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# Directed evolution of enzymes and biosynthetic pathways

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Directed evolution is an important tool for overcoming the limitations of natural enzymes as biocatalysts. Recent advances have focused on applying directed evolution to a variety of enzymes, such as epoxide hydrolase, glyphosate N-acetyltransferase, xylanase and phosphotriesterase, in order to improve their activity, selectivity, stability and solubility. The focus has also shifted to manipulating biosynthetic pathways for the production of many naturally synthesized compounds, as well as the production of novel 'unnatural' compounds. A combined directed evolution and computational design approach is becoming increasingly important in exploring enzyme sequence-space and creating improved or novel enzymes. Fueled by recent breakthroughs in genomics and metagenomics, these developments should help expand the use of biocatalysts in industry.

## Addresses

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## Introduction

Biocatalysts are increasingly used in industrial synthetic chemistry, particularly in cases in which chemical routes are difficult to implement [1]. Currently, more than 500 commercial products use enzymes in their synthesis, including such well known examples as the low-calorie sweetener aspartame and the antibiotic amoxicillin [1,2]. The majority (~65%) of industrial enzymes are hydrolytic in action and are widely used in the textile, starch, pulp and paper, leather, personal care and detergent industries; the remainder are primarily used in food processing (~25%) or in animal feed supplements (~10%) [2]. Enzymes offer several important advantages over traditional chemical catalysts in that enzymes are biodegradable, they typically offer exquisite selectivity — stereoselectivity, regioselectivity, and chemoselectivity,

and hence they produce enantiomerically pure products — and they function under relatively mild temperatures, pHs and pressures. Unfortunately, there often exists a functional gap between what nature provides us with and what we require from an enzyme in order for it to function correctly under industrial process conditions. Enzyme characteristics such as their activity, stability, selectivity, solubility and pH optima frequently need to be optimized for a given process. Several techniques have been developed and are used to tailor enzymes for specific industrial applications. Some of them are highlighted in this review.

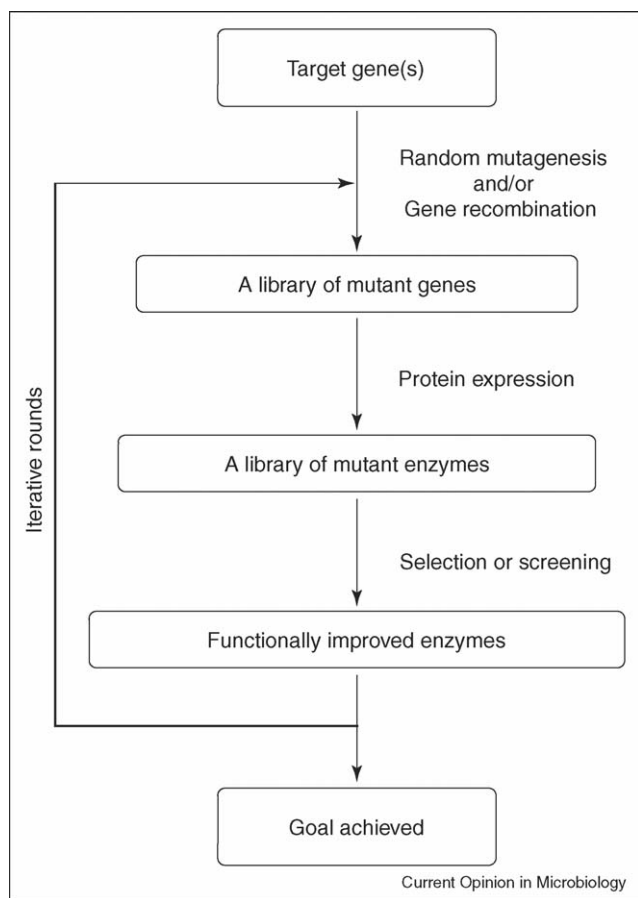
Rational protein-design uses enzyme structural information, such as crystal structure data or data obtained from homology modeling, to identify specific residue(s) that can be mutated in order to improve a specific property, such as substrate specificity or thermostability. Unlike rational design, directed evolution does not rely on a detailed understanding of the relationship between enzyme structure and function; rather, it relies on the simple yet powerful Darwinian principles of mutation and selection. As shown in **Figure 1**, starting from a single target gene or a set of target genes, a library of mutant genes is created through random mutagenesis and/or gene recombination. The resulting library is then cloned into an expression vector and inserted into microorganisms for protein expression. Functionally improved mutant proteins are identified through a carefully designed selection or high-throughput screening method, and further used as the templates for the next round of improvement. The same process is repeated until the goal is achieved or no further improvement is possible. As a result, the choice of diversity-generation methods and suitable screening or selection methods are often crucial to the success of directed evolution experiments.

This review focuses on recent advances made in the directed evolution of industrial enzymes and biosynthetic pathways. Several other related reviews have been recently published covering metabolic pathways [3•], enzymes for organic synthesis [1], improvement in the heterologous expression and stability of proteins [4•], and the use of protein engineering and directed evolution for the improvement of industrial enzymes [1,2,5,6].

## Directed evolution of enzymes

In the past decade, directed evolution has become a powerful tool for tailoring various enzyme functions. It enables the development of suitable enzymes with improved specificity, activity, stability and solubility that operate under industrial process conditions.

Figure 1



General scheme of directed evolution. Starting from the target gene(s), repeated cycles of random mutagenesis and/or gene recombination coupled with selection or high through screening are used to isolate functionally improved variants.

### Specificity

The enzymatic synthesis of enantiomerically pure compounds in the pharmaceutical, chemical and agricultural industries has become increasingly attractive because the resolution of racemates is costly and often difficult. As a result, directed evolution has become a valuable tool in enhancing or altering the enantioselectivity of target enzymes. The enantioselectivity of the *Aspergillus niger* epoxide hydrolase for phenyl glycidyl ether was recently improved twofold using only one round of epPCR — from  $E = 4.6$  [56% enantiomeric excess (*ee*)] to  $E = 10.4$  (74% *ee*), where  $E$  is the enantioselective factor [7]. The improved mutant enzyme contains three amino acid substitutions (Ala217Val, Lys332Glu and Ala390Glu), two of which are far from the enzyme's active-site. Similarly, the enantioselectivity of the epoxide hydrolase from *Agrobacterium radiobacter* was improved 13-fold using epPCR, DNA shuffling and high-throughput screening [8]. In other studies, Turner *et al.* [9,10] used random mutagenesis and an agar plate-based colorimetric

selection to focus on the evolution of an amine oxidase for the practical deracemisation of cyclic secondary amines. They found that screening a library of mutant amine oxidases for activity against one enantiomer of a specific substrate led them to the identification of an enzyme possessing much broader substrate specificity while retaining high enantioselectivity.

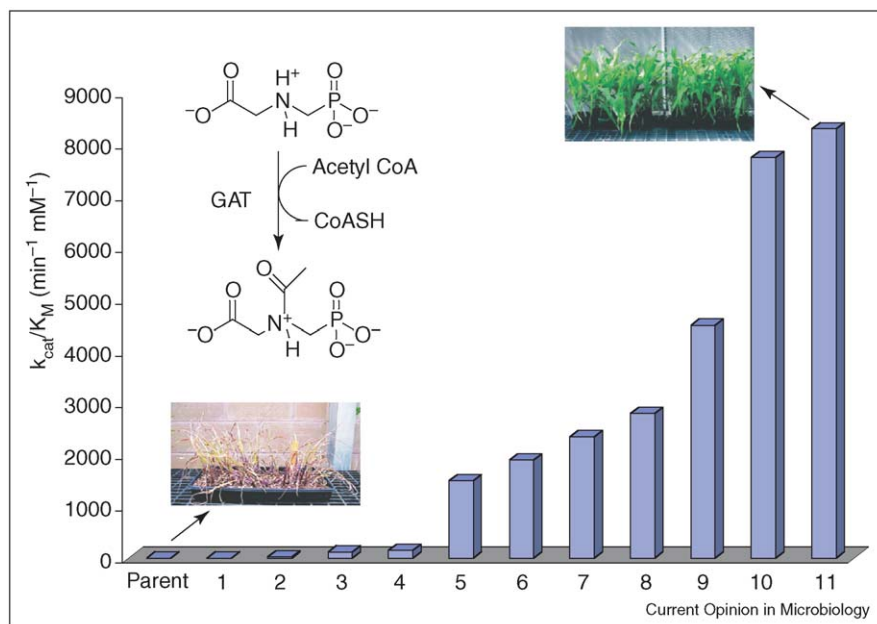
Directed evolution has been used successfully to alter the substrate specificity of several distinct enzymes. Using two rounds of DNA shuffling, Chen *et al.* [11] increased the activity of the enzyme organophosphorus hydrolase on the commercial pesticide chlorpyrifos (Dursban). The promiscuous activity of human carbonic anhydrase II on the ester substrate 2-naphthyl acetate was increased 40-fold through three rounds of mutagenesis, selection and recombination [12]. Using sequential rounds of mutagenesis, *in vitro* recombination and high-throughput screening, the enzyme cytochrome P450 BM-3 has been converted into a highly efficient catalyst for the conversion of alkanes to alcohols [13,14]. Similar results were reported by Wong *et al.* [15] by engineering the active site of cytochrome P450. Other studies dealing with the modification of substrate specificity include the directed evolution of *Escherichia coli* endopeptidase OmpT to alter its substrate preference to a different peptide [16•], engineering toluene *para*-monooxygenase for the regio-specific oxidation of toluene and naphthalene [17], DNA shuffling of a toluene-*o*-xylene monooxygenase to create variants with increased aromatic degradation capabilities and the ability to synthesize novel benzene and toluene derivatives [18–20], converting a  $\beta$ -glycosidase into a  $\beta$ -transglycosidase [21], and engineering a 2,4-dinitrotoluene dioxygenase to enhance its ability to degrade nitroaromatic compounds [22].

### Activity

Often, the activity of a natural enzyme is too low to be of commercial or therapeutic use; therefore, it becomes necessary to enhance enzyme activity by rational design or directed evolution. As an impressive example of this approach, eleven rounds of DNA shuffling of the enzyme glyphosate *N*-acetyltransferase (GAT) resulted in a variant with a 10 000-fold improvement in catalytic efficiency compared with that of the parent enzyme (Figure 2) [23••]. Interestingly, the improvement in catalytic efficiency was also accompanied by an increase in thermostability. The evolved GAT variants had half-lives that ranged from twofold to fivefold longer (at 37.7 °C) than the native GAT [24]. The engineered GAT variant should enable the development of new crops that exhibit tolerance to the herbicide glyphosate, and this would be an important transgenic phenotype in global agriculture.

Lipases are versatile acyl-transfer catalysts that are involved in the inter-conversion of structurally diverse esters, alcohols and carboxylic acids and are often useful

Figure 2



The evolution of a glyphosate acetyltransferase. Eleven rounds of DNA shuffling were used to improve the catalytic efficiency ( $k_{cat}/K_M$ ; [catalytic rate constant/Michaelis-Menten equilibrium constant]) dramatically. The eleventh round mutant was tolerant to  $1.09 \text{ g m}^{-2}$  (156 ounces per acre) Roundup UltraMAX. Parent in the figure refers to the native GAT and 11 to the eleventh round mutant.

in producing optically active pharmaceuticals and fine chemicals. Various mutagenesis approaches (e.g. epPCR, DNA shuffling, DNA family shuffling and saturation mutagenesis) have recently been used to produce lipases with improved amide-hydrolyzing activity [25], improved activity on and enantioselectivity for bulky substrates [26], and improved activity in the hydrolysis of diethyl 3-(3',4'-dichlorophenyl) glutarate [27]. Another target of recent directed evolution has been barley  $\alpha$ -amylase, which could potentially be engineered for cold starch-hydrolysis for use in the production of corn sweeteners and bioethanol [28]. Three rounds of epPCR and DNA shuffling were used to engineer a barley  $\alpha$ -amylase with a total activity 1000-fold higher than that of the wild type enzyme [29].

### Stability

Many industrial applications require enzymes that retain their function at elevated temperatures or in non-natural environments such as in organic solvents. Diversa's Gene Site Saturation Mutagenesis<sup>TM</sup> technology has been used to increase the thermostabilities of several different enzymes by direct evolution. The melting temperatures of these evolved enzymes were improved dramatically when compared with those of the wild type enzymes: pectate lyase was  $16^\circ\text{C}$  higher [30]; bacterial phytase was  $12^\circ\text{C}$  higher [31]; and xylanase was  $35^\circ\text{C}$  higher [32]. This powerful approach involves individually randomizing the amino acid at each position in a protein with all 20 amino acids in order to generate a complete library of

variants. Other studies have focused on improving the thermostability of the cofactor-regeneration enzyme phosphite dehydrogenase [33], the thermo-stabilization of the potential gene-selection marker hygromycin B phosphotransferase [34], and the increased stability in organic solvents of fructose bisphosphate aldolase [35] and cytochrome P450 BM-3 monooxygenase [36].

### Solubility and heterologous expression

Achieving a high level of expression of soluble proteins in a heterologous host is highly desirable for basic research and biotechnological applications. An excellent review has recently been published on the use of directed evolution to increase heterologous expression of proteins [4<sup>\*</sup>]; therefore, this review focuses only on advances made within the past year. Using epPCR and DNA shuffling to mutate seven residues, the solubility of phosphotriesterase from *Pseudomonas diminuta* was increased 20-fold [37]. Directed evolution was also successfully used to increase the soluble expression of the phosphotriesterase from *Enterobacter aerogenes* [38]. Phosphotriesterase catalyzes the detoxification of a wide variety of organophosphate compounds and seems to have huge potential in the decontamination of hazardous compounds. Berglund *et al.* [39] increased the soluble expression of tobacco etch virus protease by epPCR, gene shuffling and fluorescence-based solubility screening. The evolved protease should be useful in the cleavage of recombinant fusion proteins. Two other studies focused on increasing the expression level of a bacterial alanine racemase [40], and

the heterologous overexpression of a termite cellulase gene [41].

### Directed evolution of biosynthetic pathways

Compared to the numerous examples of the directed evolution of single enzymes, fewer examples exist for the directed evolution of biosynthetic pathways. Several directed evolution formats can be used to improve an isolated enzyme in a pathway, but the evolution of biosynthetic pathway components in the context of cellular metabolism has been much more difficult. This is not surprising, given the complexity of coordinated improvement of contiguous or dispersed genes in a metabolic pathway towards a desired phenotype. Despite these difficulties, directed evolution has been successfully applied to natural or engineered metabolic pathways, multiple enzyme systems and even whole organisms [3]. In recent years, the engineering of the carotenoid biosynthetic pathways in *E. coli* has received much attention because it is possible to modify these naturally occurring pathways for the creation of new natural products. For example, novel C<sub>45</sub> and C<sub>50</sub> carotenoids have been synthesized by directed evolution of the C<sub>30</sub> carotenoid synthase *crtM* gene [42]. New carotenoid products were also obtained by DNA shuffling of a *Staphylococcus aureus* C<sub>30</sub> carotenoid oxygenase gene, *crtOx*, and cloning this gene into *E. coli* for the production of oxygenated linear C<sub>30</sub> and C<sub>40</sub> carotenoids [43]. Directed evolution of two farnesylgeranyl diphosphate synthase genes was also conducted to better understand the chain-elongation mechanism of this enzyme [44,45].

The *aveC* gene from the doramectin biosynthesis pathway was subjected to iterative rounds of semi-synthetic DNA shuffling to improve the industrial scale production of the potent antiparasitic compound doramectin [46]. The *aveC* gene is thought to encode for a hydratase which helps synthesize two related compounds, CHC-B1 (cyclohexyl-B1) and CHC-B2 (cyclohexyl-B2). The best shuffled variant conferred a final CHC-B1:CHC-B2 ratio of 1:0.07, a 23-fold improvement in product specificity compared with that of the AveC wild type enzyme (Figure 3). Copley *et al.* [47] explored the use of genome shuffling to improve the degradation of the pesticide pentachlorophenol by *Sphingobium chlorophenolicum*. Three rounds of genome shuffling were used to isolate variants with the ability to grow on plates containing 6–8 mM pentachlorophenol, whereas the original strain could not grow in the presence of pentachlorophenol concentrations in excess of 0.6 mM. These examples demonstrate the power of single-gene shuffling and whole-genome shuffling to successfully improve metabolic pathways.

The directed evolution of key enzymes within metabolic pathways can benefit product yields. An improved variant of glucosamine synthase with reduced end-product

inhibition was obtained using directed evolution, and yielded an *E. coli* strain capable of producing glucosamine at 17 g l<sup>-1</sup> [48]. The yield of the aromatic amino acid L-phenylalanine was improved by performing DNA shuffling on the chorismate mutase-prephenate dehydratase enzyme to reduce feedback-inhibition, which is mediated by the allosteric binding of L-phenylalanine [49].

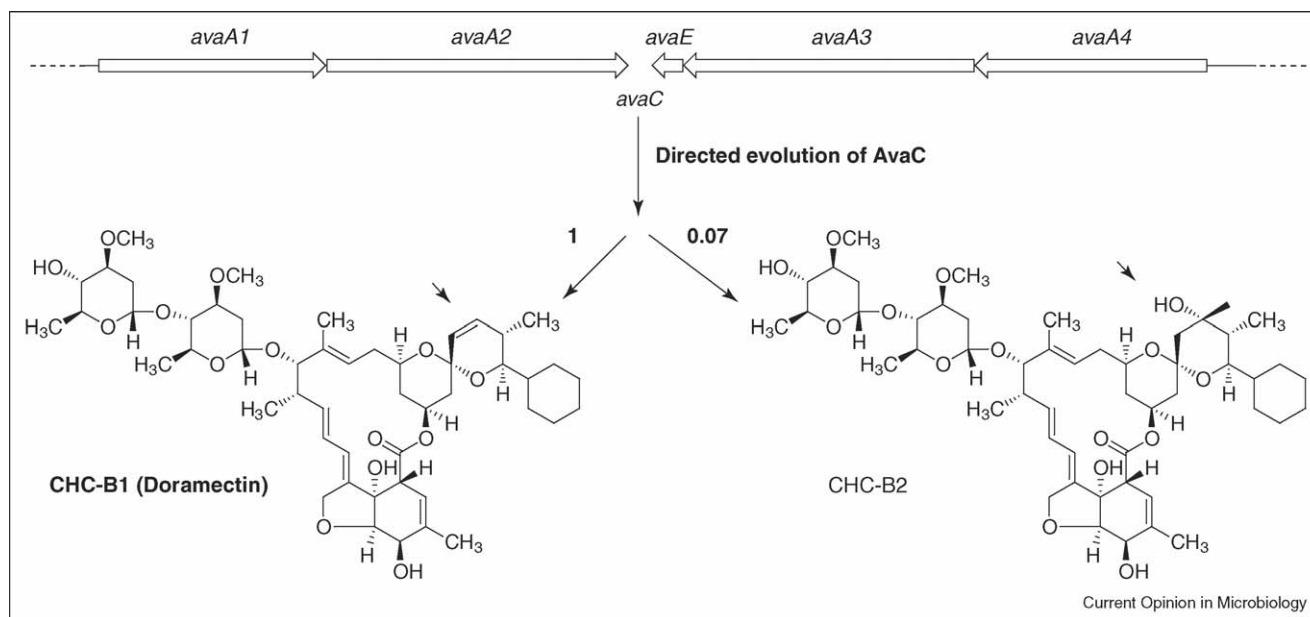
### A combined directed evolution and rational design approach

In directed evolution experiments, enzymes with altered properties are discovered frequently to have acquired these properties as a result of the alteration of amino acids in a way that would have been difficult to predict or even to justify using protein crystal structure alone. This highlights the limitations of our understanding of protein structures and of our ability to rationally design desired biocatalysts. However, directed evolution is also limited by its inability to exhaustively search the vast sequence space of proteins. Thus, the future development of many enzyme biocatalysts might depend upon the successful coupling of rational design and directed evolution approaches.

In an impressive demonstration of this combined approach, Zhao *et al.* [50] used molecular modeling to identify functionally important residues in the ligand-binding pocket of the human estrogen receptor and then used sequential saturation mutagenesis, coupled with epPCR, to alter the specificity of the receptor to a selected synthetic ligand. The resulting mutant showed a >10<sup>8</sup>-fold specificity shift in a mammalian cell assay. This approach should also be applicable to enzymes. Previously, Hayes *et al.* [51] demonstrated the feasibility of using computational design to eliminate vast regions of sequence space of the enzyme TEM-1 β-lactamase and thus to reduce the sequence space to be analyzed to a size more amenable to experimental screening. This reduced sequence-space could be screened for variants with improved resistance to the antibiotic cefotaxime.

Recently, some encouraging progress has been made in the use of computational approaches to search sequence space and predict beneficial mutations. Structure-based computational design techniques have been used in order to introduce new activity into catalytically inert protein scaffolds by the careful placement of key catalytic residues [52,53]. The uses of computational design have also been expanded to include the thermo-stabilization of enzymes without loss of catalytic efficiency [54] and the redesign of an enzyme active-site to improved catalytic efficiency [55]. Unfortunately, these are isolated successes, mainly because of the lack of sufficient computational power and the inability to apply existing algorithms to more complex enzyme mechanisms. In addition, the rationally designed novel enzymes are often less active than their natural counterparts. Thus, the development of

Figure 3



Engineering of the avermectin biosynthesis pathway. Iterative rounds of gene shuffling and high-throughput screening on the *avaC* gene led to a final CHC-B1:CHC-B2 ratio of 1:0.07, a 23-fold improvement in product specificity over the wild type gene.

more powerful computational algorithms and, in particular, the combination of computational approaches with directed evolution should make it possible to rapidly create tailor-made enzymes for various biotechnological applications.

## Conclusions

Directed evolution has become a valuable tool in engineering enzyme characteristics for industrial applications. New, efficient diversity-generation methods and high-throughput screening and selection methods combined with more sophisticated computational algorithms should accelerate the development of industrial biocatalysts. Computational approaches should be useful in creating novel enzymes as starting points for directed evolution and designing more focused directed evolution libraries. Advances in metabolic engineering have paved the way for applying directed evolution to existing pathways as well as creating new pathways capable of synthesizing novel compounds for the pharmaceutical, chemical and food industries.

## Update

As another impressive demonstration of the combined rational design and directed evolution strategy, Park *et al.* [56] created new catalytic activity in an existing protein scaffold. The authors first rationally manipulated several active site loops in the  $\alpha\beta/\beta\alpha$  metallohydrolase scaffold of glyoxalase II through amino acid insertion, deletion, and substitution, and then used directed evolution to introduce random point-mutations to fine-tune the enzyme

activity. The resulting enzyme completely lost its original activity and instead showed  $\beta$ -lactamase activity.

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