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Combinatorial Catalysis

Single-Cell High-Throughput Screening To Identify Enantioselective Hydrolytic Enzymes**

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Directed evolution has emerged as a powerful tool to optimize the stability, substrate scope, and enantioselectivity of enzymes as catalysts in biotechnology and organic chemistry.[1] The availability of efficient screening or selection systems is crucial for success. Whereas selection systems in successful cases allow for the evaluation of typically 10^6–10^8 clones, screening assays can handle only small libraries, commonly not exceeding 1000 to 10000 members.[2] Screening assays, but not selection systems, have been developed for application in the directed evolution of enantioselective enzymes with the creation of biocatalysts useful in the production of chiral pharmaceuticals and plant-protecting agents.[3] Thus, only small portions of protein sequence space can be probed, which means that numerous functionally improved variants remain unidentified.

Herein we present a high-throughput screening technique for the identification and isolation of enantioselective enzymes, which is based on fluorescence-activated cell sorting (FACS). The basic idea is to label each of the two enantiomers with a different fluorescent dye (green/red). It allows 10^8 cells, and thus this number of clones generated by directed evolution, to be screened within a few hours. To illustrate our approach, we chose as the asymmetric reaction the kinetic resolution of a racemic ester, catalyzed by the esterase from *Pseudomonas aeruginosa* (EstA). Lipolytic enzymes, such as esterases and lipases, are invaluable catalysts in the (industrial) production of chiral intermediates.[4] Substrate scope and enantioselectivity have been enhanced by directed evolution, but the number of the candidates investigated regarding the new desired property is often the deciding factor.

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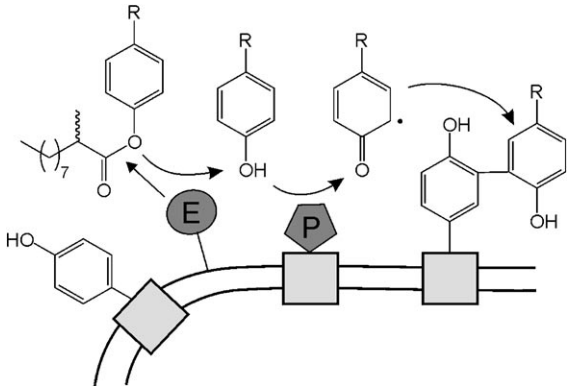
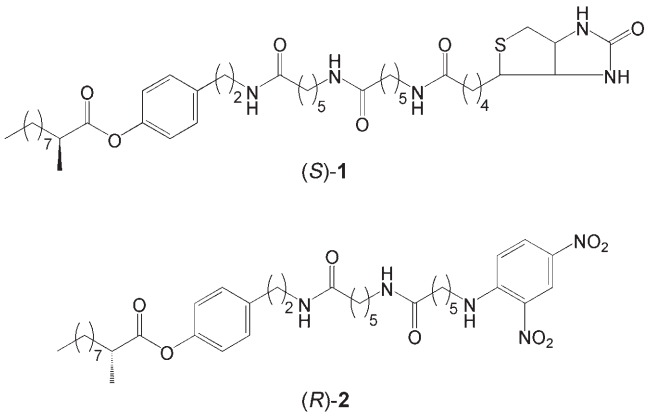
We have recently established a screening methodology to identify esterases and lipases displayed on the surface of *E. coli* cells. Tyramide ester substrates are hydrolyzed, and peroxidase-mediated radical formation ensures the immediate covalent attachment of reaction products to the surface of an esterase-proficient bacterial cell (Scheme 1).[5] After fluorescent labeling of the attached product, enzyme-reactive cells can be isolated by FACS.



Scheme 1. Representation of coupling reactions leading to covalent attachment of tyramide species to the surface of *E. coli* cells. E: esterase; P: peroxidase. R: detectable group.

This strategy was applied herein to the isolation of esterases with enhanced enantioselectivities using two different enantiomeric substrates that, upon hydrolysis, give rise to green or red cellular fluorescence (Scheme 2). The *Pseudomonas aeruginosa* esterase EstA was used as a model enzyme, as bacterial surface display of EstA in *E. coli* is well established.[6] Furthermore, the wild-type esterase shows only marginal enantioselectivity towards the *S* enantiomer of the substrate 2-methyldecanoic acid (2-MDA) *p*-nitrophenyl ester ($E_{app} = 1.2$). Esterase substrates were synthesized as tyramide esters of (*R*)- or (*S*)-2-MDA which carry as indicator groups 2,4 dinitrophenyl and biotin, respectively (Scheme 2; for the synthesis, see the Supporting Information). Substrate hydrolysis results in covalent attachment of the tyramide derivative to the cell surface by a peroxidase-mediated radical reaction. Cell labeling was performed with each substrate individually by conjugation of horseradish peroxidase to the cell surface. 2,4-Dinitrophenyl (DNP) tyramide labeling was detected with an Alexa Fluor 488-labeled anti-DNP antibody, which mediates green fluorescence, whereas biotin tyramide deposition was detected using streptavidin R-phycoerythrin conjugate (red fluorescence).



Scheme 2. Differentially labeled enantiomers of 2-MDA tyramide ester substrates.

Individually labeled cell populations were clearly distinguished by flow cytometry (Figure 1 a).

Libraries of EstA variants were generated by error-prone PCR of a DNA fragment encoding the N-terminal enzyme domain of EstA comprising amino acids 1 to 351. The resulting libraries of 6.8×10^7 and 4.0×10^7 different clones were isolated at average error rates of two and four amino acid replacements per molecule, respectively. In a first screening round, cells of the higher error-rate library were labeled with an anti-EstA antibody, followed by incubation with a biotinylated second antibody and streptavidin R-phycoerythrin conjugate. Using subsequent flow cytometric sorting, clones that contain frameshift mutations, stop codons, or entirely lack an EstA fragment were removed. The sorting gate was defined such that only those cells were isolated which displayed wild-type EstA amounts (30000 copies per cell). 1.0×10^8 cells were then subjected to two consecutive rounds of FACS (see the Supporting Information). 44% of the cells fell within the sorting window, and 1.5×10^7 EstA displaying clones were obtained. The presence of esterolytic activity in the population was confirmed by determination of octanoic acid *p*-nitrophenyl ester hydrolysis (see the Supporting Information).

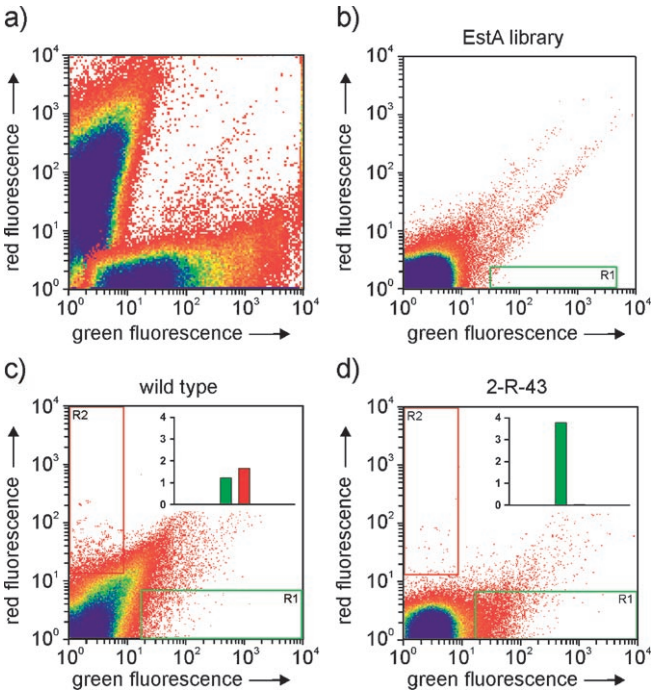


Figure 1. a) Overlay of flow-cytometry analyses of esterase-displaying cells that were incubated for 60 min with either *S* or *R* enantiomer of tyramide ester. b) EstA library sort. The green window indicates the sorting gate. c,d) FACS histogram of EstA wild-type (c) and clone 2-R-43 (d) after 5 min incubation with a 1:1 mixture of both enantiomeric substrates and fluorescence staining. The inlet shows the percentage of cells within the respective green or red gate.

Peroxidase was applied to the surface of the cells carrying the enzyme variants. A library screen for *R* enantioselectivity was performed by incubation with a 1:1 mixture of (*S*)-**1** and (*R*)-**2** followed by simultaneous labeling with streptavidin R-phycoerythrin conjugate (for detection of product formation of (*S*)-2-MDA ester hydrolysis) and Alexa Fluor 488-labeled anti-DNP antibody (for detection of product formation of (*R*)-2-MDA ester hydrolysis). Incubation time with substrates was only five minutes to eliminate variants with low activity and to avoid product formation by spontaneous ester hydrolysis. A very stringent sorting gate was defined for the enrichment of cells that carry enzyme variants with inverse enantioselectivity that preferably hydrolyze the (*R*)-2-MDA ester (Figure 1 b). From 6.8×10^7 cells that were subjected to FACS, only 86 colonies were obtained. The rate of hydrolysis of (*R*)-2-MDA and (*S*)-2-MDA *p*-nitrophenyl ester was determined for 35 individual clones, and apparent *E*-values were calculated. Of these 35 clones, four showed inversion of enantioselectivity, now preferentially hydrolyzing the *R* enantiomer. Nucleotide sequence analysis of each clone revealed one to four amino acid replacements compared to wild-type EstA (Table 1).

Clone 1-R-1, which showed the highest enantioselectivity, carried the mutation W185R, which was also identified in a second independent screen using the lower-error-rate epPCR library (data not shown). This indicates that position W185 may represent a "hot spot" for enantioselectivity of EstA. Site-saturation mutagenesis was performed at this position by a two-step overlap extension PCR using degenerated oligo-

Table 1: Enantioselectivity of EstA wild type and isolated variants.

Clone[a]	Selectivity	E_{app}	Amino acid exchanges
WT	*S*	1.2	–
1-R-1	*R*	6.7	G59S, W185R, D239S, L305P
1-R-2	*R*	3.3	L15P, A163G, P188Q
1-R-3	*R*	1.1	L150P
1-R-4	*R*	1.8	A1V, G122S, N147S
2-R-7	*R*	4.4	V184I, W185R
2-R-12	*R*	4.5	A71T, W185R, P233L
2-R-23	*R*	10.0	D169A, W185R, L249P
2-R-38	*R*	1.3	T143A, F149S, W185T
2-R-43	*R*	16.3	W185R, G224D, G263S
2-R-44	*R*	5.3	Y141H, W185R
2-R-55	*R*	5.0	W185R, E238G, A250G, F284L

[a] Clones named 1-R are the result of the error-prone PCR library screen; clones named 2-R come from site-saturation PCR at position 185.

nucleotide primers (see the Supporting Information). Sixty clones were analyzed, of which seven were *R*-selective and six carried mutation W185R together with additional mutations (Table 1). The variant showing the lowest $E_{Rapp} = 1.3$ carried the mutation W185T. These results indicate that replacement of tryptophan 185 by arginine plays a major role to shift the enantioselectivity of EstA towards the *R* substrate. All the clones we identified within the microlibrary covering amino acid position 185 carried additional mutations that most likely accumulated during PCR assembly. Variant 2-R-43 showed the highest enantioselectivity, with an E_{Rtrue} (determined by GC analysis) of 15.5 representing 81 % *ee* at 38 % conversion (see the Supporting Information). The viability of our method was convincingly demonstrated by the results shown in Figure 1c,d: EstA variant 2-R-43 was clearly distinguishable from EstA wild type in the FACS analysis using the same labeling conditions as in the initial enantioselectivity screen.

We then addressed the notion that it is mutation W185R which mainly contributes to enhanced enantioselectivity of EstA, and we additionally investigated whether expression on the cell surface had an impact on its catalytic properties. Thus, EstA wild type and variant W185R with no further amino acid substitutions were constructed by targeted mutagenesis, purified, and refolded from inclusion bodies (see the Supporting Information). Whereas isolated EstA protein again proved to be nonselective ($E_{true} \approx 1$), variant W185R displayed an E_{Rtrue} of 10, with only a slight concomitant reduction of specific activity (5.2 $U\,mg^{-1}$ vs. 4.1 $U\,mg^{-1}$ for octanoic acid *p*-nitrophenyl ester and 50 $mU\,mg^{-1}$ vs. 18 $mU\,mg^{-1}$ for (*R*)-2-MDA *p*-nitrophenyl ester), indicating that evolution of enantioselectivity does not necessarily largely compromise catalytic activity.

No previous attempts have been made to engineer EstA from *Pseudomonas aeruginosa*. However, lipase A from the same bacterial strain was extensively studied by directed evolution using epPCR, saturation mutagenesis, and DNA shuffling, medium-throughput screening being performed by a UV/Vis-based assay. This approach with iterative mutagenesis and selection of several hundred to several thousand clones per cycle led to improved enantioselectivity of the kinetic resolution of 2-methyl-decanoic acid *p*-nitrophenyl ester ($E = 1.1$ for the WT and $E = 51$ for the best mutant).[1j,2a,b] Our strategy has two major advantages: first, only those clones that display substantial enzymatic activity which is comparable to the wild-type enzyme are selected by FACS (see the Supporting Information). Second, very large libraries can be screened rapidly for enantioselective enzymes, thereby increasing the number of accessible screening events by several orders of magnitude as compared to previous *ee* screens. Therefore, the bottleneck of directed evolution of enantioselective hydrolases is no longer the screening step but the actual generation of large libraries.

In conclusion, we have developed a high-throughput screening assay for the identification and isolation of enantioselective esterase mutants from large combinatorial libraries generated by the techniques of directed evolution. Extension to other enzyme types, such as lipases, should be straightforward, as the bacterial surface display of lipases with biotechnological relevance using the same approach has been reported.[6b] At present, this technique is restricted to the enantioselectivity screen for hydrolysis of tyramide esters of chiral carboxylic acids by esterases, or for oxidation of chiral phenol derivatives by peroxidases, which has been reported previously.[7] It will be interesting to see how this technique compares to in vitro approaches that allow similar throughput,[8] and whether it can be extended to enzymes other than esterases or peroxidases by direct or indirect coupling of enzyme activity/enantioselectivity to the formation of hydrogen peroxide or tyramide.

Our results are also relevant with regard to the recent claim that massive mutagenesis might be the method of choice to introduce high functional diversity in a given library.[9] This requires high-throughput screening of large numbers of clones, as only a tiny fraction of variants that may contain over 20 mutations per gene can be expected to display a functional enzyme, a requirement that can be fulfilled by our method. The method can also be expected to be useful in iterative saturation mutagenesis when considering sites in the enzyme comprising more than four amino acid positions.[10]

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