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Directed evolution of stereoselective enzymes based on genetic selection as opposed to screening systems

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Dedicated to Karl-Erich Jaeger on the occasion of his 60th birthday.

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ABSTRACT

Directed evolution of stereoselective enzymes provides a means to generate useful biocatalysts for asymmetric transformations in organic chemistry and biotechnology. Almost all of the numerous examples reported in the literature utilize high-throughput screening systems based on suitable analytical techniques. Since the screening step is the bottleneck of the overall procedure, researchers have considered the use of genetic selection systems as an alternative to screening. In principle, selection would be the most elegant and efficient approach because it is based on growth advantage of host cells harboring stereoselective mutants, but devising such selection systems is very challenging. They must be designed so that the host organism profits from the presence of an enantioselective variant. Progress in this intriguing research area is summarized in this review, which also includes some examples of display systems designed for enantioselectivity as assayed by fluorescence-activated cell sorting (FACS). Although the combination of display systems and FACS is a powerful approach, we also envision innovative ideas combining metabolic engineering and genetic selection systems with protein directed evolution for the development of highly selective and efficient biocatalysts.

1. Introduction

Chiral compounds play a dominant role in the production of therapeutic drugs, fragrances, plant-protecting agents and special materials. The ecologically and economically most viable approach to their synthesis utilizes some form of catalysis (Blaser and Schmidt, 2004). In addition to asymmetric syntheses exploiting synthetic chiral catalysts, the use of enzymes in organic chemistry and biotechnology is continuing to gain importance (Drauz et al., 2012). Many of the traditional problems regarding the often observed limited substrate scope (rate), poor stereo- or regioselectivity and/or insufficient stability can be addressed and generally solved by directed evolution (Chaparro-Riggers et al., 2007; Tracewell and Arnold, 2009; Turner, 2009; Lutz and Bornscheuer, 2009; Bornscheuer et al., 2012; Goldsmith and Tawfik, 2012; Reetz, 2013).

Directed evolution is a protein engineering method based on iterative cycles of gene mutagenesis, expression and screening or selection. An improved mutant (hit) is then used as a template for the subsequent cycle, and so on, until the desired degree of biocatalyst improvement has been reached. Many researchers differentiate between screening and selection in a specific way as illustrated in Scheme 1. When screening is employed, the necessary linkage between genotype (DNA encoding an enzyme variant) and phenotype (enzyme activity/stereoselectivity) is ensured in appropriately designed systems. Accordingly, individual bacterial colonies on agar plates are harvested and subsequently expressed in the wells of microtiter plates, followed by high-throughput screening of the transformants using an analytical technique such as UV/vis spectroscopy, fluorescence, multiplexing mass spectrometry (MS), automated GC or HPLC (Reetz, 2003; Reymond, 2006). In the case of stereoselectivity, the enantiomeric excess (*ee*) or the selectivity factor *E* constitute the information of interest.

Genetic selection instead of screening is an appealing alternative, which has been defined by most researchers in a specific way (Lin and Cornish, 2002; Taylor et al., 2001; Aharoni et al., 2005; van Sint Fiet et al., 2006). In this case the system is constructed in a manner that ensures growth or survival advantage of the host organism as a consequence of harboring an enzyme with a desired (evolved) catalytic profile such as activity or thermostability. One of many early examples pertains to the original establishment of DNA shuffling as a mutagenesis method in directed evolution, in which the activity of *Escherichia coli*-producing TEM-1 β-lactamase as the

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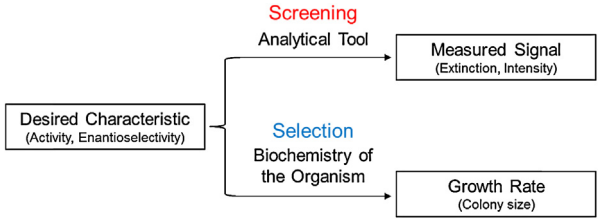
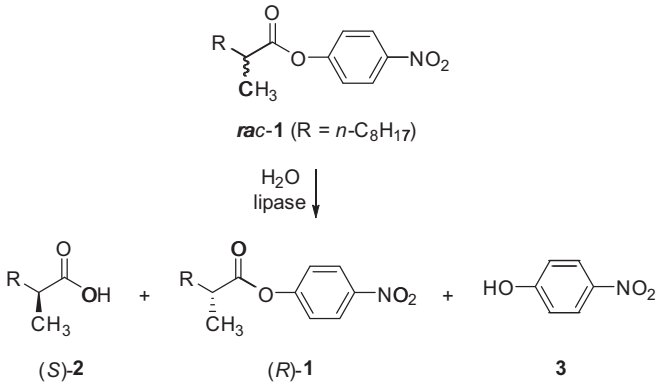


Scheme 1. Illustration of screening versus selection in directed evolution.



Scheme 2. PAL-catalyzed hydrolytic kinetic resolution of *rac*-**1** used in the original directed evolution study (Reetz et al., 1997). Compound **3** is UV/vis-active at 410 nm and can be detected easily by a plate reader.

catalyst in the inactivating hydrolysis of the antibiotic cefotaxime was increased stepwise, selection being based on the antibiotic resistance (Stemmer, 1994). An early example of selection on agar plates in the quest to generate active esterase mutants was based on a hydrolysis reaction which liberates glycerol as a carbon source (Bornscheuer et al., 1998). In another study, selection was used in the directed evolution of a (β,α)8-barrel enzyme to catalyze related reactions in two different metabolic pathways (Juergens et al., 2000). Yet another typical investigation describes the directed evolution of an aspartate aminotransferase with extended substrate acceptance in which the selection system makes use of the auxotrophy of an *E. coli* strain deficient in the branched-chain amino acid transferase gene *ilvE* (Yano et al., 1998). The enzyme catalyzes the final step of branched-chain amino acid biosynthesis, which means that the *ilvE*-deficient strain cannot grow on a minimal plate in the absence of the supplement valine, isoleucine and leucine; this forms the selection basis of active mutants. The idea of using auxotrophic strains that grow only when a (mutant) enzyme is generated, which replaces a missing cellular protein has been implemented in other studies as well. However, in those cases even low enzyme activity may be sufficient for cell growth. One way to address this problem is to tune transcription with an enzyme-degradation tag in order to reduce intracellular protein concentrations from high to low levels (Neuenschwander et al., 2007). The concept was successfully applied to a chorismate mutase.

Yet another selection method was cleverly developed in the successful attempt to increase the activity of an *N*-acyl amino acid racemase (NAAAR) by directed evolution in order to enable the efficient dynamic kinetic resolution of amino acids (Baxter et al., 2012). The actual enantio-differentiating step from a rapidly racemizing mixture of chiral *N*-acyl amino acids was ensured by a D-acylase known to be highly stereoselective. Selection for improved NAAAR variants was achieved by linking racemization rate to the viability of the *E. coli* host, which was accomplished by disabling its natural L-methionine biosynthetic pathway while also eliminating a D-amino acid racemization pathway. The combination of improved NAAAR and known D-acylase proved to be an excellent system for dynamic kinetic resolution of several different amino acids at an industrially practical level. Finally, a related method based on metabolic engineering and directed evolution was reported to increase the selectivity of an NADPH-dependent homophenylalanine dehydrogenase (Li and Liao, 2013).

In spite of these and other impressive achievements, genetic selection is not as general as one would like it to be, and for some enzyme types it is difficult to construct such experimental platforms. Devising selection systems for laboratory evolution directed toward stereoselectivity, the subject of this review, is a particularly difficult challenge.

A somewhat different directed evolution approach is based on (surface) display (microbial) systems in combination with such instrumental tools as fluorescence-activated cell sorting (FACS) (Becker et al., 2004; Aharoni et al., 2005; Farinas, 2006; Levin and Weiss, 2006; Yang and Withers, 2009; Diamante et al., 2013). As delineated previously (Yang and Withers, 2009), FACS can be used in four different situations: (1) cell surface display of active enzymes (e.g., Olsen et al., 2000); (2) reporting enzyme activity with green fluorescent protein (e.g., Santoro and Schultz, 2002; Schuster et al., 2005); (3) entrapment of products within cells (e.g., Aharoni et al., 2006; Griswold et al., 2005); and (4) *in vitro* compartmentalization as in the "oil in a droplet" approach (Griffiths and Tawfik, 2006; Mastrobattista et al., 2005). Although such techniques are sometimes called "selection systems", they are essentially a type of screening. Irrespective of the exact definition, FACS makes possible the rapid analysis of 10^7–10^8 single cells within a few hours. This allows vast portions of protein sequence space to be assessed in a single mutagenesis experiment, which in principle may cover the mutational diversity produced successively in several conventional iterative mutagenesis steps. The present review also includes those studies that have utilized these techniques in the directed evolution of stereoselective enzymes.

2. Screening systems for directed evolution of stereoselective enzymes

Before turning to the survey of genetic selection systems used in directed evolution of stereoselective enzymes, it is instructive to consider the pros and cons of screening. The first example of directed evolution of an enantioselective enzyme concerns the hydrolytic kinetic resolution of *rac*-**1** catalyzed by the lipase from *Pseudomonas aeruginosa* (Scheme 2) (Reetz et al., 1997). Since no medium- or high-throughput screening systems for enantioselectivity existed at the time, an assay utilizing a UV/vis plate reader was developed, which measures the time-dependent formation of *p*-nitrophenolate (**3**) and allows up to 700 samples per day to be pre-screened on 96- or 384-well microtiter plates. The indicated hits were then subjected to kinetic characterization for ascertaining the exact enantioselectivity as measured by the selectivity factors (*E*). Four cycles of error-prone polymerase chain reaction (epPCR) enhanced enantioselectivity stepwise from $E = 1.2$ (wild-type, WT) to $E = 11.3$. This practical assay has been used ever since in the directed evolution of lipases and esterases, some studies involving up to 50,000 samples (transformants) (Reetz, 2004). Yet it suffers from the fact that the *p*-nitrophenolate part of ester **1** constitutes a surrogate, which would not be employed in real applications. The respective methyl or ethyl ester would be the preferred substrate which often requires a mutant different from the one evolved for the reaction of the surrogate substrate. Many other surrogate substrates containing UV/vis- or fluorescence active chromophores have been devised to suit a variety of different screening needs (Reymond, 2006), yet all of them suffer from the same disadvantage. This limitation does not pertain to the use of