Directed enzyme evolution

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Laboratory evolutionists continue to generate better enzymes for industrial and research applications. Exciting developments include new biocatalysts for enantioselective carbon–carbon bond formation and fatty acid production in plants. Creative contributions to the repertoire of evolutionary methods will ensure further growth in applications and expand the scope and complexity of biological design problems that can be addressed. Researchers are also starting to elucidate mechanisms of enzyme adaptation and natural evolution by testing evolutionary scenarios in the laboratory.

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Abbreviations

CSR	compartmentalized self-replication
FACS	fluorescence-activated cell sorting
FRET	fluorescence resonance energy transfer
GAO	galactose oxidase
HRP	horseradish peroxidase
KDPG	D-2-keto-3-deoxy-6-phosphogluconate
PCR	polymerase chain reaction
RACHITT	random chimeragenesis on transient templates
SHIPREC	sequence homology independent protein recombination

Introduction

Enzymes can perform intricate regioselective and/or enantioselective chemical transformations and can accelerate reaction rates by enormous factors (up to 10^{12}), all under mild conditions [1]. Although highly attractive for chemical synthesis, enzymes almost always present problems for use on an industrial scale [2,3]. Limitations include sluggish catalysis on nonnatural substrates, low stability or little tolerance for changes in operating parameters, poor activity in nonaqueous media, and requirements for expensive cofactors. The reliable and quick identification of amino acid substitutions that generate desired changes in enzyme performance remains the ultimate goal of protein engineering research.

Evolutionary design approaches have enjoyed considerable success in recent years [4••]. Numerous enzymes have been improved by directing their evolution in the laboratory, which usually involves iterations of random mutagenesis or recombination followed by screening or selection [5]. This review covers recent progress in directed enzyme engineering applications and methods.

Evolved biocatalysts

Carbon–carbon bond formation

D-2-Keto-3-deoxy-6-phosphogluconate (KDPG) aldolase from *Escherichia coli* provides synthetic routes for asymmetric carbon–carbon bond formation. The utility of this enzyme is limited, however, by its high specificity for its natural substrates: KDPG in the cleavage reaction and D-glyceraldehyde-3-phosphate in the addition reaction. Fong *et al.* [6•] evolved the aldolase to accept the nonphosphorylated analog of KDPG and L-glyceraldehyde. Wymer *et al.* [7] also performed directed evolution on KDPG aldolase, broadening its substrate range while retaining enantioselectivity. The crystal structure of the evolved enzyme shows that directed evolution actually relocated the key catalytic lysine residue to another position. Together, these studies open important new opportunities for using aldolases in stereoselective carbon–carbon bond formation.

Oxidation

The need for functional expression in a host suitable for making mutant libraries (usually *Saccharomyces cerevisiae* or bacteria) is a serious bottleneck that has kept several enzymes out of the evolutionist's reach. Until recently, the widely used reporter enzyme horseradish peroxidase (HRP) was one such example. Morawski *et al.* were able to improve functional expression [8] and stability [9] of HRP in *S. cerevisiae* by random mutagenesis, recombination and screening for total HRP activity. The thermostable mutant is also more stable in the presence of H_2O_2 and more active with substrates frequently used in diagnostic applications.

Sun *et al.* [10] expressed the fungal enzyme galactose oxidase (GAO) in *E. coli* and increased the total GAO activity 60-fold by random mutagenesis and screening. Slightly more thermostable than the wild-type enzyme, this mutant is a good starting point for functional improvement by directed evolution. GAO is an interesting catalyst with potential applications in synthesis, biosensors and the labeling of glycoconjugate structures. Delagrave *et al.* [11] report the optimization of GAO for yet another application, selective oxidation of the natural polymer guar, for use in paper manufacturing. Random mutagenesis by error-prone polymerase chain reaction (PCR) and screening on a surrogate substrate, methylgalactose, using solid-phase digital imaging, increased total activity up to 16-fold. Screening the library using a guar-plate assay did not generate mutants that were more active than the ones found using the surrogate substrate. Characterization of the evolved GAO expressed in *Pichia pastoris* showed an approximately threefold decrease in K_m on methylgalactose.

Directed evolution is also being use to expand the substrate ranges of monooxygenases and dioxygenases. Cytochrome P450 BM-3 hydroxylates fatty acids with chain lengths of between C12 and C18. Farinas *et al.* [12] report that P450 BM-3 also slowly oxidizes octane (C8) and that directed evolution can improve this alkane hydroxylation activity. Dioxygenases are useful for the production of

chiral dihydrodiols from a range of aromatic substrates. Sakamoto *et al.* [13] used random and saturation mutagenesis to increase the activity of toluene dioxygenase from *Pseudomonas putida* on a more polar heterocyclic substrate, 4-picoline (see also Update).

Metabolic engineering

The discovery of new natural products continues to be of great interest for the production of pharmaceuticals, new materials, agrochemicals, and consumer products. An exciting approach to generating new natural products is to evolve biosynthetic pathways in microorganisms. For example, torulene was created for the first time in *E. coli* by the recombination of genes encoding carotenoid biosynthetic enzymes followed by screening for novel compounds [14,15]. Evolutionary methods can also be applied to improving production of known compounds. Wang *et al.* [16] previously introduced the biosynthetic genes for enhanced production of isoprenoids into *E. coli*. Geranylgeranyl diphosphate synthase, found to be rate-limiting in the isoprenoid biosynthetic pathway, was targeted for directed evolution [17]. Clones with increased lycopene production of up to 100% were identified by visual screening for orange-colored colonies. One higher-producing variant had mutations only upstream from the coding region, and the enhanced lycopene synthesis was due to increased enzyme expression. Shuffling the best eight variants did not yield further improvements in lycopene production, which was attributed to the limited dynamic range of the visual screen at these high production levels (see also Update).

Metabolically engineered plants are also highly attractive for the production of modified natural products. Plant biosynthetic enzymes can be evolved conveniently in microorganisms and transferred to plants for production. Cahoon and Shanklin [18••] used this strategy to tailor fatty acid production in *Arabidopsis fab1* plants by engineering the acyl-acyl carrier protein desaturase that desaturates C18 fatty acids. Mutagenesis and selection in *E. coli* yielded variants with greater specific activity for shorter-chain C16 fatty acids. One mutant showed a 25-fold preference for C16 chains as compared with the 70-fold preference of the wild type for longer chains. Expression in the plants resulted in an accumulation of unusual monounsaturated fatty acids, which formed more than 25% of the seed oil.

Enzyme adaptation

This review period has seen several interesting applications of laboratory evolution methods to address fundamental questions of enzyme function and evolution. In the laboratory, a particular evolutionary scenario can be simulated by imposing specific selective pressures. In contrast to natural evolution, however, all the intermediates along the evolutionary trajectory are available for study to identify mechanisms of adaptation. The enzymes β-galactosidase and β-glucuronidase from *E. coli* evolved from a common ancestor. Matsumura and Ellington [19•] sought to identify the structural determinants of their substrate specificities by evolving β-glucuronidase in the laboratory to hydrolyze β-galactosides. Error-prone PCR and DNA shuffling generated a variant that hydrolyzed *para*-nitrophenyl-β-D-galactoside 500 times better than wild type and with a strong preference for this substrate over β-glucuronides. (The evolved β-galactosidase activity was still 10 000-fold less active than the natural β-galactosidase.) According to the patchwork hypothesis, modern enzymes evolved by gene duplication and diversification from ancient enzymes with broad substrate specificity [20]. Intermediate variants of β-glucuronidase showed broadened substrate specificity, and the authors propose that adaptation to new substrates was achieved by passing through a functional intermediate similar to their ancestral states [19•].

Jurgens *et al.* [21•] used directed evolution to create an enzyme that might resemble the common ancestor of two isomerases that catalyze similar reactions during the biosynthesis of histidine and tryptophan. Their laboratory-evolved HisA (*N*′-[(5′phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide isomerase) mutant has low but equal activity in both pathways, which they claim to support a patchwork hypothesis. But a single amino acid exchange in wild-type HisA isomerase was also shown to change the substrate specificity from one reaction pathway to the other. This switch of specificity without a generalist intermediate demonstrates that a specialized enzyme can also evolve directly from another specialist (see also Update).

Directed evolution can anticipate natural evolution. The development of β-lactam antibiotic resistance in microorganisms is a product of the rapid evolution of β-lactamase to catabolyze the drugs, and laboratory evolution experiments [22,23] have generated a mutant that was subsequently found in clinical isolates [22,23,24•]. In recent work, Orencia *et al.* [24•] combined directed evolution and structural analysis to elucidate the mechanism of β-lactam antibiotic resistance and its development by stepwise mutations. Using an *E. coli* mutator strain and selection on cefotaxime, they generated the same β-lactamase triple mutant (Glu104Lys, Gly238Ser, Met182Thr) whose emergence had been predicted by previous studies using high mutation rates [22] and DNA shuffling [23]. The crystal structure of the evolved β-lactamase revealed that the first two mutations improved access to the active site, whereas the third mutation (Met182Thr) compensates for the deleterious side-effects of the first ones. Understanding the emergence of antibiotic resistance will allow the prediction of mutations in the clinic and should aid in the development of new antibiotics.

Another area where evolutionary experiments have been able to shed light on ancient processes and adaptive mechanisms is adaptation to different temperatures [25–27]. Enzymes are readily evolved in the laboratory for higher

thermostability and high-temperature activity [28•,29]; activity at low temperatures can also be improved. Because relatively few amino acid substitutions (1–5% of the sequence) can convert a mesophilic enzyme into its thermophilic or psychrophilic counterpart, laboratory-evolved enzymes provide a superb, almost noise-free dataset for comparative studies to uncover adaptive mechanisms. Also of great interest is the fact that thermostability can be improved at no cost to activity at low temperature and vice versa. Naturally occurring homologous enzymes adapted to different temperature niches often show trade-offs in activity and stability, which are thought to reflect conflicting demands of enzyme flexibility. The directed evolution studies clearly show that any trade-offs are much more likely to reflect a lack of selective pressure on both properties and genetic drift [26].

The room temperature activity of a hyperthermophilic β-glucosidase from *Pyrococcus furiosis* (CelB) was improved by directed evolution [30]. After error-prone PCR, DNA shuffling, and screening on *p*-nitrophenol-β-D-glucopyranoside, mutants with up to three times more activity at 20°C than wild type were identified. 3-Isopropylmalate dehydrogenase from *Thermus thermophilus* (LeuB) was also adapted to lower temperatures [31•]. To select for enzyme function, Suzuki *et al.* [31•] replaced the *leuB* locus of *E. coli* with the genes coding for *T. thermophilus* LeuB and kanamycin nucleotidyl transferase. The resulting *E. coli* strain only grew at 42°C and above in the absence of leucine, because the dehydrogenase was inactive at lower temperatures. The strain was treated with a chemical mutagen and screened on leucine-free plates containing kanamycin at 40°C. After three days, approximately 100 colonies appeared on the plates. Sequencing *leuB* from five randomly picked colonies revealed 0–3 base substitutions per gene. One gene had no mutations at all, illustrating the potential hazards of using a growth-based selection and mutagenesis that is not limited to the gene of interest, but which instead targets the entire genome. The remaining four mutant enzymes showed increases of up to 4.1-fold in specific activity at 40°C. Two had melting temperatures similar to wild type, demonstrating that activity at lower temperatures can be acquired without sacrificing the high thermostability of the thermophilic enzyme.

Several evolution experiments have increased enzyme thermostability. A psychrophilic subtilisin from Antarctic *Bacillus* TA41 (S41) was converted into its thermophilic counterpart over eight generations [28•,32]. The final variant was as stable as a thermophilic subtilisin while retaining, and even improving, low-temperature activity towards a peptide substrate. Sequencing revealed 13 amino acid substitutions, some of which are believed to stabilize a surface loop and increase calcium affinity. Notably, six of the 13 were substitutions of serine by another amino acid. A reduced occurrence of serine in thermophilic enzymes was observed when sequences and structures were compared with their naturally occurring mesophilic homologs [33,34].

Prolyl endopeptidase from *Flavobacterium meningosepticum* cleaves specifically at proline residues; no other endopeptidase has shown this selectivity. The low stability of prolyl endopeptidase compared with other peptidases such as subtilisin and trypsin made it a target for laboratory evolution [29]. The best mutant after three cycles of random mutagenesis and screening in *E. coli* increased its half-life 60-fold at 60°C. Furthermore, the thermostable mutant retained 50% of its activity after 3 h in 50% glycerol (necessary for *in vitro* amidation), whereas the wild-type enzyme rapidly lost activity.

New methods for directed evolution

Although widely used for improving biocatalysts, directed evolution is still an emerging technology. Methods for mutagenesis and recombination are constantly developing. Identifying improved clones from a vast pool of mutants is only feasible with a sensitive screen or selection. Screening methods are of great importance, but have been reviewed recently [35–37] and will not be covered here.

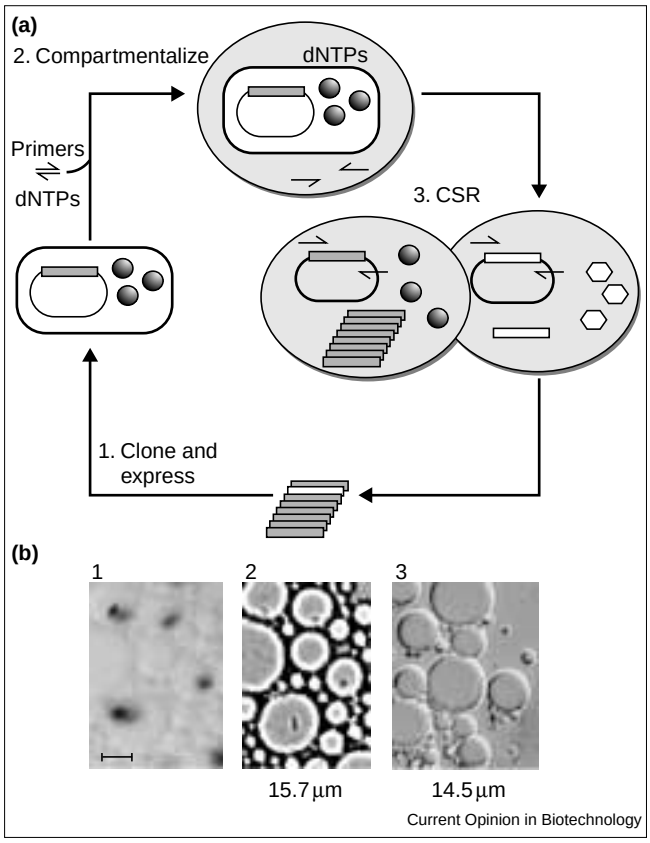
An elegant strategy called CSR (compartmentalized self-replication) has been demonstrated for directed evolution of enzymes involved in DNA replication (e.g. polymerases) [38••] (Figure 1). *E. coli* cells were used to deliver a library of *Taq* DNA polymerases into separate aqueous compartments, formed from a water-in-oil emulsion [39], which also contained flanking PCR primers and deoxynucleotide triphosphates. PCR of these samples amplified mutant *Taq* DNA polymerase genes, depending on the activity of their gene product. CSR increased the thermostability of *Taq* DNA polymerase 11-fold and enhanced resistance to heparin 130-fold, without reducing catalytic activity. CSR could be extended to the evolution of other enzymes that can couple to the gene replication reaction, for example, by providing substrates required for replication of their own genes [38••].

Cell-surface and phage-display methods have been limited mostly to evolution of binding rather than catalysis. Olsen *et al.* [40•] devised a strategy to screen large enzyme libraries displayed on bacterial cell surfaces by fluorescence-activated cell sorting (FACS). The *E. coli* cell-surface protease OmpT was evolved for novel substrate specificity using a specially designed fluorescence resonance energy transfer (FRET) substrate that allows the genotype to remain linked to the phenotype by association with the cell surface. The peptide substrate contained a fluorophore, the scissile bond, a quenching fluorophore, and an overall +3 charge that retains the fluorescent product on the cell surface. This FRET/FACS system nicely exploits the high-throughput of FACS screening for catalysis; however, limitations on surface expression of enzymes and constraints on substrate design make this approach somewhat specialized.

New methods to create enzyme libraries

The method used to create enzyme diversity determines the quality of the mutant library and strongly influences the

Figure 1



Evolution of polymerases by compartmentalized self-replication (CSR) [38••]. (a) *E. coli* cells expressing a library of polymerases are separated into heat stable, aqueous compartments of an oil-in-water emulsion together with flanking primers and deoxynucleotide triphosphates (dNTPs) (steps 1 and 2). Thermocycling lyses the cells and empties them into the individual compartments where the polymerase variants amplify their coding genes (step 3). Genes amplified by the most active polymerase variants are selectively enriched, isolated from the emulsion and subjected to the next CSR cycle. (b) Panels 1, 2 and 3 illustrate the aqueous compartments of an oil-in-water emulsion. Scale bar 10 µm; the size of the compartments is indicated below. (The figure was reproduced from [38••] with permission 2001 National Academy of Sciences USA.)

efficiency of the evolutionary search. Methods for gene diversification are far from perfect. *In vitro* recombination by DNA shuffling [41] creates libraries of chimeric genes from any number of parent sequences, including naturally occurring homologous genes. A recently published method, RACHITT (random chimeragenesis on transient templates) [42•], claims to overcome some limitations of previous shuffling protocols. In RACHITT one of the parent genes is used as a transient scaffold to which complementary fragments of the other parents anneal. Unhybridized fragment termini are trimmed and gaps are filled enzymatically. These trimming reactions have the potential to reduce the size of the shuffled fragments and increase the number of recombinations per gene. The transient single-stranded scaffold is expected to avoid amplification of parent sequences. However, the choice of the scaffold will probably influence the outcome of the experiment, as sequences that are similar to the scaffold will be incorporated more frequently into the chimeras. When used to shuffle two bacterial monooxygenases, RACHITT generated an average of 14 crossovers per gene, which is higher than that reported for other *in vitro* recombination methods. However, the benefit of such a high crossover frequency remains to be demonstrated. Coco *et al.* [42•] found 29% of their crossovers in regions of 10 or fewer bases of sequence identity; however, DNA shuffling of dioxygenases using Stemmer's approach [41] also generated a similar frequency (32%) of crossovers in regions of 10 or fewer bases (J Joern, FH Arnold, personal communication).

Another recombination method, CLERY (combinatorial libraries enhanced by recombination in yeast), combines DNA shuffling *in vitro* and *in vivo* shuffling in yeast [43•]. The procedure was used to shuffle two human cytochrome P450 enzymes. The average number of crossovers was around 4.4 and the library contained only 14% nonchimeric (parental) sequences. Requiring two hosts, this procedure is most suitable for shuffling eukaryotic genes that cannot be expressed in bacteria. A high mutation rate associated with the *in vitro* shuffling reduced the fraction of functional clones (12%). This paper also describes a useful probe hybridization method for characterizing large numbers of sequences from the chimeric library.

All the available methods for DNA shuffling require high sequence similarity for recombination (> ~60%). There is some interest, however, in shuffling parent sequences of lower identity or even with no discernible sequence homology [44,45]. During this period, Sieber *et al.* [46•] introduced SHIPREC (sequence homology independent protein recombination). To maximize the abundance of functional hybrids in a library made by recombining two homologous genes of low sequence identity, the chimeras are selected by size to retain the length of the parent genes. SHIPREC therefore combines the N-terminal fragment of the parent protein with the appropriate C-terminal fragment and, in principle, makes all such possible combinations. With a chloramphenicol acetyl transferase preselection for properly folded and soluble variants, SHIPREC was able to generate hybrids that combined the activity of a membrane-bound human cytochrome P450 enzyme with the increased solubility of a bacterial cytochrome P450. The two P450s share only 16% amino acid identity. The method as described is limited to creating libraries containing only one crossover between two parent genes.

It is proposed that proteins evolved naturally by the assembly of nonhomologous genes. Protein domains might also have developed by the exchange and assembly of gene segments [47]. One natural process that leads to nonhomologous recombination is exon shuffling. The *in vitro* recombination of domain-encoding exons might prove useful for creating enzymes with novel functions [48], although this approach would be limited to modular proteins which contribute only a small subset to the diversity of enzymes.

One shortcoming of *in vitro* mutagenesis is the limited size of the variant libraries created ($<10^9$ and usually considerably smaller). Fabret *et al.* [49•] report an *in vivo* mutagenesis method that is capable of generating large collections of mutants (10^{12}–10^{13} per litre of cell culture). In contrast to mutator strains commonly used, the *E. coli* strain employed in this approach is genetically stable. The target gene is propagated in a ColE1-type plasmid in which DNA polymerase I is essential for the initiation of replication. After replication of the initial 1 kb of the plasmid insertion, polymerase I is replaced by polymerase III, which completes the replication. The *E. coli* strain expresses a mutant polymerase I with negligible proof-reading activity, and the mismatch repair system is disabled. As polymerase I replicates only 1% of the host chromosome, the target gene was mutagenized without changing the cell phenotype. This makes it possible to cultivate the cells for a longer time, so that ~10% of the cells in the culture are mutated after 150 generations. The claim that the method can generate libraries of 10^{12} mutants is overstated, because the library diversity is limited by the low mutation rate and by the restriction that only 1 kb can be mutated. Achieving this with the reported system does not seem practical owing to the low error rate. Also because of the low mutation rate, this approach is suitable for selections but not screening (see also Update).

Computational approaches to evolution

Screening is usually the most labor-intensive part of the evolution experiment, and there is considerable value in finding ways to use structural or mechanistic information to increase the efficiency of the search for improved mutants. This will reduce the cost of obtaining a given functional improvement or, even better, lead to much greater improvements for the same amount of effort. Structural information is therefore often used to choose regions or even individual amino acids for mutagenesis. The risk, of course, is that targeting specific sites for mutation is based on limited understanding of the molecular basis for the desired function and often fails. Directed evolution experiments have shown time and time again that beneficial mutations often occur where least expected.

A new, computational approach to the problem uses powerful protein design software to identify sites on proteins that are most likely to accept mutations. Voigt *et al.* [50•] observed that sites calculated to be highly tolerant to amino acid substitution are very often the sites where beneficial mutations are found during directed evolution. If one can identify a limited number of mutation sites computationally, simultaneous saturation mutagenesis becomes feasible. This has the benefits that all possible amino acid substitutions can be accessed (rather than the 5.7 on average that are available by point-mutation [51]) and that multiple synergistic mutations can be discovered. This method requires a crystal structure or a good homology model, but does not make *a priori* decisions as to where beneficial mutations will appear (e.g. in a substrate recognition site).

Conclusions

Over the past decade, different implementations of directed evolution have improved the specific properties of a wide variety of proteins [4••], offering a robust means to transfer enzymes out of the laboratory and into industrial processes. More recently, these same approaches have provided insights into past as well as future evolutionary events. Directed evolution is merging molecular biology, chemistry and engineering to forge many opportunities for further growth.

Update

In recent work, Suenaga *et al.* [52] shuffled the biphenyl dioxygenase genes from *Pseudomonas pseudoalcaligenes* and *Burkholderia cepacia*. *E. coli* expressing biphenyl dioxygenase chimeric genes showed increased activity for monocyclic aromatic compounds (benzene, toluene and alkylbenzenes) that are poor substrates for the wild-type enzymes. Wang and Liao [53] used directed evolution to alter the product specificity of phytoene desaturase from *Rhodobacter sphaeroides* from neurosporin to lycopene. The gene was expressed in *E. coli* together with another plasmid containing the necessary isoprenoid pathway genes. Two rounds of directed evolution and visual selection of the colored colonies followed by recombination of the mutations by site-directed mutagenesis resulted in a mutant that produced 90% lycopene.

Raillard *et al.* [54•] recently published their *tour de force* study of an enzyme library made by shuffling two very closely related enzymes involved in s-triazine hydrolysis. The resulting library exhibited considerable functional diversity, including enzymes that hydrolyze substrates not accepted by either parent. 'Reverse engineering' the functional enzymes provided useful and comprehensive information on sequence–function relationships.

Using saturation mutagenesis at every site in the protein, Gray *et al.* [55•] dramatically increased the thermostability of an haloalkane dehalogenase from *Rhodococcus rhodochrous*, without compromising its activity. The enzyme also became fully reversible in its denaturation. Eight single-site mutations were combined by site-directed mutagenesis. The variant was immobilized on alumina, and the enzymatic conversion of 1,2,3-trichloropropane to 2,3-dichloropropanol in a bioreactor was investigated at 55°C. The operational productivity of the mutant (lb product/ lb of enzyme) was 25-fold greater and the half-life was 18 times longer compared with wild type.

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