly folded and overexpressed at high levels in the cytoplasm. One disulfide bond is formed in the evolved protein, while the other exists as free cysteine residues, even under oxidative conditions.

Enzymes adapted to different temperatures: activity versus thermostability

Each enzyme exhibits optimal activity in a characteristic temperature range. Increasing or decreasing the temperature invariably results in decreased performance. Enzyme activity/temperature profiles change during evolution, in nature and in the laboratory. Directed evolution yielded a very significant increase in the activity of subtilisin E from *Bacillus subtilis* over a wide range of temperatures (a 17°C increase in temperature optimum) and a > 200-fold increase in half-life at 65°C [11•]. This result was achieved using successive cycles of error-prone PCR (to introduce random point mutations) and DNA recombination by the staggered extension process (StEP) method [19••], combined with screening for residual activity after inactivation at a high temperature (elevated with each successive generation). The resulting enzyme is functionally equivalent to the thermophilic subtilisin E homolog thermitase from *Thermoactinomyces vulgaris*. Only eight amino acid substitutions were sufficient to create the new 'thermophilic' subtilisin E. (The naturally occurring homologs thermitase and subtilisin E differ at 157 amino acids.)

Applications for cold-adapted enzymes are appearing in food processing, laundry, environmental remediation and biosynthetic processes. Directed evolution was used recently to create a cold-adapted subtilisin BPN′ with twice the activity of the wild type enzyme at 10°C [5].

Directed evolution has been used to probe the relationship between thermostability and catalytic activity during the evolution of a thermophilic version of the *p*-nitrobenzyl esterase from *B. subtilis* [12••]. A 14°C increase in melting temperature (Tm) was achieved after four cycles of random mutagenesis and one of DNA shuffling. By screening simultaneously for thermostability and activity at 30°C, it was possible to create a variant that was both thermostable and retained its activity at lower temperatures. Catalytic activity increased with temperature, as expected, such that at high temperatures (near the Tm) the activity was ~20-fold that of wild type at low temperatures. This, and the study of subtilisin E [11•], clearly demonstrate that thermostability and catalytic activity at low temperatures are not mutually exclusive: acquisition of thermostability need not come at the cost of catalytic activity, for example. Furthermore, it is not necessary to screen directly for activity at high temperatures in order to obtain 'thermophilic' enzymes. Screening for improved thermostability with retention or improvement of activity at lower temperature should be sufficient.

The thermostability of 3-isopropylmalate dehydrogenase (IPMDH) from *B. subtilis* was improved by directed evolution using an *in vivo* selection [13•]. This dehydrogenase gene, when integrated into the chromosome of an auxotrophic strain of *Thermus thermophilus*, complements a defective *leuB* gene and supports cell growth in minimal media. Spontaneous mutations were accumulated at lower temperatures; thermostable variants were then selected by

growth at elevated temperatures. After three cycles, an IPMDH variant allowed bacterial growth at 70°C, a 14°C increase over the starting conditions. The increased thermostability of this mutant, which had three amino acid substitutions, also did not come at the cost of its activity at lower temperatures.

Improving enzyme performance in non-natural applications

FLP recombinase from *Saccharomyces cerevisiae* is used in genetic experiments involving mutations such as chromosomal translocations, large deletions and site-specific integrations in order to manipulate DNA in a site-specific fashion. This yeast enzyme performs poorly at physiological temperatures in mammalian systems, however. Repeated cycles of error-prone PCR, DNA shuffling and screening were used to improve the performance of FLP recombinase [2•]. The best variant showed a fourfold increase in recombinase activity at 37°C and a tenfold increase at 40°C. This improved enzyme did not start to denature until ~37°C, while wild type FLP recombinase already shows signs of denaturation at 20°C. Increased recombination efficiency was confirmed in mammalian cells.

Laundry applications make tough demands on enzymes, molecular solutions for which are not always apparent. The lipase from *Thermomyces lanuginosa* was successfully evolved to improve its performance in laundry detergents [P2,P3]. A combination of random mutagenesis of the entire gene and site-directed mutagenesis of selected regions of the protein, followed by *in vivo* recombination in *Saccharomyces cerevisiae*, produced a variant with the desired wash properties.

New methods for DNA recombination

Recombination of mutations identified in parallel screens or selections can significantly accelerate laboratory evolution efforts. The Stemmer DNA shuffling method [20,P4] has been supplemented with two new methods for *in vitro* recombination, the StEP [19••] and random-priming recombination [21•]. StEP is based on a PCR-like reaction with very short annealing and extension steps. The very short cycles prematurely terminate primer extension and create truncated products that randomly anneal to the parent strands in the next cycle, creating recombinant sequences. This method offers the advantage over DNA shuffling that there is no need to fragment and reassemble the genes, procedures which often present technical difficulties. Instead, a PCR reaction containing the parental genes is run with multiple very short cycles until the primers extend to form a full-length product. The optimal cycling conditions depend on the base composition and the degree of recombination desired. In general, the annealing temperature should be decreased with decreasing sequence identity between parental genes, and cycles should be shortened for recombining closely spaced mutations.

Wilson and co-workers [22] reported a conceptually similar approach using a double-cycle method involving regular PCR cycles preceded by very short cycles with only the annealing step. This paper compares the recombination frequency in traditional PCR with double-cycle amplification. Recombination frequency for two markers 282 bp apart increases from 1% and 7% in PCR to 14% and 19% in the double-cycle protocol, using Taq and Vent DNA polymerases, respectively. Vent polymerase is known to be slower than Taq polymerase, which explains the higher recombination frequencies when using Vent polymerase.

Random-priming recombination [21•] offers an alternative to the DNase digestion step in the Stemmer DNA shuffling method. Short, random primers are annealed to the template and extended by polymerase to create random fragments suitable for reassembly. The fragment length can be controlled by changing the concentration of primers in the reaction mixture. Unlike DNA shuffling and StEP recombination, this method does not require a thermostable polymerase — any polymerase with the desired fidelity and processivity can be used. Furthermore, RNA templates or DNA–RNA hybrids can be recombined, at least in principle, by this approach.

In yeast, *in vivo* recombination is particularly simple [P2,P3]. Transformation of *S. cerevisiae* cells with a linearized vector and PCR fragments of the target genes yields a high level of recombined products by homologous recombination.

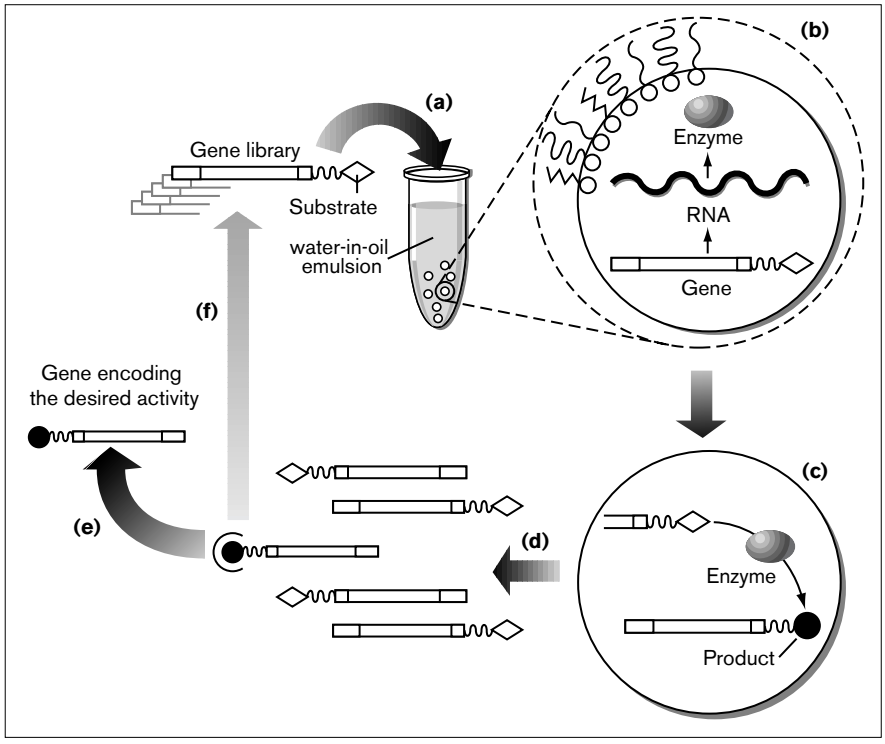
In vitro DNA recombination has mainly been used to recombine gene variants generated by random mutagenesis; however, naturally occurring homologous genes with highly similar sequences and functions present an enormous 'database' for directed evolution by recombination to create new, chimeric enzymes [6••,8••,23]. This approach of 'family shuffling' exploits existing sources of genetic variability that have already been selected for functionality in nature. It is possible that shuffling genes within families to access remote, unexplored regions of sequence space will yield proteins with a variety of desirable, but as yet undiscovered, functions.

Screening and selection

A major challenge in directed evolution is to establish a screen or selection that is sensitive to the properties of interest. True selections in which only those clones carrying a desired improvement survive or grow faster are rare, usually highly specific to one problem, and difficult to implement productively in directed evolution. When available and validated, however, they can deliver dramatic results [4•,15••,18•]. Genetic complementations, which provide a rapid way of identifying clones that retain at least some level of activity, have also been useful, particularly for identifying the relatively few functional sequences in heavily mutated proteins [24,25].

Selection is normally used for evolving intracellular enzymes, for which the required link between cell survival and enzyme activity is easier to implement. A recent study shows one relatively simple way of linking

Figure 1



A novel approach to compartmentalization and selection for *in vitro* evolution. **(a)** A gene library is expressed in an *in vitro* transcription/translation reaction and **(b)** distributed in a water-in-oil emulsion. **(c)** In the proposed selection, active enzyme variants modify the DNA template to which they are coupled through compartmentalization in the droplet. **(d)** The emulsion is broken and **(e)** the modified DNA templates purified to enrich for genes coding for active enzymes. **(f)** The entire cycle can be repeated to achieve higher enrichment. Reproduced with permission from [28•].

cell growth to a secreted enzyme (i.e. subtilisin) [17•]. Cells were grown in hollow fibers at an average density of one clone per fiber, using bovine serum albumin as the sole source of nitrogen. Cells secreting more subtilisin or more active enzyme should be able to obtain more nitrogen and grow faster. The hollow fibers provide tiny compartments to limit cross-feeding. One round of mutagenesis and enrichment produced a clone with fivefold increased protease production, found to arise from mutations outside the target gene.

Selection also requires that the target enzymatic activity does not interfere with cellular metabolism and that it can be distinguished from the background of all other cell reactions. *In vitro* translation systems such as those recently reported [26,27] may provide the basis for protein evolution in the absence of cells. A recent paper describes how *in vitro* evolution might be carried out in a water-in-oil emulsion and proposes a selection strategy based on gene modification by its product [28•] (Figure 1). The tiny aqueous droplets in water-in-oil emulsions can provide compartmentation that is needed to link the genetic material to the expressed enzyme. In this study, a cell-free transcription/translation-coupled system was mixed with mineral oil to form an emulsion containing one gene (on average) per droplet. Expression of a functional methyltransferase resulted in site-specific methylation of the template gene and its protection from digestion by *HaeIII* endonuclease. One cycle was able to achieve an enrichment factor of at least 5,000, and two cycles were enough to select the *HaeIII* methyltransferase gene from a 10^7-fold excess of other DNA.

An interesting new approach to selection for stabilized proteins links the protease resistance of the target protein to the infectivity of a filamentous phage [29•]. The protein of interest is fused with the phage minor coat protein in such a way that the phage will infect cells only if the minor coat protein is intact and folded [30]. Selection of stabilized variants then consists of proteolytic treatment, infection and phage propagation. Application of one round of this selection to a library of RNaseT1 mutants yielded several stabilized proteins. This method does not depend in any way on activity, and further screening of the stabilized variants would be necessary to retain the catalytic activity during evolution of thermostability (see above).

A screen is required when the desired activity or feature cannot be linked easily to cell survival. Screens are more versatile than selections. The throughput, however, is generally relatively low and inversely proportional to sensitivity to the desired property. A quantitative, colorimetric pH-based assay was developed for screening enantioselectivity of hydrolytic reactions [31•]. 72 commercial lipases and esterases were screened using this approach in order to identify the one with the best enantioselectivity toward solketal butyrate, an important building block in the synthesis of pharmaceuticals and biologically active compounds. The authors provide a

lucid discussion of the sensitivity of the assay and the selection of proper buffer–indicator pairs.

Conclusions

Despite significant progress during the review period, directed evolution is still clearly in its infancy; many new applications will appear in coming years, as will better technologies and evolutionary strategies. The potential to solve difficult design problems such as the evolution of new catalytic activities, has still hardly been tested. The progress this field has enjoyed reflects the rapid development of new experimental methods for creating genetic diversity and for searching through large populations for improved functions. Further advances in screening and selection technologies will reduce the time and cost of the experiments and will make it possible to tackle ever more difficult problems involving multiple enzymes, multicomponent enzymes, and the creation of new functional molecules.

Acknowledgements

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