Assembly of Designed Oligonucleotides as an Efficient Method for Gene Recombination: A New Tool in Directed Evolution

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A new and practical method for gene recombination with formation of libraries of mutant genes is presented. The method is based on the assembly of appropriately prepared oligonucleotides whose design is guided by sequence information. High recombination frequency with formation of full-length products is achieved by controlled overlapping of the designed oligomers. This process (ADO) minimizes self-hybridization of parental genes, which constitutes a significant advantage over conventional family shuffling as used in the directed evolution of functional enzymes. ADO was applied to the recombination of two lipase family genes from Bacillus subtilis *(LipA and LipB). In a library of 3000 lipase variants created by this method, several were found that display increased enantioselectivity in a model reaction involving the hydrolysis of a* meso*-diacetate.*

KEYWORDS:

asymmetric catalysis · directed evolution · enzymes · mutagenesis · oligonucleotides

Introduction

Directed evolution of functional proteins has emerged as a powerful alternative to traditional forms of protein engineering such as structure-based site-specific mutagenesis.[1] Directed evolution involves multiple cycles of random gene mutagenesis and expression, followed by high-throughput screening (or selection); knowledge of the structure or mechanism of the enzyme is not necessary. We recently demonstrated that the methods of in vitro directed evolution can be applied successfully in the quest to create enantioselective enzymes for use in synthetic organic chemistry.[2] Specifically, highly enantioselective lipases were evolved as catalysts in the kinetic resolution of a chiral ester. Other examples have since followed.[3] This fundamentally new approach to asymmetric catalysis promises to have considerable practical significance, for example, in the synthesis of chiral intermediates for therapeutic drugs.[4]

The success of any attempt to apply directed evolution of proteins depends upon the availability of efficient mutagenesis methods[1] and rapid high-throughput screening systems.[5] A number of novel mutagenesis methods have been developed and applied in recent years,[1] such as error-prone polymerase chain reaction (epPCR), saturation mutagenesis, and cassette mutagenesis. Although epPCR is applied widely, it is not a truly random process because only single bases are replaced within the triplet codons, which results in amino acid bias and therefore in limited diversity.

In earlier work the idea of assembling DNA sequences from oligodeoxyribonucleotides (oligos) found application in DNA synthesis and in mutagenesis (in vitro). For example, ligation was first described as a method to assemble DNA sequences from oligos in the 1970s,[6] followed by the FokI method of gene synthesis[7] and the process of self-priming PCR.[8] Another advancement was made by the introduction of DNA shuffling, a method of in vitro recombination that results in the formation of gene libraries from random fragments, which in turn are generated by partial DNaseI digestion of appropriate "starting" genes.[1c, 9] Relatively high diversity is achieved, although reassembly relies on homologous recombination during the PCR step, which precludes the creation of crossovers between genes at loci of low homology. The most severe bias toward parental recombination is believed to occur when sequence identity is less than 70%. Recently, Arnold showed that conventional family shuffling methods lead to high background self-hybridization patterns that do not reveal any crossovers among the parental genes.[10] According to these results, at least 20% of the created library consists of redundant parental background, which makes the need to screen large libraries inevitable.

Further developments concerning shuffling led to the use of mixtures of oligos and random gene fragments in the form of combinatorial multiple cassette mutagenesis [2e, 11] and of single-step assembly of genes and entire plasmids from large numbers of oligos.[12] A different approach, the so-called staggered extension process (StEP) was developed, that does not involve DNA cleavage.[13] This method relies only on PCR, but requires high homology. The problem of low recombination rate as a consequence of insufficient homology was addressed by several

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research groups. The method of degenerate oligonucleotide gene shuffling, for example, does not involve gene cleavage and was recently introduced in order to enhance the frequency of recombination in family shuffling.[14] However, the authors state several disadvantages, which include limited diversity and the necessity to use a large number of primers, which complicates the PCR process.[14] Another interesting concept is the creation of hybrid proteins by in vitro exon shuffling.[15]

In order to recombine family genes with low, or without any, sequence homology, Benkovic and co-workers developed a combinatorial approach to create hybrid proteins independent of DNA homology, termed ITCHY.[16] Later, several variations such as Thio-ITCHY, TV-ITCHY, CP-ITCHY and SCRATCHY were also reported.[17] The Arnold group developed a related homology independent protein recombination method called SHIPREC.[18] Their methodology leads to fusion of parental genes in a relatively nonbiased manner, not based on homology. However, only two parental genes can be recombined and the created hybrids are limited to one crossover point per library member.

A more effective family shuffling method that uses single-stranded DNA (ssDNA) was developed by Harayama in order to increase the recombination rate.[19] In comparison to normal double-stranded DNA shuffling, chimeric genes were obtained at a 14-fold higher frequency. However, the process of making ssDNA needs special phage techniques. Another method termed RACHITT also takes advantage of single-strand hybridization.[20] As opposed to the outcome of the thermocycling steps used in PCR-based methods, the results of RACHITT demonstrate a high recombinant rate compared to conventional DNA shuffling.

Most recently, a useful method, termed random insertion/deletion (RID), for the introduction of codon-based mutations at random positions along the entire range of a gene was developed by Sisido.[21] RID allows an arbitrary number of consecutive bases to be deleted at random positions. At the same time, random sequences of an arbitrary number of bases can be inserted into the same position. Anchor ligation to ssDNA with an unknown terminal sequence was successfully carried out by using an anchor with a 10-base random tail.[21]

The present study was initiated with the aim of developing a new and practical method for gene recombination with a high frequency rate that can also be applied to genes with low homology and that results in libraries of mutant genes. A major goal was to minimize self-hybridization of parental genes in relevant cases, which would constitute a significant advantage over conventional family shuffling. Instead of cleaving and recombining two or more family genes, we rely on the assembly of appropriately prepared oligonucleotides whose design is guided by sequence information. Controlled overlapping of these oligos leads to the full-length product with high recombination frequency. The assembly of designed oligonucleotides (ADO) is illustrated by its application to the recombination of two lipase family genes from *Bacillus subtilis* (LipA and LipB). Moreover, we show that in a library as small as 3000 lipase variants created by this method, several were found that show increased enantioselectivity in a model reaction involving the hydrolytic desymmetrization of a *meso*-diacetate.

Results and Discussion

Description of the method

The ADO method is best illustrated by considering a process equivalent to the recombination of two genes of the same family, with formation of a library of mutant genes, although the technique is not restricted to this simplest case. Instead of using the actual genes in a cleavage process, we utilize the information stored by the sequences. This allows for the design and preparation of appropriate oligos, which can then be assembled. In order to assemble the full-length oligo fragments, conserved areas need to be identified. This is achieved by gene alignment.

Figure 1 illustrates the general concept. Two strategies of assembly can be used, depending on the homology between the sequences. In case I, the two genes A and B to be virtually recombined are aligned; the different colored stars in the figure refer to different encoded amino acids. Oligonucleotide fragments shown with both colored stars in the same position of the parent gene denote the synthetic oligonucleotide fragment with

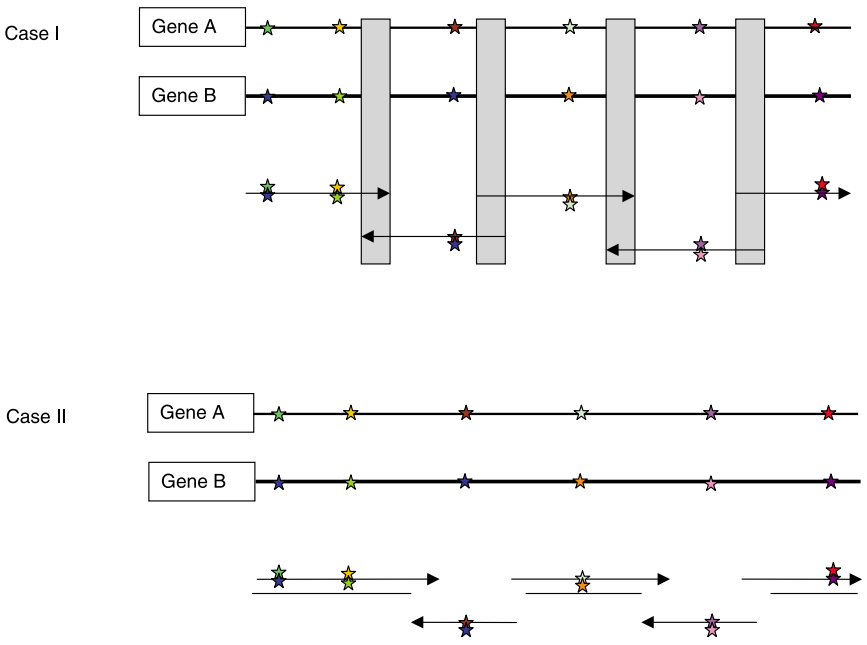


Figure 1. *General concept of ADO. Two strategies for the linking of fragments are possible: Case I: the two genes A and B to be virtually shuffled are aligned. The different colored stars refer to codons that encode different amino acids, while oligonucleotide fragments with both colored stars in the same position of the parent gene denote the synthetic oligonucleotide fragment with degenerate nucleotides. The gray blocks denote conserved regions of sequence that can be used as links for homologous recombination. Case II: there is no homology between flanking oligos, which can be assembled by ligation between ssDNA strands with unknown terminal sequences.*

degenerate nucleotides. The gray blocks denote conserved regions of sequence, which can be used as linkers in homologous recombination. In case II, there is no homology between flanking oligos. The ligation method of Sisido[21] used for ssDNA with an unknown terminal sequence can be adapted to extend the oligos. The double and single strands with colored stars in this case refer to designed oligos according to gene alignment.

The BLAST computer program can be used to align two genes.[22] The software ClustalW is available from the European Bioinformatics Institute[23] for alignment of more than two genes. In both cases, default parameters are used. Thereafter, oligonucleotide fragments are designed according to the protein sequence alignment and the following guidelines. If the amino acid residues encoded in the two sequences are the same, then the encoding nucleotide is the same and is selected in accordance with the gene sequence. If the amino acid residues are different at a given position, then degenerate nucleotides are used. This strategy is related to long degenerate PCR primer design, which means that PCR primer design software can also be applied in a modified form.

Normal PCR primer length ranges between 15 and 70 bps, whereas in our method, any length of oligonucleotide can be designed to any length, as long as it can be synthesized. Commercially synthesized oligonucleotides usually do not exceed 140 bps. However, if a conserved sequence exists between two flanking fragments, then efficient assembly is to be expected. Otherwise fragment ligation is necessary.

The design of the oligonucleotides is accomplished in the following way. The amino acid sequences are divided into five blocks according to the protein alignment of LipA and LipB. The amino acid residues are reversibly translated into nucleotides according to standard code usage, while the original DNA sequences of LipA and LipB are used as references. Five fragments of oligonucleotides named Lip1 to Lip5 are designed according to the block sequences. We retain codons for those identical amino acid residues that are encoded by both the original nucleotides. When the amino acids in the two sequences are different, degenerate nucleotides need to be used. For example, if serine is encoded at a certain position by one codon (TCT) and in the second parental gene lysine is encoded at that position by another codon (AAA), then the nucleotide for this position is chosen to be (T/A) (C/A) (T/A). In this case, the nucleotides that encode amino acids at this position will be WMW according to the International Union of Biochemistry (IUB) Code (see the Experimental Section). The five oligonucleotides are 132 bps, 132 bps, 114 bps, 138 bps, and 81 bps long and encode 44, 44, 38, 46, and 27 amino acid residues, respectively. The lengths of the oligos are chosen according to the conserved regions in the parental genes. Generally, the favored length of the designed oligos ranges between 50 bps and 150 bps. The orientation of the two flanked oligonucleotides is opposite, and oligonucleotides of 18 bps to 33 bps at the end of each fragment are overlapped by reversed complement oligonucleotides.

The degeneracy of the genetic code may lead to the introduction of extra coded amino acids at a given position, for example, TA(T/A) could give a tyrosine residue or form a stop codon and AC(T/A) could give threonine. Such "extra" amino acids enhance coding diversity. However, this introduction of new residues does not always occur. For example, if the codes at a particular position in the two sequences are TTT and GTT (phenylalanine and valine, respectively), then the degenerate code should be (G/T)TT, and the two originally encoded amino acids are in fact the only possibilities.

The PCR assembly of the fragments is performed in a straightforward manner, normally in two steps. In a primerless PCR, the synthesized oligonucleotide fragments Lip1 – Lip5 represent single-stranded designed synthetic oligos. The orientation of flanking fragments are opposite, for example, Lip1 is 5′ – 3′, Lip2 is 3′ – 5′, and Lip3 is 5′-3′ again, and so on. Additional deoxynucleotide triphosphates (dNTPs), buffer, and a polymerase are processed. After several cycles of amplification, the fragments are linked with each other and form double-stranded DNA. Amplification of the whole gene follows (Figure 2). Full-length gene products are ligated into the commercially available overexpression vector pET22b(+) under the PelB signal sequence and are transformed into *Escherischia coli* BL21(DE3) according to the method described by Jaeger et al.[24a]

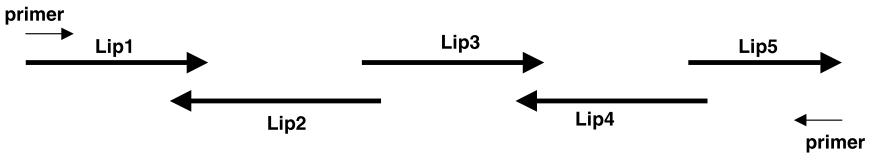
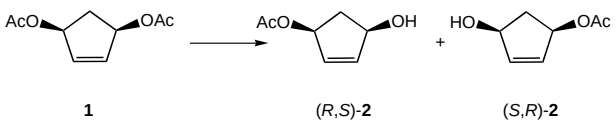


Figure 2. *Illustration of the process of assembly of designed oligonucleotides. Lip1 – Lip5 represent the designed oligonucleotides; arrows indicate the orientation of the fragments.*

Application to the evolution of enantioselective lipase variants

Two related lipases from *Bacillus subtilis*, LipA and LipB,[24] were chosen to test the ADO method. Following expression in *E. coli* and plating on agar plates, a prescreen based on the tributyrin plate assay[5a] was applied to test the lipase activity of approximately 3000 clones. About 25 of these clones turned out to be active catalysts. This result (about 1% active clones) is to be expected in experiments of this kind, which involve the recombination of family genes, and is expected in traditional DNA shuffling experiments.[1c, 9–12] The active clones (hits) were then used as catalysts in the hydrolytic desymmetrization of 1,4-diacetoxycyclopent-2-ene (**1**) to form the enantiomers (*S*,*R*)-**2** and (*R*,*S*)-**2** (Scheme 1). The wild-type lipA was also tested for



Scheme 1. *Lipase-catalyzed hydrolysis of* meso*-1,4-diacetoxy-cyclopent-2-ene (**1**) to afford the enantiomers (*R,S*)-**2** and (*S,R*)-**2**.*

Table 1. *Identified variants with improved enantioselectivity.*[a]

Variant	Conversion [%]	*ee* [%]
wild type	40	38
no. 3	3	45
no. 4	15	58
no. 5	12	42
no. 6	85	48
no. 7	70	54
no. 13	31	56

[a] *ee* values were determined by chiral GC; precision = ± 0.1 %.

Table 2. *Amino acid substitutions in sequenced variants and their origin.*

Variant	Amino Acid Substitutions		
	From LipA	From LipB	Others
no. 1	20	20	4
no. 3	18	16	9
no. 4	21	17	8
no. 6	42	1	1
no. 7	20	20	8
no. 8	17	21	6
no. 10	19	21	5
no. 12	19	23	4
no. 13	17	18	12

comparison and showed an enantiomeric excess (*ee*) of 38 % and a conversion of about 40 % under the conditions used. LipB shows no activity in the test with substrate **1**. Table 1 shows the six best lipase variants with improved enantioselectivity. Enantioselectivities were determined by ESI MS[25] and checked by chiral GC with an error of less than 0.1 % *ee*. In all cases the *ee* value was found to be independent of the degree of conversion. Variants number 6 and 7 (Table 1) appear to be most active, with *ee* values of 48 % and 54 %, respectively

In order to test whether the assembly of designed oligonucleotides had actually worked, sequence analyses of the recombinant mutants were performed on 15 of the variants that tested positive in the tributyrin plate assay. These 15 examples were chosen at random. Table 2 shows that recombination between LipA and LipB occurs with high efficiency. For example, variant number 7 has 20 mutations from LipA and 20 from LipB. The eight other point mutations are due to the degeneracy of the genetic code or are introduced by PCR. The latter effect is to be expected and is similar to the situation in standard DNA shuffling. The remarkable result is the distinct recombination, that is, we can "cut" the gene codon by codon, which is impossible by conventional family shuffling methods. The sequences of the variants in Table 2 are identified in Figure 3. No parental genes were found among the sequenced active mutants. This is a remarkable effect and illustrates the efficiency of ADO. Moreover, the sequences of all of the randomly selected clones are different, which indicates that the 3000-member library consists of individual clones. It is also interesting to note that, in spite of

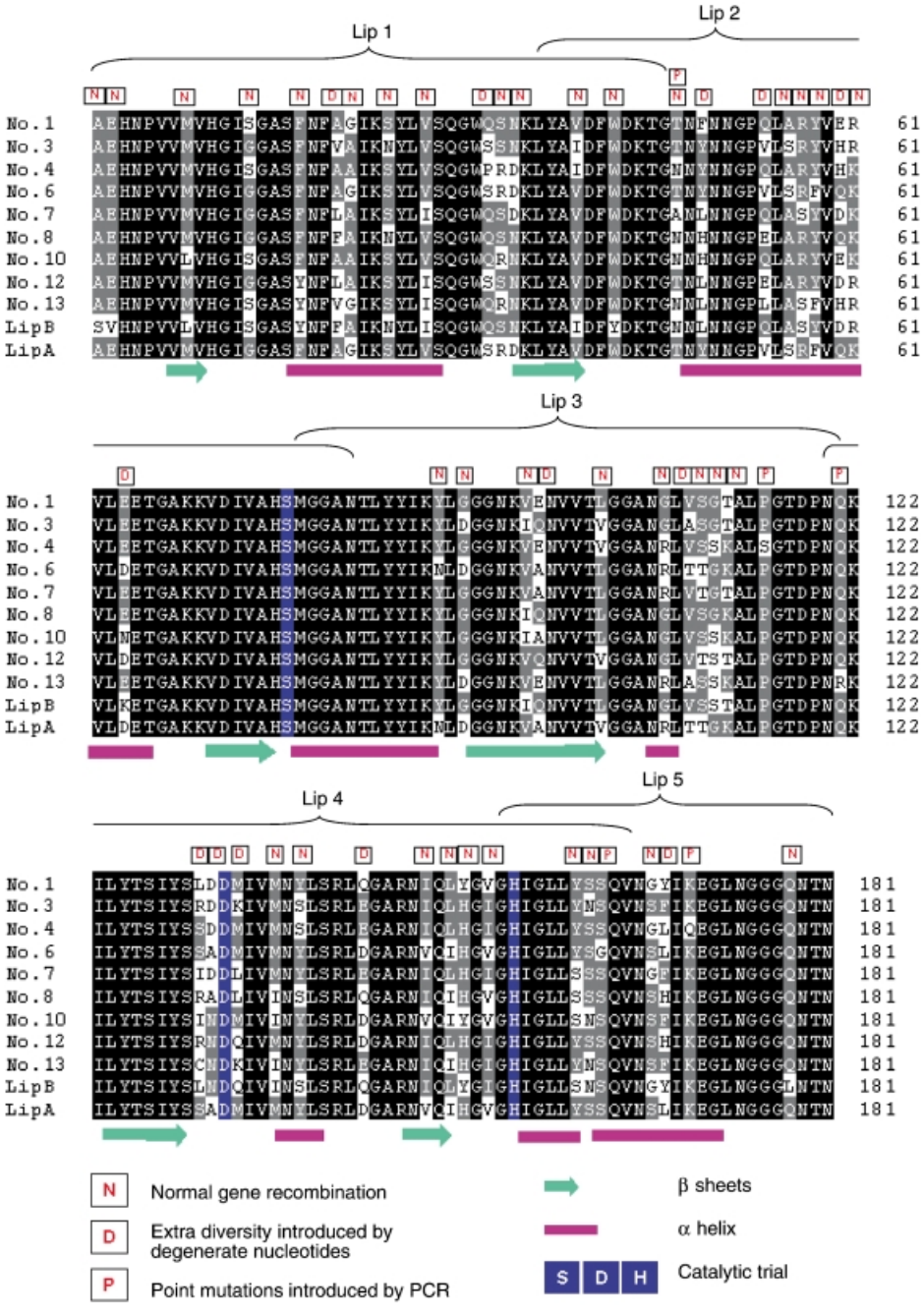


Figure 3. *Sequence alignment of clones derived from parental LipA and LipB chosen at random.*

the fact that the wild-type LipB is inactive in the catalysis of the hydrolysis of **1**, almost all of the active clones are true hybrids. Only variant number 6 is derived mainly from one parent (LipA).

The purpose of the present study was to develop and illustrate the ADO method. Further optimization of enantioselectivity by carrying out several cycles of mutagenesis/expression/screening with ADO and/or other methods is the subject of an ongoing study, as are kinetic experiments.

Conclusions

We have developed a practical method for gene recombination that results in libraries of mutant genes. The process is based on the assembly of appropriately designed synthetic oligonucleotides (ADO). The design of the oligonucleotides is guided by information derived from gene alignment. The major advantage of the method is that self-hybridization of parental genes is minimized or eliminated. This outcome is achieved for the first time in the recombination of two lipase family genes from *Bacillus subtilis*, but extension to more than two genes should pose no problems.[26] The exclusion of parental genes as an undesired background increases the quality of the library in evolution experiments and thereby reduces the effort necessary in screening, although the oligonucleotides must be synthesized. ADO can thus be expected to be an attractive method in the directed evolution of functional proteins and in metabolic engineering.

A paper by Richardson et. al. has just appeared in which gene recombination based on the reassembly of oligos prepared by restriction-enzyme-catalyzed cleavage of parental genes is described.[27] This method is different from our approach.

Experimental Section

Materials: Restriction enzymes were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and were used according to the manufacturer's instructions. The expression vector pET22b(+) was purchased from Novagen (Bad Soden, Germany) and the host strain *E. coli* BL21(DE3) from Invitrogen GmbH (Karlsruhe, Germany).

Expression of the recombinant lipase gene: The recombinant lipase mutants were cloned into the vector pET22b(+) and expressed in *E. coli* BL21(DE3) under control of strong bacteriophage T7 transcription and the *pel* B translation signal.[24] The recombinant library was prescreened for activity on tributyrin agar plates;[5a] active clones formed clear halos. Tributyrin plates were prepared as follows: Arabic Gum (0.75 g; Sigma, Germany) was dissolved in sterilized distilled water (7.5 mL) and mixed with tributyrin (7.5 mL; Sigma). The mixture was added to LB agar (500 mL) and emulsified with an Ultraturrax T25 instrument (IKA Labortechnik, Germany) for 1 min at 24000 U Min^{-1} prior to pouring the plates.

Oligonucleotide fragment design: The oligos were designed according to the alignment of the protein sequences of LipA and LipB. Five fragments named Lip1, Lip2, Lip3, Lip4, and Lip5 were synthesized (Thermo Hybrid GmbH, Germany) that together cover the open reading frames of both LipA and LipB. For mixed bases, the IUB Code was used (M = A + C, R = A + G, W = A + T, S = G + C, Y = C + T, H = A + C + T, D = A + G + T, B = G + T + C, N = A + A + A + G + C + T, K = G + T). The sequences are listed as following: **Lip1**: 5′-CAC AAT CCA GTC GTT MTG GTT CAC GGT ATT RGT GGG GCA TCA TWC AAT TTT KYS GSA ATT AAG ARC TAT CTC RTA TCT CAG GGC TGG YMG MGT RAC AAG CTG TAT GCA RTT GAT TTT TGG GAC AAG ACA GGC-3′; **Lip2**: 5′-CGC GCC CCC CAT GCT GTG AGC GAC AAT ATC CAC TTT TTT CGC ACC CGT TTC MTY TAA AAC CYT KTS CAC AWA KCT TGM TAA TWS CGG TCC ATT GTT AWR ATT AKT GCC TGT CTT GTC CCA AAA ATC AAY TGC ATA CAG-3′; **Lip3**: 5′-CAC AGC ATG GGG GGC GCG AAC ACA CTT TAC TAC ATA AAA WAT CTG GRC GGC GGA AAT AAA RTT SMA AAC GTC GTG ACG STT GGC GGC GCG AAC SGT TTG RYG WCA RGC AMG GCG CTT CCG GGA ACA GAT CCA AAT CAA-3′; **Lip4**: 5′-CAG AAG GCC GAT GTG TCC AAY GCC ATR GAK TTG AAY GTT TCT AGC ACC WTS TAA TCT TGA TAA GKA ATT YAT GAC AAT CWK ATC GKY AMK GCT GTA AAT GGA TGT GTA TAA AAT CTT TTG ATT TGG ATC TGT TCC CGG-3′; **Lip5**: 5′-GGA CAC ATC GGC CTT CTG TMC ARC AGC CAA GTC AAC RGC YWT ATT AAA GAA GGG CTG AAC GGC GGG GGC CWG AAT ACG AAT-3′. Two primers were used for amplification of the assembled gene; the primer located upstream was 5′-CAC AAT CCA GTC GTT MTG GTT CAC GGT ATT-3′, that located downstream was 5′-AAT ACC GTG AAC CAK AAC GAC TGG ATT GTG-3′.

Fragment assembly and PCR amplification: Fragments Lip1 – Lip5 were dissolved in distilled water to a concentration of 10 μM. A primerless PCR reaction was carried out in a total reaction volume of 50 μL, which contained each fragment (2 μL), 1X Taq polymerase buffer (75 mM tris(hydroxymethyl)aminomethane – HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 1.25 mM $MgCl_2$), dNTP (5 μL, 2 mM), and Taq polymerase (2.5 U; Eurogentec, Belgium). 20 cycles were carried out at 94 °C min^{-1}, 50 °C min^{-1}, and 72 °C/1.5 min. After the primerless PCR, the reaction solution (1 μL) was taken as the template for a standard PCR experiment in a 50 μL solution volume under the same conditions as used in the primerless PCR but with two primers, one up- and one downstream of the assembled fragment. The amount of recombinant DNA obtained is similar to that usually obtained in DNA shuffling procedures.

Screening process: The mutant variants were cultured at 37 °C in 96-well microtiter plates filled with LB/M9 (1 mL; 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, and additionally 4 g L^{-1} glucose, 0.25 g L^{-1} $MgSO_4$, 0.02 g L^{-1} $CaCl_2$, 7 g L^{-1} Na_2HPO_4, 3.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl) with carbenicillin (100 μg mL^{-1}) as the antibiotic. After 6 hours shaking at 37 °C, lipase expression was induced by addition of isopropyl-β-D-thiogalactosid (10 μg) and the culture was grown overnight by shaking at 37 °C. Cells were separated from the culture by centrifugation at 5000 × g for 10 min. An aliquot (100 μL) was taken from the culture supernatant in each well and pipetted into another 96-well microtiter plate containing aqueous Na_2HPO_4/KH_2PO_4 buffer (800 μL, pH 7.5) and the substrate (**1**; 100 μL) dissolved in dimethylsulfoxide (100 mM). After 24 hours of shaking at room temperature, the reaction solution was extracted with ethyl acetate and analyzed by ESI MS by using the method of Reetz et al.[25] Those variants showing an improved enantioselectivity were further analyzed by chiral GC. The genes encoding lipase variants with improved enantioselectivity in the model reaction were sequenced (Medigenomix, Martinsried, Germany).

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