Milestones in directed enzyme evolution

Haiyan Tao* and Virginia W Cornish†

Directed evolution has now been used for over two decades as an alternative to rational design for protein engineering. Protein function, however, is complex, and modifying enzyme activity is a tall order. We can now improve existing enzyme activity, change enzyme selectivity and evolve function *de novo* using directed evolution. Although directed evolution is now used routinely to improve existing enzyme activity, there are still only a handful of examples where substrate selectivity has been modified sufficiently for practical application, and the *de novo* evolution of function largely eludes us.

Addresses
Department of Chemistry, Columbia University, New York, NY 10027, USA
*e-mail: ht265@columbia.edu
†Correspondence: e-mail: vc114@columbia.edu

Current Opinion in Chemical Biology 2002, 6:858–864

1367-5931/02/$ – see front matter

Abbreviations

AATase	aspartate aminotransferase
β-ATase	β-branched aminotransferase
DsRed	*Discosoma* red fluorescent protein
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
RE	restriction endonuclease
SLiPE	substrate-linked protein evolution

Introduction

Enzymes are able to catalyze a broad range of chemical transformations, not only with impressive rate enhancements but also with both regioselectivity and stereoselectivity and are therefore attractive candidates as practical alternatives to traditional small-molecule catalysts. With applications as diverse as chemical synthesis, reagents for commercial products and biomedical research, and even therapeutics, there is a great demand for enzymes with both improved activity and novel catalytic function. In theory, the properties of an enzyme can be altered by rational design; however, rational design is greatly hindered in practice by the complexity of protein function. With advances in molecular biology, the possibility has arisen that an enzyme with the desired catalytic property can instead be isolated by directed evolution.

Mimicking natural evolution, directed evolution aims to generate a protein with new activity by screening or selecting for the desired function from a large pool of protein variants (Figure 1) [1–5]. Having been optimized over the past two decades, here we look at what can now be achieved with directed evolution experiments — improving enzyme fitness for a given application, changing substrate selectivity and even evolving function *de novo*.

Improving robustness and activity

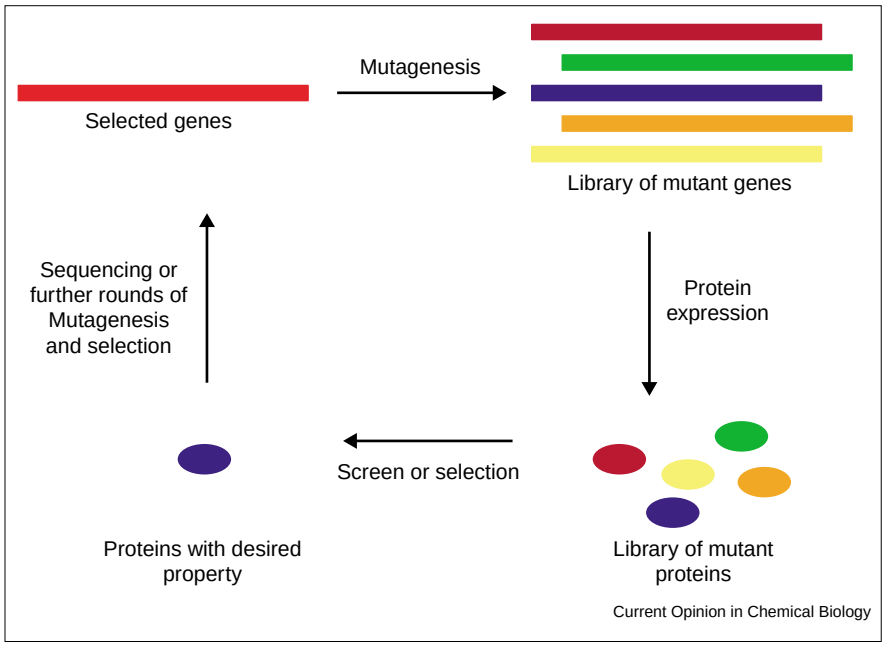
Enzymes generally show optimal activity in aqueous solution at neutral pH, and often need to be modified for use in industrial and biotechnology applications. In 1993, Arnold and co-workers [6] reported that directed evolution could be used to improve the activity of the protease subtilisin E in organic co-solvents. Fewer than 10^5 variants needed to be screened to generate a subtilisin E variant whose activity in 60% aqueous dimethylformamide was improved 256-fold compared with the activity of the wild-type enzyme. The mutations were introduced randomly throughout the structure, and 0.1–1% of the mutations were beneficial. Many experiments have now confirmed that with an appropriate screen or selection, libraries on the order of 10^4 variants are sufficient to improve enzyme activity at extremes of temperature or pH, in organic co-solvents, or other desired conditions.

Directed evolution is now used routinely to improve enzyme fitness for a given application. This past year, Kichise *et al.* [7] reported *Aeromonas caviae* FA440 synthase mutants with improved synthesis yields of the biodegradable polyester polyhydroxyalkanoate. Canada *et al.* [8] improved the activity of toluene *ortho*-monooxygenase for 1-naphthol synthesis and degradation of chlorinated ethene pollutants. Bosma *et al.* [9] increased the activity of haloalkane dehalogenase for the degradation of the pollutant 1,2,3-trichloropropane. The activity and thermostability of β-glucuronidase, endoglucanase EngB and luciferase were all improved using directed evolution [10–12]. Impressively, most of these experiments required only two to three rounds of mutagenesis and library sizes of 10^3 to 10^5 variants.

A particularly useful recent example is the generation of a red fluorescent protein suitable for live cell imaging [13•,14•]. Green fluorescent protein (GFP) has emerged as an essential research tool for tracking protein localization *in vivo*. A red fluorescent variant would be ideal for co-localization and fluorescence resonance energy transfer experiments (FRET), but has proven difficult to engineer. *Discosoma* red fluorescent protein (DsRed) showed promise as such a reagent. There are two major problems that restrict the use of DsRed, however — its chromophore maturation is slow and its tetrameric state can perturb the function and localization of tagged proteins. Bevis and Glick [13•] used seven rounds of both directed and random mutagenesis, with library sizes from 10^3 to 10^5 variants, followed by a high-throughput screen for fluorescence to create DsRed variants that mature 15 times faster than the wild-type protein. Illustrating a difficulty with directed evolution experiments, the potential loss of properties not selected for, the variant with the optimal maturation half-life has compromised spectral properties. Whereas the maturation half-life is improved from 11 h to less than 1 h,

Figure 1

General steps of directed enzyme evolution. The gene encoding the protein of interest is mutated to generate a library of mutant genes. Expression of the mutant genes provides the library of mutant proteins. The proteins are screened or selected based on a desired property, and the variants with modified activity are sequenced or used for further rounds of mutagenesis and selection.



the maximal extinction coefficient is decreased from 52 000 $M^{-1}cm^{-1}$ to 30 100 $M^{-1}cm^{-1}$, the quantum yield from 0.68 to 0.42, and the relative brightness from 1.00 to 0.36 compared with the starting DsRed protein. Campbell *et al.* [14•] further evolved this DsRed variant to be monomeric using a combination of structure-based design and directed evolution. The final variant is a monomer and has a maturation half-life of less than 1 h, an extinction coefficient of 44 000 $M^{-1}cm^{-1}$, and a quantum yield of 0.25. This evolved DsRed variant should provide the needed complement to GFP.

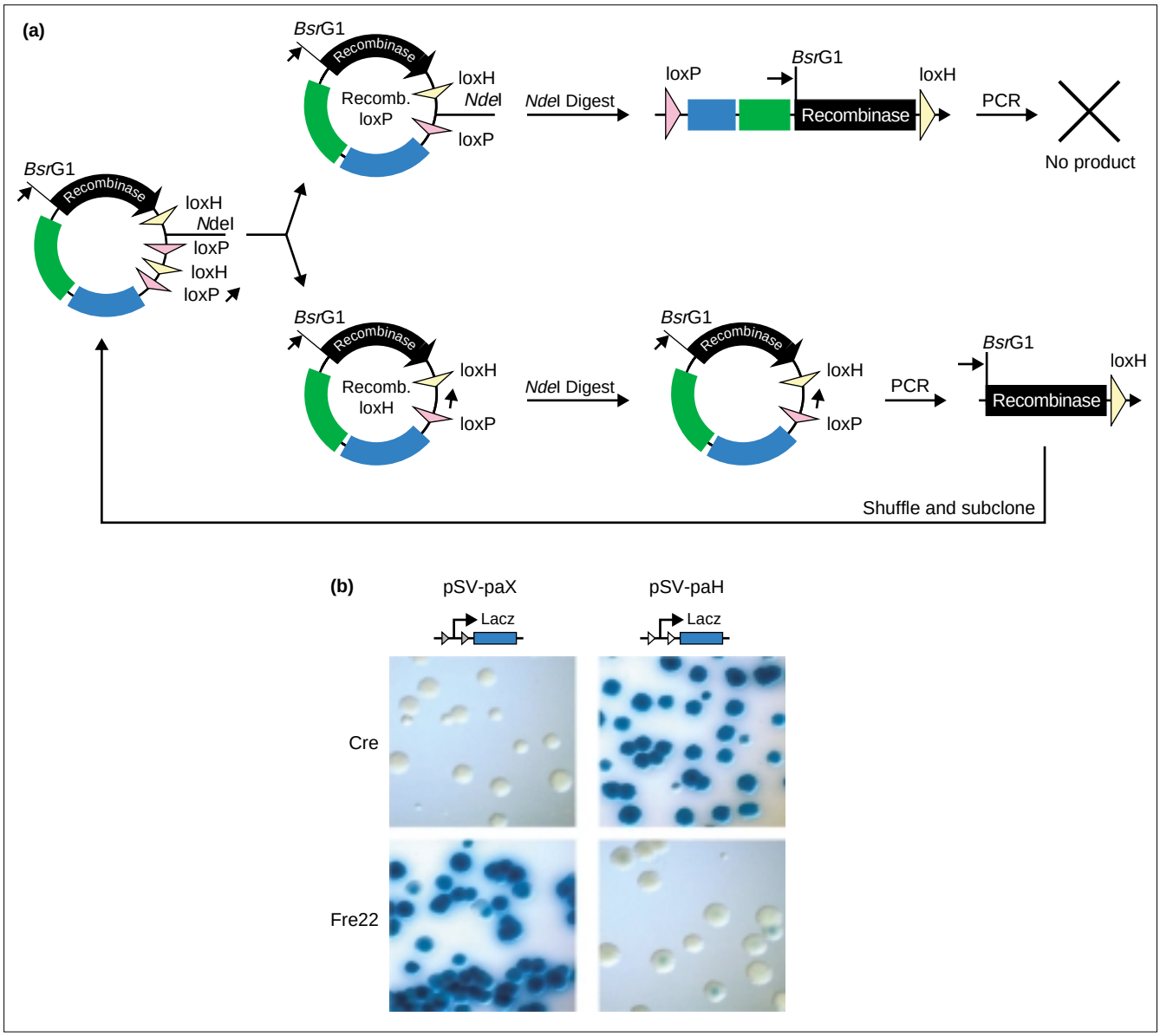
Modifying substrate selectivity

Natural enzymes typically are quite specific for their intended substrates, and it is often necessary to modify or broaden their substrate selectivity for industrial or biotechnology applications. The relationship between enzyme structure and selectivity is complex, however, and modifying the substrate selectivity of an enzyme is more difficult than improving the activity of an enzyme in different environments. There are some impressive examples of modifying enzyme selectivity. For example, in 1998 Yano *et al.* [15] reported the successful re-engineering of an aspartate aminotransferase (AATase) to a β-branched aminotransferase (β-ATase) using random mutagenesis and a cell-based selection for complementation of an engineered deficiency in the endogenous β-ATase. Much larger libraries were required than in the previous examples of increasing enzyme activity — five cycles of mutation and selection with 10^6–10^7 variants in each cycle. An AATase variant was generated that utilizes 2-oxo acids 10^5 times more efficiently than the wild-type AATase, with k_{cat} on the order of 10^2 s^{-1}. Despite such successes, however, directed evolution of substrate selectivity still is far from a solved problem.

A representative area to consider is the directed evolution of restriction endonucleases (REs) with defined selectivities. Particularly given the near completion of the human genome sequencing project, there is strong impetus to develop REs with a variety of selectivities. Despite significant effort, however, directed evolution has yet to provide a general solution to RE engineering. A recent example is an effort to modify the RE *Bst*YI, which recognizes the site A/GGATCC/T, to selectively cleave AGATCT sites [16•]. A genetic selection and screen for resistance to DNA damage in the presence of *Bgl*II methylase was used to assay 10^7 random *Bst*YI variants. The best mutations were combined to give a *Bst*YI variant that cleaves AGATCT 12 times more efficiently than either AGATCC or GGATCT and shows no detectable activity towards GGATCC. Although encouraging, this selectivity is not sufficient for biotechnology applications and illustrates the difficulty in evolving not only activity, but also selectivity.

Related to this, Buchholz *et al.* [17••] and Santoro *et al.* [18•] successfully altered Cre recombinase site selectivity using directed evolution. Cre recombinase is used routinely for conditional mutagenesis and gene replacement experiments, but applications of Cre are restricted to the natural loxP sites. Buchholz *et al.* [17••] developed a novel substrate-linked protein evolution (SLiPE) strategy, by which only recombinases with the desired substrate selectivity can be amplified by PCR for subsequent rounds of mutagenesis and selection (Figure 2). Using SLiPE, two Cre recombinase variants with loxH, but not loxP, site recombinase activity were isolated after 35 cycles of selection with >10^6 variants per cycle. The evolved Cre variants displayed dramatically increased activity for loxH sites and decreased activity for loxP sites, and could be used *in vivo*

Figure 2

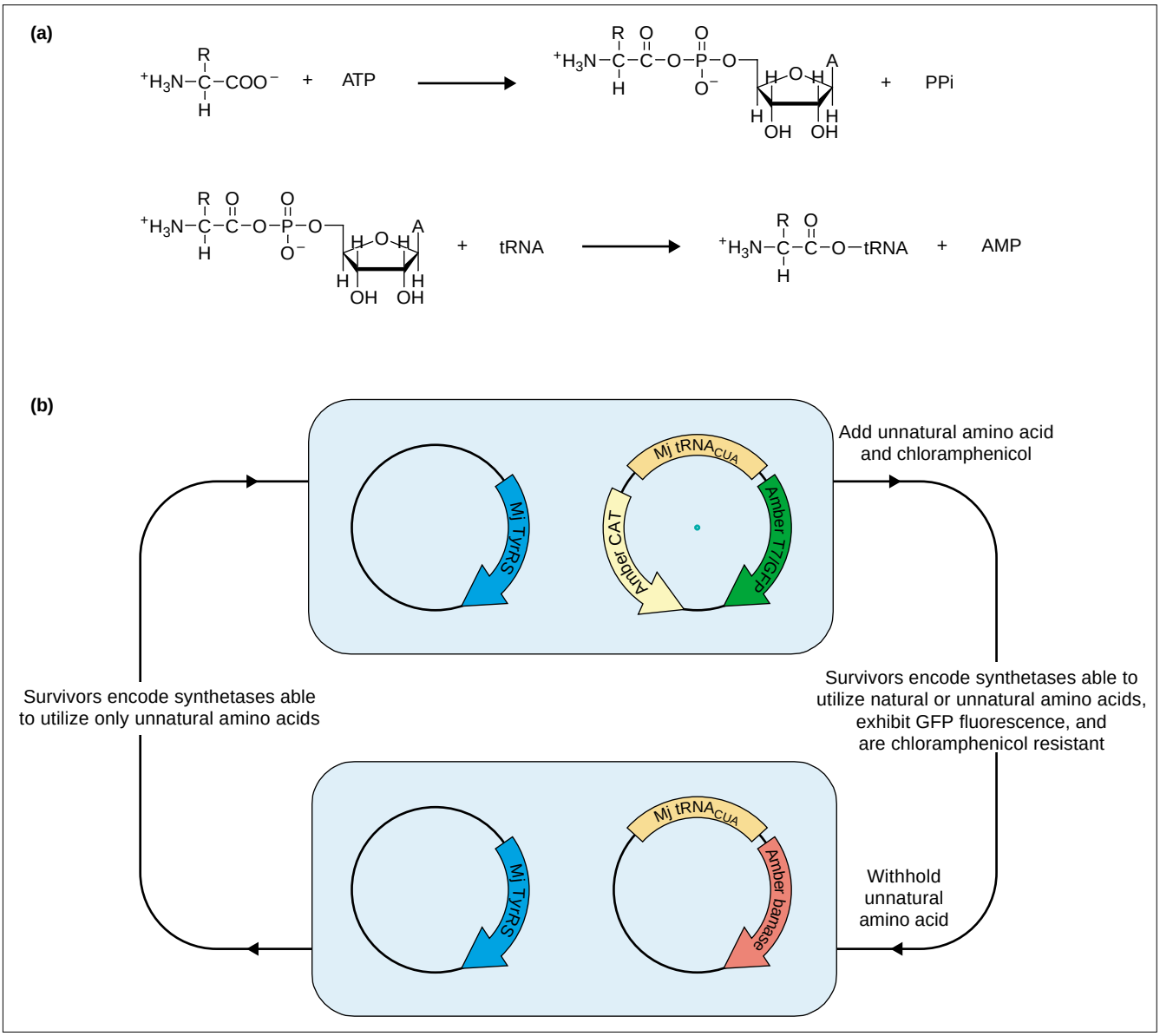


A Cre recombinase variant (Fre22) with loxH, as opposed to the natural loxP, site selectivity. (a) Substrate-Linked Protein Evolution (SLiPE) selection. The vector encoding Cre recombinase contains two loxP sites, two loxH sites, and one *NdeI* site. PCR primers for amplifying the Cre recombinase gene after recombination are designed such that recombination at the loxP site deletes the binding site for the downstream primer, while recombination at the new loxH sites deletes the *NdeI* site. Thus, genes encoding Cre recombinase variants that selectively cleave the loxH, but not the loxP sites, are preferentially amplified. (b) *lac*Z recombination reporter assay. In plasmid pSV-paX the *lacZ* reporter gene promoter is flanked by two loxP sites, while in plasmid pSV-paH the promoter is flanked by two loxH sites. Recombination at the loxP or loxH sites deletes the promoter, disrupting transcription of the *lacZ* reporter gene. Cre recombinase recognizes the loxP sites. The evolved Cre variant Fre22 recognizes the loxH sites. Adapted from [17••] with permission. Copyright 2001, Macmillan Publishing.

to selectively recombine loxH sites. Whether because of the large library size and multiple cycles of evolution or a powerful selection method, SLiPE appears to provide a robust method for engineering recombinases with new site selectivities. Directed evolution has also been used to modify the site selectivity of a transposase [19].

DNA modification enzymes provide natural selections for activity — one would expect other types of enzyme activities to be harder to assay and, hence, evolve. Recent efforts include modifying the substrate selectivity of 2-hydroxybiphenyl 3-monooxygenase and biphenyl dioxygenase [20,21]. A particularly successful example is the directed evolution of an orthogonal aminoacyl-tRNA synthetase suitable for incorporating synthetic amino acids into proteins via an amber suppressor tRNA *in vivo* [22••,23•]. Tyrosyl-tRNA synthetase from *Methanococcus jannaschii* was evolved to an efficient *O*-methyl-L-tyrosine tRNA

Figure 3



Directed evolution of an orthogonal aminoacyl-tRNA synthetase with altered amino acid selectivity. (a) Aminoacyl-tRNA synthetase reaction scheme. The enzyme catalyzes both steps of the two-step reaction. In the first step, aminoacyl-tRNA synthetase catalyzes the formation of an activated aminoacyl adenylate. In the second step, aminoacyl-tRNA synthetase couples the activated amino acid to tRNA releasing AMP. (b) A general selection for aminoacyl-tRNA synthetase with altered amino acid selectivity. The key to this strategy is both a positive selection for incorporation of the unnatural amino acid and a negative selection against incorporation of the natural amino acid. First, aminoacyl-tRNA synthetase that can charge the amber suppressor tRNA with both the natural and unnatural amino acids are selected for using an antibiotic resistance gene, chloramphenicol acetyltransferase (CAT), with an amber stop codon engineered in the coding region. Efficient incorporation of an amino acid in response to the amber stop codon is required for gene expression and hence antibiotic resistance. Then, aminoacyl-tRNA synthetases that utilize the natural amino acid are selected against using a toxic reporter gene, barnase. Cells expressing the aminoacyl-tRNA synthetase variants are grown in the presence of only the natural amino acid; incorporation of the natural amino acid in response to the stop codon engineered in the barnase coding sequence is lethal. Part (b) adapted from [23•] with permission. Copyright 2001, National Academy of Sciences, USA.

synthetase [22••]. Again, a large library size and a powerful *in vivo* selection seem the key to this successful enzyme evolution experiment. Three generations of 10^9 variants were taken through both a positive selection for suppression of an amber stop codon in the gene encoding chloramphenicol acetyltransferase and a negative screen to eliminate evolved variants with residual activity with tyrosine. The combination of an assay for the new amino acid substrate and an assay against the natural amino acid substrate seems critical for ensuring that the evolved synthetase is selective for its intended substrate. The negative assay is refined in subsequent applications (Figure 3) [23•]. The

evolved tRNA synthetase variant prefers *O*-methyl-L-tyrosine to L-tyrosine by 100-fold in catalytic efficiency. The evolved variant could incorporate *O*-methyl-L-tyrosine into dihydrofolate reductase in response to an amber codon with high fidelity *in vivo*.

All of the experiments discussed thus far hinged on an assay designed specifically for the desired activity. However, many enzyme activities do not provide an obvious high-throughput assay for function and developing each new assay may take several years. An exciting recent development, therefore, is the introduction of general selections for enzyme catalysis that theoretically can be applied to a variety of diverse chemical reactions [1–5]. To date, these general selections had been demonstrated as proof-of-principle experiments with well-studied natural enzymes. But this past year Xia *et al.* [24••] demonstrated that a general selection method using co-expression of the enzyme and substrate on the phage surface could be used to convert a DNA polymerase to an RNA polymerase. Four rounds of selection were used to assay $>10^8$ variants. DNA polymerase variants were isolated that incorporate nucleoside triphosphate substrates as efficiently as the wild-type DNA polymerase incorporates deoxynucleoside triphosphate substrates, although the evolved variants have limited processivity.

Evolving activity *de novo*

Although natural enzymes already catalyze many diverse chemical transformations, including carbon–carbon bond forming reactions, hydrolysis reactions and oxidation reactions, the ultimate goal of directed evolution from both a practical and an academic perspective is in many ways the *de novo* evolution of activity. On the one hand, this goal has already been achieved with catalytic antibodies [25,26]. By eliciting an immune response to tetrahedral phosphinate or phosphonate ester transition-state analogs, it was shown in 1986 that antibodies could be generated that catalyzed ester or carbonate hydrolysis, respectively. Since these first reports, transition-state analogs and other molecules designed to elicit catalytic residues in the antibody combining site have been used to evolve antibodies that catalyze not only hydrolysis reactions, but also the Aldol reaction, the Diels–Alder reaction, the oxy-Cope reaction, and the enantioselective reduction of ketones. This research certainly has proven that protein catalysts can be evolved *de novo*. However, catalytic antibodies generally have not achieved the catalytic efficiencies (k_{cat}/K_M) observed for natural enzymes, and antibodies are typically difficult to produce on an industrial scale.

With the advent of *in vitro* approaches for linking phenotype to genotype, such as phage display, ribosome display and, more recently, *in vitro* compartmentalization, it seemed obvious that transition-state analogs and other suitable small-molecule traps could be extended to *in vitro* evolution. Interestingly, this promise has not been realized. Existing catalytic antibodies have been improved modestly using phage display, and antibody libraries generated from mice pre-immunized with the given transition-state analog have been panned using phage display. But this approach has been proven difficult to extend to these *in vitro* technologies or to scaffolds other than antibodies. A good representative example of using *in vitro* evolution to improve the activity of a catalytic antibody is provided by Wells and co-workers [27]. They grafted the antigen-binding loop of immunoglobulin G 17E8 onto a humanized Fab fragment expressed on the surface of phage. Directed mutations based on the high resolution structure of 17E8 were then panned against the norleucine phosphonate transition-state analog originally used to generate antibody 17E8. Although variants were isolated with increased affinity for the transition-state analog, none showed improvements in k_{cat}/K_M over an order of magnitude from the starting Fab. Moreover, the authors emphasized that increases in affinity for the transition-state analog did not correlate with increases in catalytic efficiency. Other than a handful of examples of catalytic RNAs [28], transition-state analogs have not proven useful with *in vitro* evolution technologies.

There are only smatterings of reports that suggest that protein catalysts can be generated *de novo* without the aid of the immune system. The most success, in fact, has come with mechanism-based random peptide libraries [29–32]. An Aldol catalyst based on formation of a Schiff base intermediate was evolved by Tanaka and Barbas [32] using phage display. Miller and co-workers [31] have used on-bead screens to generate a series of imidazole-based catalysts for acyl-transfer reactions.

Conclusion

Directed evolution is now used routinely to improve enzyme fitness for a given application. Libraries on the order of 10^4 variants seem sufficient for these applications, and directed evolution offers a practical route to engineering protein variants for industrial and biotechnology applications. Changing the substrate selectivity of an enzyme seems to be a bigger hurdle. Although there are a few impressive examples, it remains difficult to achieve the selectivity required for practical applications. In the most successful examples of changing substrate selectivity, large libraries of 10^6–10^9 variants have been assayed using powerful *in vivo* selections. Other than in the catalytic antibody field, the *de novo* evolution of function remains elusive. An interesting intellectual, as well as practical, question remains: what is required to create function *de novo*? Exciting recent advances include the development of general assays for enzyme catalysis that can be applied to a variety of chemical transformations [33–38], and the extension of directed evolution to whole organisms [39,40].

Acknowledgements

VWC is a recipient of a Beckman Young Investigator Award, a Burroughs–Wellcome Fund New Investigator Award in the Toxological Sciences, a Camille and Henry Dreyfus New Faculty Award, and a National Science Foundation Career Award. In addition, the authors are grateful

for financial support for their research from the National Institutes of Health (GM 62867).

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Amstutz P, Forrer P, Zahnd C, Pluckthun A: ***In vitro*** display technologies: novel developments and applications. *Curr Opin Biotechnol* 2001, 12:400-405.

2. Farinas ET, Bulter T, Arnold FH: Directed enzyme evolution. *Curr Opin Biotechnol* 2001, 12:545-551.

3. Kurtzman AL, Govindarajan S, Vahle K, Jones JT, Heinrichs V, Patten PA: Advances in directed protein evolution by recursive genetic recombination: applications to therapeutic proteins. *Curr Opin Biotechnol* 2001, 12:361-370.

4. Lin H, Cornish VW: Screening and selection methods for large-scale analysis of protein function. *Angew Chem Int Ed Engl* 2002, in press.

5. Taylo SV, Kast P, Hilvert D: Investigating and engineering enzymes by genetic selection. *Angew Chem Int Ed Engl* 2001, 40:3310-3335.

6. Chen K, Arnold FH: Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide. *Proc Natl Acad Sci USA* 1993, 90:5618-5622.

7. Kichise T, Taguchi S, Doi Y: Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by ***in vitro*** evolution of Aeromonas caviae PHA synthase. *Appl Environ Microbiol* 2002, 68:2411-2419.

8. Canada KA, Iwashita S, Shim H, Wood TK: Directed evolution of toluene ortho-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *J Bacteriol* 2002, 184:344-349.

9. Bosma T, Damborsky J, Stucki G, Janssen DB: Biodegradation of 1,2,3-trichloropropane through directed evolution and heterologous expression of a haloalkane dehalogenase gene. *Appl Environ Microbiol* 2002, 68:3582-3587.

10. Flores H, Ellington AD: Increasing the thermal stability of an oligomeric protein, beta-glucuronidase. *J Mol Biol* 2002, 315:325-337.

11. Murashima K, Kosugi A, Doi RH: Thermostabilization of cellulosomal endoglucanase EngB from ***Clostridium cellulovorans*** by ***in vitro*** DNA recombination with non-cellulosomal endoglucanase EngD. *Mol Microbiol* 2002, 45:617-626.

12. Hirokawa K, Kajiyama N, Murakami S: Improved practical usefulness of firefly luciferase by gene chimerization and random mutagenesis. *Biochim Biophys Acta* 2002, 1597:271-279.

13. • Bevis BJ, Glick BS: Rapidly maturing variants of the discosoma red fluorescent protein (DsRed). *Nat Biotechnol* 2002, 20:83-87.
The maturation half-life of DsRed is reduced from 11 h to less than 1 h using a combination of rational design and directed evolution. Combined with [14•], this evolved variant should provide the needed complement to GFP for co-localization and FRET experiments.

14. • Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, Tsien RY: A monomeric red fluorescent protein. *Proc Natl Acad Sci USA* 2002, 99:7877-7882.
Using a combination of rational design and directed evolution, DsRed, which is naturally tetrameric, was evolved to be a monomer. Combined with [13•], this evolved variant should provide the needed complement to GFP for co-localization and FRET experiments.

15. Yano T, Oue S, Kagamiyama H: Directed evolution of an aspartate aminotransferase with new substrate specificities. *Proc Natl Acad Sci USA* 1998, 95:5511-5515.

16. • Samuelson JC, Xu SY: Directed evolution of restriction endonuclease BstYI to achieve increased substrate specificity. *J Mol Biol* 2002, 319:673-683.
Directed evolution is used to change the substrate selectivity of the restriction endonuclease *Bst*YI from A/GGATCC/T to AGATCT. This paper illustrates the difficulty in engineering endonuclease variants with sufficient selectivity for practical applications.

17. •• Buchholz F, Stewart AF: Alteration of Cre recombinase site specificity by substrate-linked protein evolution. *Nat Biotechnol* 2001, 19:1047-1052.
The site selectivity of Cre recombinase is successfully changed to provide a useful reagent for biotechnology applications. The key to the success of this experiment is a powerful selection strategy – SLiPE.

18. • Santoro SW, Schultz PG: Directed evolution of the site specificity of Cre recombinase. *Proc Natl Acad Sci USA* 2002, 99:4185-4190.
An alternative strategy for altering the site selectivity of Cre recombinase using a fluorescence-activated cell sorting screen.

19. Naumann TA, Reznikoff WS: Tn5 transposase with an altered specificity for transposon ends. *J Bacteriol* 2002, 184:233-240.

20. Meyer A, Schmid A, Held M, Westphal AH, Rothlisberger M, Kohler HP, van Berkel WJ, Witholt B: Changing the substrate reactivity of 2-hydroxybiphenyl 3-monooxygenase from Pseudomonas azelaica HBP1 by directed evolution. *J Biol Chem* 2002, 277:5575-5582.

21. Barriault D, Plante MM, Sylvestre M: Family shuffling of a targeted bphA region to engineer biphenyl dioxygenase. *J Bacteriol* 2002, 184:3794-3800.

22. •• Wang L, Brock A, Herberich B, Schultz PG: Expanding the genetic code of ***Escherichia coli***. *Science* 2001, 292:498-500.
An orthogonal tRNA synthetase is evolved that allows an 'unnatural' amino acid to be incorporated in response to an amber stop codon *in vivo*. This paper provides an excellent example of using a combination of a positive selection and a negative screen to successfully switch enzyme selectivity.

23. • Chin JW, Martin AB, King DS, Wang L, Schultz PG: Addition of a photocrosslinking amino acid to the genetic code of ***Escherichia coli***. *Proc Natl Acad Sci USA* 2002, 99:11020-11024.
In vivo suppression of an amber stop codon is extended to *p*-benzoyl-L-phenylalanine. The selection strategy for the aminoacyl-tRNA synthetase is modified such that there are growth selections both for the new amino acid and against the natural amino acid substrate.

24. •• Xia G, Chen L, Sera T, Fa M, Schultz PG, Romesberg FE: Directed evolution of novel polymerase activities: mutation of a DNA polymerase into an efficient RNA polymerase. *Proc Natl Acad Sci USA* 2002, 99:6597-6602.
A DNA polymerase is evolved to an efficient RNA polymerase. This experiment is the first application of a reaction-independent assay for directed evolution.

25. Schultz PG, Lerner RA: From molecular diversity to catalysis: lessons from the immune system. *Science* 1995, 269:1835-1842.

26. Hilvert D: Critical analysis of antibody catalysis. *Annu Rev Biochem* 2000, 69:751-793.

27. Baca M, Scanlan TS, Stephenson RC, Wells JA: Phage display of a catalytic antibody to optimize affinity for transition-state analog binding. *Proc Natl Acad Sci USA* 1997, 94:10063-10068.

28. Conn MM, Prudent JR, Schultz PG: Porphyrin metalation catalyzed by a small RNA molecule. *J Am Chem Soc* 1996, 118:7012-7013.

29. Yamauchi A, Nakashima T, Tokuriki N, Hosokawa M, Nogami H, Arioka S, Urabe I, Yomo T: Evolvability of random polypeptides through functional selection within a small library. *Protein Eng* 2002, 15:619-626.

30. Goud GN, Artsaenko O, Bols M, Sierks M: Specific glycosidase activity isolated from a random phage display antibody library. *Biotechnol Prog* 2001, 17:197-202.

31. Copeland GT, Miller SJ: Selection of enantioselective acyl transfer catalysts from a pooled peptide library through a fluorescence-based activity assay: an approach to kinetic resolution of secondary alcohols of broad structural scope. *J Am Chem Soc* 2001, 123:6496-6502.

32. Tanaka F, Barbas CF III: Phage display selection of peptides possessing aldolase activity. *Chem Commun* 2001:769-770.

33. Koltermann A, Kettling U, Bieschke J, Winkler T, Eigen M: Rapid assay processing by integration of dual colour fluorescence cross-correlation spectroscopy: high throughput screening for enzyme activity. *Proc Natl Acad Sci USA* 1998, 95:1421-1426.

34. Pedersen H, Holder S, Sutherlin D, Schwitter U, King D, Schultz P: A method for directed evolution and functional cloning of enzymes. *Proc Natl Acad Sci USA* 1998, 95:10523-10528.

35. Liu Y, Patricelli M, Cravatt B: Activity-based protein profiling: the serine hydrolases. *Proc Natl Acad Sci USA* 1999, 96:14694-14699.

36. Olsen M, Stephens D, Griffiths D, Daugherty P, Georgiou G, Iverson B: Function-based isolation of novel enzymes from a large library. *Nat Biotechnol* 2000, 18:1071-1074.

37. Firestine S, Salinas F, Nixon A, Baker S, Benkovic S: Using an AraC-based three-hybrid system to detect biocatalysis ***in vivo***. *Nat Biotechnol* 2000, 18:544-547.

38. Baker K, Bleczinski C, Salazar-Jimenez G, Lin H, Sengupta D, Krane S, Cornish V: Chemical complementation: a reaction-independent genetic assay for enzyme catalysis. *Proc Natl Acad Sci USA* 2002, in press.

39. Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WP, Ryan CM, Del Cardayre S: Genome shuffling of Lactobacillus for improved acid tolerance. *Nat Biotechnol* 2002, 20:707-712.

40. Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WP, del Cardayre SB: Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature* 2002, 415:644-646.