Protein Engineering

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A Combination of In Vivo Selection and Cell Sorting for the Identification of Enantioselective Biocatalysts**

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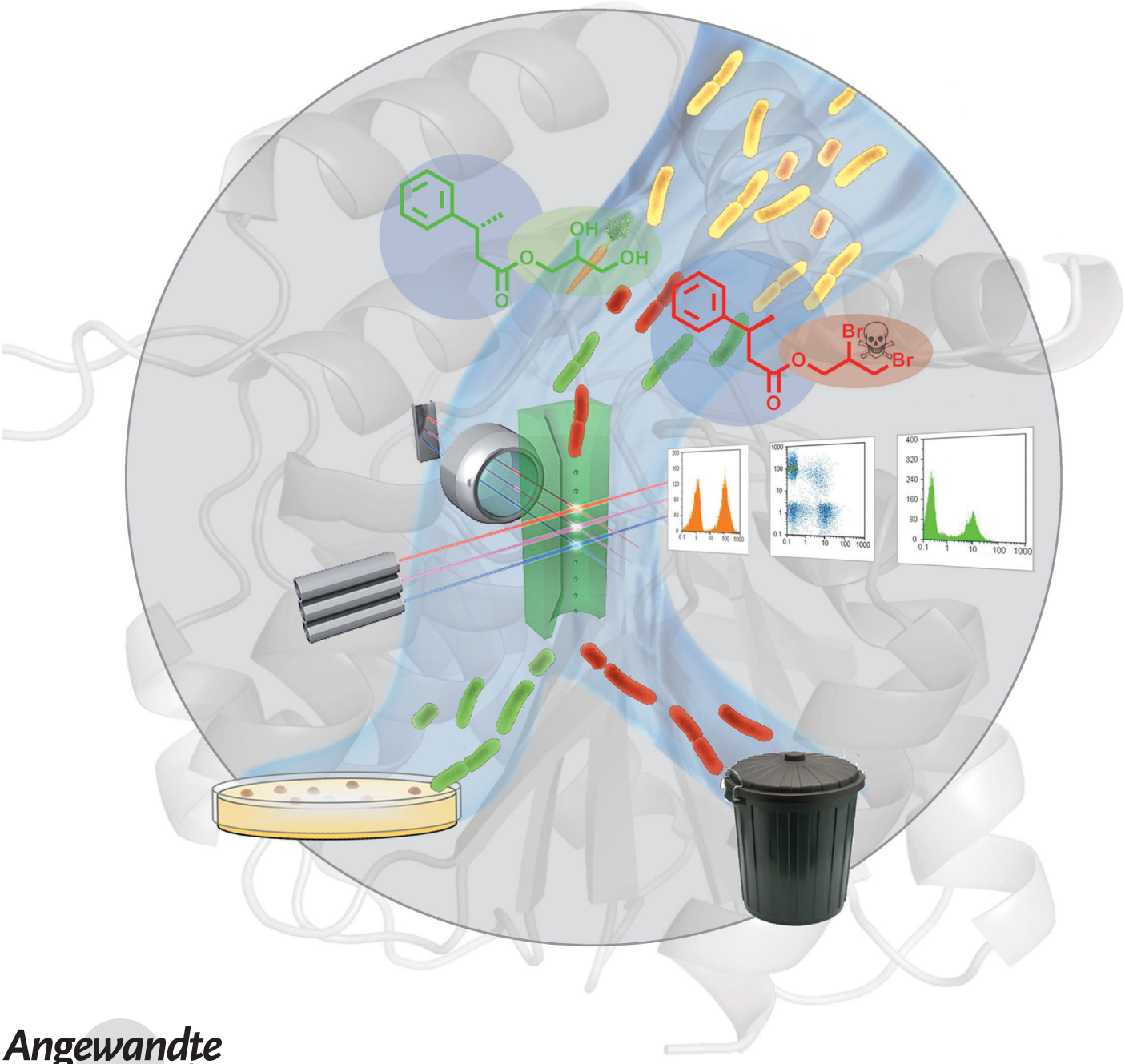

Protein engineering is an important methodology to investigate and understand the basic function of proteins, but especially to change their properties as enzymes are frequently used in biocatalysis.[1] Rational protein design (in which distinct amino acid substitutions are introduced guided by computer modeling based on the 3D structure of the protein) and directed evolution (the creation of random mutant libraries followed by screening or selection to identify desired variants) are the two major concepts used for protein engineering. Whereas rational design is limited by the available information (such as, structure, knowledge about mechanism, substrate binding mode), directed evolution approaches are often hampered by the huge sequence space making high-throughput screening or selection methods a necessity.[2] Screening is usually performed in 96-well microtiter plates and hence most researchers usually analyze only a few thousand clones per directed evolution round. Selection methods allow for a much higher throughput (10^5–10^8 clones), but are mostly restricted to problems, where complementation of a key step in the metabolism needs to take place. One exception is the use of in vitro compartmentalization (IVC) or single-cell compartmentalization in combination with fluorescence activated cell sorting (FACS),[3] but this requires in vitro protein biosynthesis of the enzyme of interest (in case of IVC), stable substrates over the entire procedure, and the generation of a fluorescent product. Moreover, only enzymes with novel activities can be discovered, but the identification of variants with improved properties is difficult to establish with this system. Another recently published alternative is the use of cell surface display in combination with FACS.[4] Although this method could successfully be used to identify more enantioselective variants of an esterase or lipase, the required biotin tyramides are laborious to synthesize and the protein must be processed and displayed in an active form on the surface of the cell.[4]

Another limitation in typical directed evolution experiments is that all variants generated in a library need to be investigated. In some cases only the active mutants are studied, but still the majority of them do not possess the desired property and hence time and consumables are spent on them. A genetic selection system would enable this limitation to be overcome and allow the investigation of a much larger sequence space. In previous work, we could successfully establish an agar plate based selection method to identify variants of an esterase from *Pseudomonas fluorescens* (PFE) capable of hydrolyzing a sterically hindered 3-hydroxyester. Active mutants can be identified by either enhanced growth (due to release of carbon source; glycerol) or by monitoring of the pH shift (due to release of acid).[5]

Possibly the most useful property of enzymes in biocatalysis is their enantioselectivity (E value). However, enzymes often do not display the desired enantioselectivity towards industrially interesting chiral compounds. Consequently improvement of enantioselectivity[6] or even inversion of enantiopreference[7] have been extensively studied by directed evolution, but solely by screening in microtiter plates. Alternatively, inhibition of enzymes by enantiomers of sulfoxides was used for enantioselective screening, as one enantiomer was a better inhibitor than the other.[8] Selective binding of antibodies was also described as principle to determine the enantiomeric excess.[9]

One concept to establish an in vivo selection system to discover mutants with altered enantioselectivity is based on linking the survival of the microbial host with one enantiomer and to cause cell death by the opposite enantiomer. Thus, with this "carrot and stick" approach desired variants should, in principle, be accessible because only surviving cells need to be further analyzed. Reetz and Rüggeberg could show that for yeast strains in the presence of either an acetic acid ester of pantolactone (supporting growth) or a fluoroacetic acid ester of pantolactone (causing cell death) differential growth occurs, but no improvement in *ee* value was reported.[10] In a follow-up study, they could further refine the principle and identified variants of lipase CAL-B with slightly inverted but not high enantioselectivity towards 1,2-*O*-isopropylidene glycerol (IPG, WT (wild type): $E = 1.9$, favoring the (*R*)-enantiomer to mutants with $E = 3$–8 favoring the (*S*)-enantiomer).[11] Similarly, Quax and co-workers[12] investigated a lipase from *Bacillus subtilis* using IPG, but linked either to aspartate or a phosphonate. Release of the amino acid supported growth of an aspartate auxotroph *E. coli* strain whereas the phosphonate lead to inhibition of the lipase. They also reported a mutant with inverted enantioselectivity (up to 73% *ee*); improved *E*-values were not reported.

In both examples, the throughput was very low (80 or 2500 colonies, respectively) as selection was still based on agar plate screening. In addition, the success was only partial, as the mutants identified displayed only moderate enantioselectivity ($E < 10$).

Herein, we report an in vivo selection method coupled with flow cytometric[13] analysis to allow ultra-high throughput identification of esterase variants with altered enantioselectivity in the kinetic resolution of 3-phenyl butyric acid by coupling one enantiomer of the carboxylic acid to either glycerol—serving as carbon source—or 2,3-dibromopropanol[14]—serving as toxic compound (Scheme 1). The use of this pair has the additional advantage, that both alcohols are highly similar with respect to steric demands and hence the important requirement "you get what you screen for" for successful directed evolution experiments is fulfilled too. Further advantages of our selection system are that no auxothroph *E. coli* strains are needed and the substrates are cleaved by intracellularly expressed enzymes; hence a surface display technique is not required.

Incubation of this pseudo-racemic mixture with *E. coli* liquid cultures containing the esterase mutant libraries should

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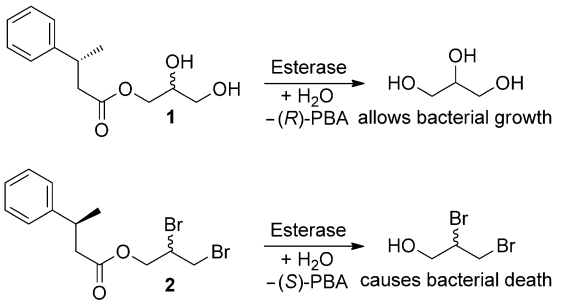

Scheme 1. General scheme for the in vivo selection concept. Hydrolysis of **1** generates the carbon source glycerol supporting bacterial growth whereas hydrolysis of **2** releases 2,3-dibromopropanol resulting in cell death. PBA: 3-phenyl butyric acid.

enable facilitated growth of only those *E. coli* expressing highly selective esterases, while clones with non-selective esterase die or at least show restricted growth. In combination with cell viability measurements, only the desired clones are then sorted out by FACS analysis. Thus, evolutionary pressure in a Darwinian sense of "survival of the fittest" is established in an in vivo liquid culture system.

For the validation of this concept we used several esterases displaying different enantioselectivities towards the model substrate 3-phenyl butyric acid (see Supporting Information). Esterase BS2[15] served as non-enantioselective ($E \approx 1$–3) control, while esterases PestE[16] and CL1[17] exhibit high enantioselectivity ($E > 100$, (*R*)-preference, see Supporting Information) and served as positive control. Next, preliminary experiments were performed to investigate the growth behavior of *E. coli* expressing these esterases in medium supplemented with either (*R*)-**1**/(*S*)-**2** (selection medium) or (*S*)-**1**/(*R*)-**2** (anti-selection medium) as substrates (see Supporting Information).

The selection media contained 5 mM **1** and 20 mM **2**. Compound **1** could not be added in higher concentration because inhibition of *E. coli* growth was observed. The concentration of **2** was optimized to achieve complete growth inhibition in case of non-enantioselective enzymes.

As expected from its (*R*)-preference, only in the culture supplemented with (*R*)-**1**/(*S*)-**2** substrate pairs and expressing PestE or CL1 bacterial growth was observed (Figure 1 c and Supporting Information). The control strain expressing the non-selective esterase BS2 was unable to grow (Figure 1 d and Supporting Information).

Flow cytometry using fluorescent dyes (propidium iodide and Syto9) was applied to evaluate the viability of the cells. Figure 1 shows the principle with dual staining of cultures expressing PestE (Figure 1 c) and BS2 (Figure 1 d) esterases in medium supplemented with (*R*)-**1**/(*S*)-**2**-pairs after 24 h incubation. Controls were established to differentiate between cells with intact membrane (Figure 1 a, viable *E. coli* cells, without esterase expression) and non-intact membrane (Figure 1 b, dead *E. coli* cells). Mixtures of the control esterases (BS2 together with either PestE or CL1) were incubated in selection medium, and after flow cytometry and cell sorting of the viable population, a clear enrichment of

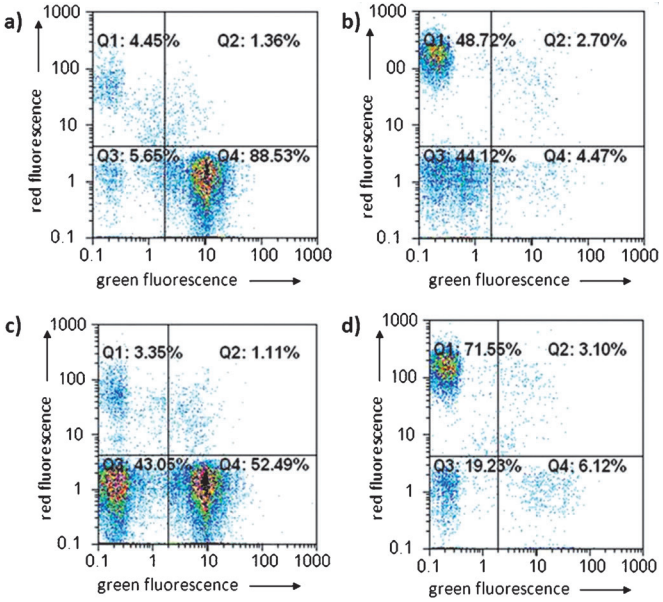

Figure 1. Syto9/PI staining of cultures to differentiate between viable and dead *E. coli* cells. Q1: dead cells; Q2: cells in intermediate status; Q3: background; Q4: viable cells; a) cells from exponentially grown culture (viable cells). b) cells after treatment at 95 °C for 15 min (dead cells). c) PestE culture grown in selection medium. d) BS2 culture grown in selection medium.

cells expressing the enantioselective esterase was observed (see Supporting Information).

PFE was chosen as a model catalyst for the creation of a mutant library, as it has a low *E* value towards the target substrate ($E = 3.5$ towards the ethyl ester, favoring the (*R*)-enantiomer)[18] and important amino acid substitutions to increase the enantioselectivity of this enzyme towards carboxylic acids have been described.[19] Thus, an esterase mutant library[20] was constructed by site-directed mutagenesis using degenerate primers, and transformed into *E. coli* JM109-(DE3). The whole PFE library was expressed in one liquid culture and afterwards 10^8 clones were washed and incubated in the selection or anti-selection media for 24 h. Viable cells expressing mutated and presumably selective PFE variants were sorted out by the cell sorter, spread onto agar plates and 28 clones were able to grow using the (*R*)-**1**/(*S*)-**2** pair. A few false positive clones found contained truncated and inactive esterase variants and they could be excluded by a prior enrichment using (*R*,*S*)-**1** and subsequent flow cytometric sorting.

The 28 selected clones were cultivated in 96-well microtiter plates and prescreened for activity using racemic 3-phenyl butyric acid *p*-nitrophenyl ester.[21] Four different clones were further characterized in the kinetic resolution of racemic ethyl- and glycerol esters of 3-phenyl butyric acid using crude cell lysate or purified enzyme and *E* values were calculated from chiral HPLC (or GC) analysis (Table 1).

As expected from the selection design, all four mutants had (*R*)-enantiopreference. Variants E8 and F5 exhibited high enantioselectivity towards the glycerol ester used in the selection and excellent *E* values for the ethyl ester, which is the compound used in kinetic resolution in biocatalysis.

Table 1: Results from biocatalysis with selected PFE mutants towards the glycerol (**1**) or the ethyl ester (**3**) of 3-phenylbutyric acid.

Mutant	$E^{[a]}$	$E^{[b]}$	$E^{[c]}$	$E^{[d]}$	Mutations
C4	4	4	3	1	V121I, F198G, V225 A
E7	2	n.d.	3	n.d.	V121S
E8	25	16	50	>100	V121S, F198G, V225 A
F5	13	16	18	80	V121I, F198C

[a] Towards **1** with crude cell lysate or [b] purified enzyme. [c] Towards **3** with crude cell lysate or [d] purified enzyme. $\%ee_S$ (enantiomeric excess of substrate) and $\%ee_P$ (enantiomeric excess of product) values were determined by HPLC. n.d. not determined owing to enzyme instability after purification.

Clones C4 and E7 showed only low enantioselectivity in the same range as the wild-type enzyme.

In conclusion, we have developed an in vivo selection system in combination with cell sorting which is highly useful for the discovery of enantioselective enzymes. By applying a mixture of pseudo-enantiomers—one acting as a potential energy source for the host organism and the opposite as a potential growth inhibitor—*E. coli* clones harboring the desired enantioselective esterases were identified through growth and cell sorting. These were confirmed by subsequent kinetic resolutions as exemplified for 3-phenyl butyric acid. With appropriate selection substrates, this methodology should be generally applicable to other compounds or enzyme classes.

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[21] E_{app} values (apparent E values) determined using optically pure (*R*)- and (*S*)-esters of the *p*-nitrophenol(*p*NP) esters differed substantially from the E_{true} values (true E values) determined by kinetic resolution of the racemic glycerol or ethyl esters. This difference can be due to the known discrepancies, which can occur between E_{app} and E_{true} values (L. E. Janes, R. J. Kazlauskas, *J. Org. Chem.* **1997**, *62*, 4560–4561) and because with substrates **1** and **2** we selected substrates structurally close to the ethyl ester **3**, but significantly different from the *p*NP esters confirming the "you get what you screen for" rule.

