Multiplex-PCR-Based Recombination as a Novel High-Fidelity Method for Directed Evolution

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A new and convenient method for the in vitro recombination of single point mutations is presented. This method efficiently reduces the introduction of novel point mutations, which usually occur during recombination processes. A multiplex polymerase chain reaction (multiplex-PCR) generates gene fragments that contain preformed point mutations. These fragments are subsequently assembled into full-length genes by a recombination-PCR step. The process of multiplex-PCR-based recombination (MUPREC) does not require DNase I digestion for gene-fragmentation and is therefore easy to perform, even with small amounts of target DNA. The protocol yields high frequencies of recombination without creating a wild-type background. Furthermore, the low error rate results in high-quality variant libraries of true recombinants, thereby minimizing the screening efforts and saving time and money. The MUPREC method was used in the directed evolution of a Bacillus subtilis *lipase that can catalyse the enantioselective hydrolysis of a model* meso*-compound. Thereby, the method was proved to be useful in producing a reliable second-generation library of true recombinants from which better performing variants were identified by using a high-throughput electrospray ionization mass spectrometry (ESI-MS) screening system.*

Introduction

Directed evolution has matured during the last decade to become a key technology in the field of molecular enzyme engineering, in particular, when neither the 3D structures nor the catalytic mechanisms of the enzymes are known. However, even if crystal structures are available and reaction mechanisms are well understood, directed evolution often provides alternative solutions in comparison to rational-design experiments.[1–3]

The creation of diversity is a crucial step in each directed-evolution experiment. Diversity can either be directly retrieved from nature by isolation of homologous but not identical genes or artificially generated by introducing random point mutations into a target gene. Moreover, subsequent recombination of this diversity has proved to be a very effective strategy for combining advantageous mutations and separating out deleterious ones. Today, at least twelve in vitro recombination methods have been published, which are summarized in two excellent review articles.[2,4] Among these approaches DNA-shuffling is still the method of choice for most directed-evolution experiments. Other methods, which include staggered extension process (StEP),[5] random priming recombination (RPR),[6] heteroduplex recombination,[7] ssDNA-family shuffling,[8] degenerate oligonucleotide gene shuffling (DOGS),[9] random chimeragenesis on transient templates (RACHITT),[10] mutagenic and unidirectional reassembly (MURA),[11] synthetic shuffling,[12] assembly of designed oligonucleotides (ADO)[13] and recombined extension on truncated templates (RETT)[14] use different experimental strategies to ensure the exchange of DNA fragments between different variants. Slight variations in these methods have been published by different groups.[15–17] All these methods result in a significant improvement in the efficiency to create novel enzymes by directed evolution. However, they also have major drawbacks, including i) a recombinational bias depending on the target DNA and ii) the creation of additional diversity by introducing novel point mutations during recombination, a process that could result in a library far too large to be screened by available methods. Therefore, novel developments in directed-evolution methodology focus on improving library quality instead of quantity.[2]

A major strategy to reduce the size of a library is based on increasing the fidelity of the recombination process. The original DNA-shuffling protocol led to the introduction of an average of seven novel point mutations per kilobase (kb), which results in extra diversity.[18,19] This effect is favoured when screening capacity is not a limiting factor as it is for powerful selection systems like phage display[20] or fluorescence-activated cell sorting (FACS).[21] Unfortunately, such systems are not available as yet to select for enzyme properties like enantioselectivity. Zhao and Arnold modified the DNA-shuffling protocol to reduce the rate of newly introduced point mutations by using

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  DOI: 10.1002/cbic.200400417

different DNA polymerases during fragment reassembly.[22] The creation of DNA fragments by using restriction endonucleases also reduced the number of novel point mutations; however, it also increased the bias of recombination.[8] Nevertheless, the methods based on DNase I digestion have in common the facts that large amounts of DNA are needed and that the frequency of recombination is very low for neighbouring mutations.

Here, we describe a high-fidelity method for the recombination of point mutations that introduces a single, novel point mutation per 10 kb (mutation rate 1×10^{-4}) but results in a high frequency of recombination independent of the amino-acid positions to be recombined. Furthermore, the protocol for this multiplex-PCR-based recombination method is simple and generally applicable. The versatility of this method was tested by the recombination of point mutations that had been introduced into the *Bacillus subtilis* lipase A (BSLA) gene and subsequent screening for the enantioselective hydrolytic desymmetrization of a model *meso*-compound.

Table 1. Oligonucleotides used in this study.

Primer	Sequence[a]	Modifications
mut1-up	5′-ctcctcgctgcccagccggcgatggccatg-3′	*Mls*I
mutS-low	5′-atataagcttcagcaaacagctatgaccatgattacgaattc-3′	*Hin*dIII
N18X-up	5′-ggaggggcatcatcattnnstttgcgggaattaag-3′	N18 saturation primer
N18X-low	5′-cttaattcccgcaaasnngaatgatgatgcccctcc-3′	N18 saturation primer
I22T-up	5′-ttcaattttgcgggaactaagagctatctcg-3′	I22T mutation
I22T-low	5′-cgagatagctcttagttcccgcaaaattg-3′	I22T mutation
Y49C-up	5′-aagacaggcacaaattgtaacaatggaccggta-3′	Y49C mutation
Y49C-low	5′-taccggtccattgttacaatttgtgcctgtctt-3′	Y49C mutation
Y49I-up	5′-aagacaggcacaaatatcaacaatggaccggta-3′	Y49I mutation
Y49I-low	5′-taccggtccattgttgatatttgtgcctgtctt-3′	Y49I mutation
Y49V-up	5′-aagacaggcacaaatgtcaacaatggaccggta-3′	Y49V mutation
Y49V-low	5′-taccggtccattgttgacatttgtgcctgtctt-3′	Y49V mutation
N50S-up	5′-acaggcacaaattatagcaatggaccggtattatc-3′	D50S mutation
N50S-low	5′-taataccggtccattgctataatttgtgcctgt-3′	N50S mutation
F58L-up	5′-ccggtattatcacgacttgtgcaaaaggttttag-3′	F58L mutation
F58L-low	5′-taaaacctttgcacaagtcgtgataataccgg-3′	F58L mutation
Q60L-up	5′-ttatcacgatttgtgttgaaggttttagatgaa-3′	Q60L mutation
Q60L-low	5′-catctaaaaccttcaacacaaatcgtgataa-3′	Q60L mutation
Q60N-up	5′-ttatcacgatttgtgaacaaggttttagatg-3′	Q60N mutation
Q60N-low	5′-catctaaaaccttgttcacaaatcgtgataa-3′	Q60N mutation
L114P-up	5′-acgacaggcaaggcgcctccgggaacagatcc-3′	L114P mutation
L114P-low	5′-tggatctgttcccggaggcgccttgcctgtcg-3′	L114P mutation
C124S-up	5′-ccaaatcaaaagatttcatacacatccatttac-3′	C124S mutation
C124S-low	5′-gtaaatggatgtgtatgaaatcttttgatttgg-3′	C124S mutation
A132D-up	5′-tccatttacagcagtgacgatatgattgtcatg-3′	A132D mutation
A132D-low	5′-catgacaatcatatcgtcactgctgtaaatgg-3′	A132D mutation
I157N-up	5′-caaatccatggcgttggagacggccttctgtacagc-3′	I157N mutation
I157N-low	5′-gctgtacagaaggccgtctccaacgccatggatttg-3′	I157N mutation
N166Y-up	5′-tacagcagccaagtctacagcctgattaaagaag-3′	N166Y mutation
N166Y-low	5′-ttctttaatcaggctgtagacttggctgctgtac-3′	N166Y mutation

[a] Mutated codon unterlined.

Results and Discussion

Multiplex-PCR-based recombination (MUPREC)

A protocol was developed for the efficient recombination of single point mutations that are generated by directed evolution methods. This protocol is based on multiplex-PCR for the amplification of those fragments that carry point mutations for recombination.

As a starting point we used two multiplex-PCR reactions that were performed simultaneously by using two different template plasmids of the target gene. In one reaction, a set of mutagenesis primers that were designed as lower primers were applied together with a universal upper primer (Table 1). This resulted in the formation of a mixture of different megaprimers, each containing a single point mutation. The other multiplex-PCR reaction produces the complementary megaprimers by applying a set of mutagenesis primers that are designed as upper primers and amplify gene fragments along with a universal lower primer. In a third PCR reaction, these megaprimers were used together with the flanking primers (mut1-up and mutS-low; Table 1) to produce the full-length gene that carried the desired point mutations. The high concentration of megaprimers in comparison to flanking primers resulted in megaprimer overlaps and subsequent elongations, which led to the random recombination of the desired point mutations (Figure 1). Theoretically, template switching occurs during megaprimer-annealing and -elongation processes and results in the formation of all possible combinations of point mutations. In practice, however, we have observed an accumulation of mutants that carry two or three point mutations (data not shown). Fortunately, these recombinants were randomly generated, nevertheless, recombinants with more than four point mutations were relatively rare.

This result forced us to change the protocol so that a larger number of small fragments would be formed in the first multiplex-PCR reaction. Here, a single, universal, upper primer (mut1-up) was used together with sense and antisense mutagenesis primers, which again produced megaprimer fragments. In addition, small fragments that were complementary to the middle of the gene and carried two point mutations were amplified. This fragment mixture together with a new gene template, which has different flanking regions, was then used along with a universal lower primer in a second so-called recombination-PCR reaction (Figure 2). The modification of the original protocol (Figure 1) resulted in the formation of an increased number of recombinants that carried multiple point mutations. This protocol is recommended for five or more point mutations that are to be recombined. The results shown in Table 2 indicate that up to eleven point mutations can be randomly recombined. This suggests that this method could also allow the recombination of an even higher number of point mutations. The limitation of this method is set solely by the size of the created library. As an example, the recombination of 20 point mutations will generate a library consisting of 1.05×10^{6} different variants (see formula in Table 3), a number that exceeds the capacity of most high-throughput screening methods.

The correct formation of DNA fragments during a multiplex-PCR reaction, as shown by agarose gel electrophoresis (Figure 3), indicated that the protocol could be further simpli-

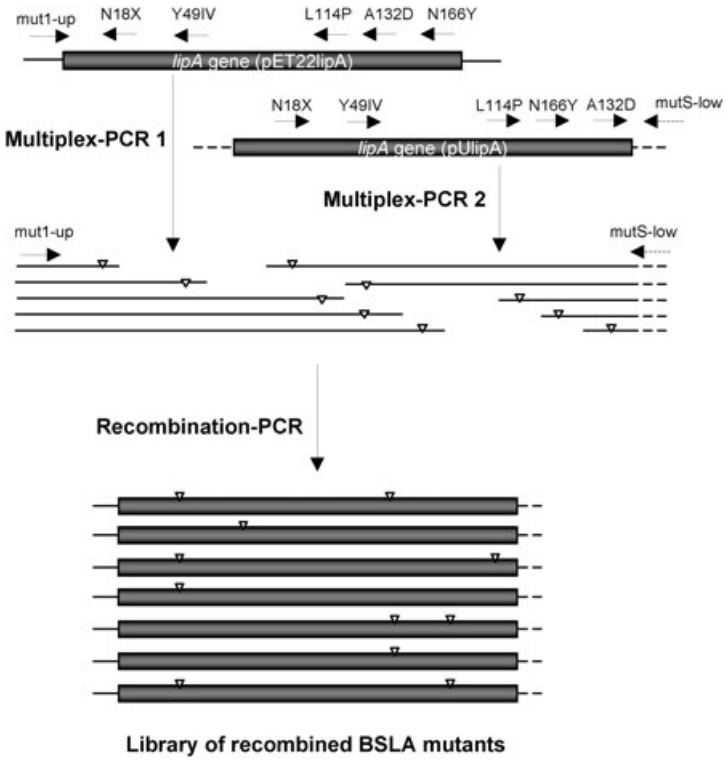


Figure 1. Initial experimental approach for the directed recombination of existing point mutations. This protocol comprised three independent PCRs and led to recombinant genes that contained combinations of up to three mutations at maximum. Triangles indicate point mutations.

Table 3. Theoretical library sizes generated by randomly recombined point mutations.

Number of single point mutations[a]	Number of recombinants[b]
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024

[a] Number of single point mutations to be recombined. [b] Number of true recombinants without any new point mutation, calculated by using the formula $\sum_{k=0}^{n}\binom{n}{k}=2^n$ where n = number of single point mutations to be recombined and k = overall number of point mutations present in a variant protein.

fied by performing just a single PCR reaction. In this procedure (an all-in-one PCR), the amplification of wild-type sequences was excluded by using a universal upper primer, mut1-up, that only hybridized to pET22 lipA1 (template 1) and a universal lower primer, mutS-low, that only hybridized to pUlipA (template 2; Figure 2). We call these primers "universal" due to their sequence independence towards the gene to be mutated. Primer mut1-up ("universal" upper primer) hybridizes to the vector sequence of pET22b upstream of the target gene, whereas mutS-low ("universal" lower primer) anneals downstream of the target gene in the pUC18 vector. By using the two "universal" primers together with two different template vectors, the amplification of unwanted wild-type DNA is efficiently prevented for site-directed mutagenesis by using the megaprimer PCR technique.[23–25]

It should be noted that the mutagenesis and the "universal" primers must be carefully designed so that they have comparable melting temperatures, and equimolar concentrations of primers should be used in the reaction mixture. If a specific mutation is to be favoured, then the corresponding mutagenesis primer can be used in higher molar concentrations and will therefore be incorporated into the respective fragments at a statistically higher rate.

Table 2. Mutations and amino-acid substitutions found in randomly chosen variants[a] that were generated by the MUPREC process.

Variant no.	Mutation[b]
	Recombination of point-mutations I22T (att-act), Y49C (tat-tgt), N50S (aac-agc), F58L (ttt-ctt), Q60N (caa-aac), Q60L (caa-ttg), L114P (ctt-cct), C124S (tta-tca), A132D (gcc-gac), I157N (atc-gac), N166Y (aac-tac)
1	I22T, Y49C, N50S, L114P, C124S
2	I22T, N50S, L114P, C124S, **S24I (agc-atg)**
3	I22T, N50S
4	N50S, F58L, L114P
5	I22T, N50S, Q60N, L114P, C124S, I157N
6	I22T, Y49C, F58L, L114P
7	Y49C, L114P

[a] All variants showed lipolytic activity towards the substrate tributyrin. [b] Recombined point mutations are given as amino-acid exchanges; newly generated point mutations are indicated in bold; base substitutions are written in brackets.

Application of MUPREC to evolve enantioselective lipase variants

Extracellular BSLA was optimized by directed evolution so that it catalyzed the enantioselective hydrolytic desymmetrization of 1,4-diacetoxycyclopentene (Scheme 1). Variant libraries were generated by error-prone PCR (epPCR), and by complete saturation mutagenesis.[26] During this project, we observed that several newly isolated enantioselective lipase variants showed a reduced thermostability. Therefore, we chose to recombine several mutations that lead to higher enantioselectivity with others previously shown to increase the thermostability of BSLA.[27] L114P, A132D and N166Y amino-acid substitutions were chosen for recombination as they resulted in increased thermostability, and several different substitutions at positions N18 and Y49 were chosen since they all resulted in increased enantioselectivity.[28] For position N18, a primer mix was used that encoded all 20 amino acids, and at position 49, the substitutions Y49I and Y49V were chosen. These had been identified during previous screenings (data not shown).

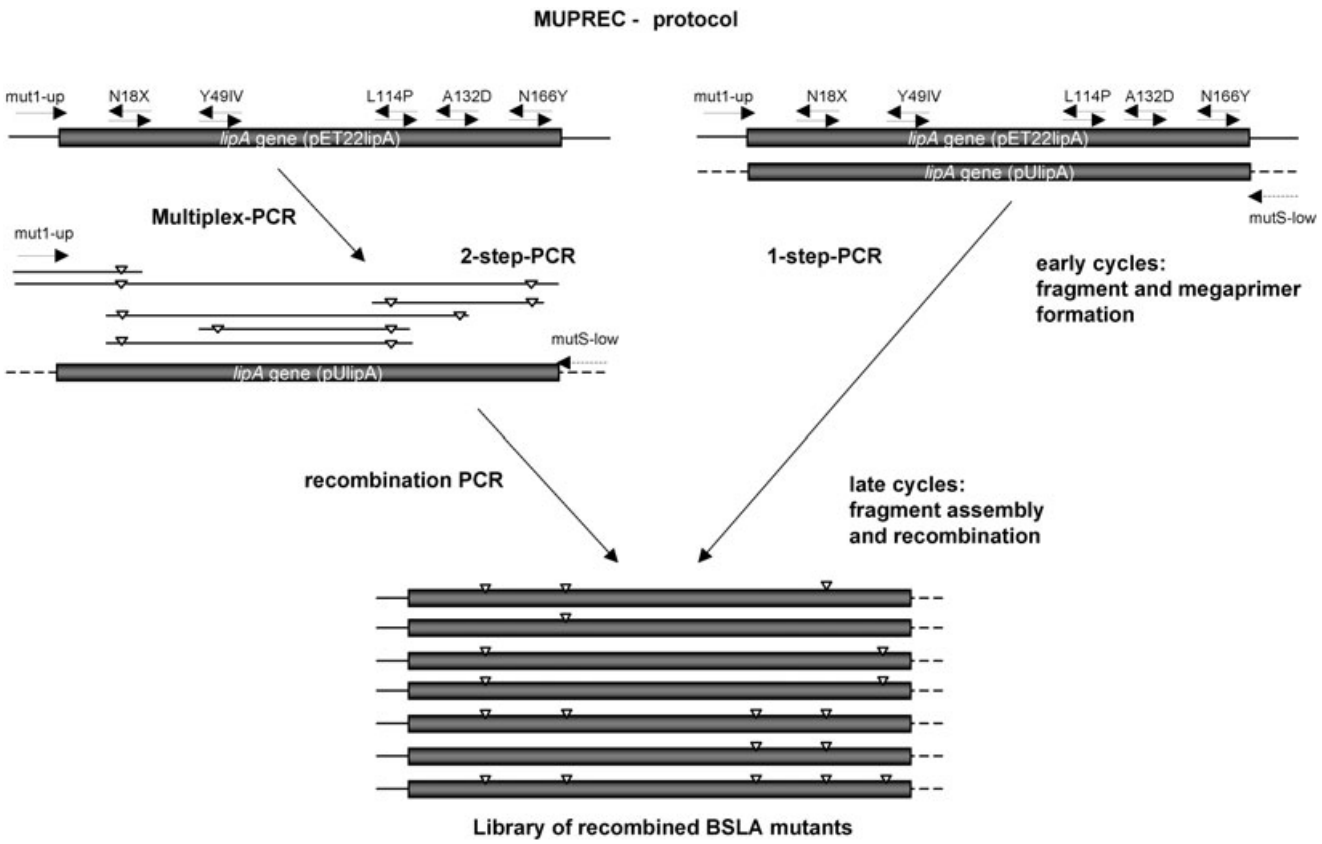


Figure 2. The MUPREC process. Mixtures of upper and lower primer pairs that carry the point mutations to be recombined are used in a multiplex PCR to amplify gene fragments, which are recombined in a second PCR. The efficiencies of fragment formation during the multiplex-PCR, which are mainly determined by the melting temperature of the mutagenesis primers, can be directly monitored by using the two-step method. Alternatively, the one-step protocol can be applied for convenient and high-fidelity recombination. More experimental details are given in the text. Triangles indicate point mutations.

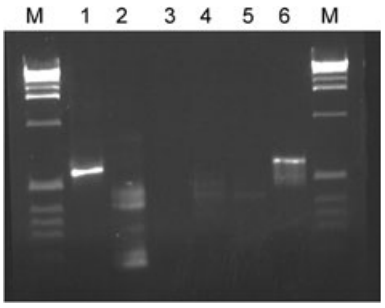
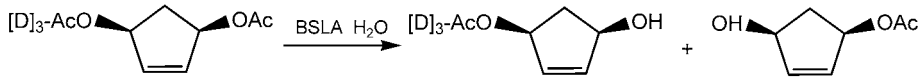


Figure 3. Gel electrophoretic analysis of a MUPREC experiment. Lane 1: Full-length BSLA gene amplified by standard PCR (positive control); lane 2: fragment mixture after multiplex PCR that contained megaprimer and internal PCR fragments that carried the point mutations to be recombined; lane 3: negative control for the recombination-PCR reaction using pUlipA as the template along with primers mut1-up and mutS-low; lane 4: negative control for the recombination PCR with pUlipA as the template and the fragment mixture shown in lane 2, but omitting the universal primers mut1-up and mutS-low; lane 5: negative control for the recombination PCR by using the fragment mixture shown in lane 2 and the universal primers mut1-up and mutS-low, but omitting the template pUlipA; lane 6: full-length PCR product after the recombination PCR.

The two-step MUPREC protocol described above (Figure 2, left) was used to monitor whether the correct formation of fragments and megaprimers occurred after the multiplex-PCR reaction or not (Figure 3). Afterwards, the one-step MUPREC protocol (Figure 2, right) was used to essentially yield the same results. The amplified full-length genes were cloned into the expression vector pET22b by using the unique restriction sites *Mls*I and *Hin*dIII, which were introduced into the fragments during the recombination-PCR reaction. After over-expression in *E. coli*, a library of about 390 enzymatically active lipase variants was created and screened for enantioselectivity by using ESI-MS.[29] Nine BSLA variants were identified that showed inverse enantioselectivities to the wild-type enzyme (Table 4) and of which variant 37-01-G5 (N18Q, Y49V) also showed a much higher enzymatic activity when grown on tributyrin-indicator plates (Figure 4). Interestingly, for all variants, the increase in enantioselectivity was accompanied by a decrease in thermostability, although amino-acid substitutions were incorporated that were previously shown to increase the thermostability of BSLA. At present, the number of screened variants is still too low to conclude that a general incompatibility exists for combining thermostability and enantioselectivity in this lipase.

The efficiency of the MUPREC method

The efficiency of the MUPREC method was analysed by determination of the DNA sequences from randomly chosen re-



pseudo-*meso*-1,4-diacetoxy-cyclopentene (**1**) | 1*R*,4*S*-enantiomer (**2**) | 1*S*,4*R*-enantiomer (**3**)

Scheme 1. The model reaction used to identify enantioselective variants of BSLA. The asymmetric hydrolysis of the model compound *meso*-1,4 diacetoxy-2-cyclopentene, was determined by a high-throughput ESI-MS screening system. The deuterium-labelled substrate pseudo-*meso*-1,4 diacetoxy-2-cyclopentene allows the formation of chiral alcohol products (**2**) and (**3**) to be identified by their mass differences.

Table 4. BSLA variants with improved enantioselectivity

Variant	amino-acid exchanges	*ee* [%][a]	conversion [%][a]
wild-type	–	45 (1*R*,4*S*)	100
thermostable	L114P, A132D, N166Y	52 (1*R*,4*S*)	100
First generation (complete saturation mutagenesis library)			
144-F7	N18I	14 (1*S*,4*R*)	90
133A6	N18A	21 (1*S*,4*R*)	100
195-E8	N18L	65 (1*S*,4*R*)	75
22-N18C	N18C	72 (1*S*,4*R*)	85
145-F4	N18Q	82 (1*S*,4*R*)	75
133-H12	N18S	83 (1*S*,4*R*)	50
196-C2	Y49I	16 (1*S*,4*R*)	5
Second generation (MUPREC library)			
16-02-D1	N18L, N166Y	68 (1*S*,4*R*)	n.d.
16-02-B1	N18S, L114P, N166Y	23 (1*S*,4*R*)	n.d.
37-02-F2	N18H, N166Y	61 (1*S*,4*R*)	n.d.
37-02-B12	N18Q, Y49V	82 (1*S*,4*R*)	85
16-02-F1	N18Q, L114P	85 (1*S*,4*R*)	n.d.
16-02-G1	N18Q, L114P, A132D; N166Y	85 (1*S*,4*R*)	n.d.
37-03-A3	N18S, Y49I, L114P	30 (1*S*,4*R*)	n.d.
37-01-G5	N18Q, Y49V	82 (1*S*,4*R*)	85
37-02-E2	N18Q, Y49I	87 (1*S*,4*R*)	n.d.

[a] Enantioselectivity and conversion rate of the substrate pseudo-*meso*-1,4 diacetoxy-2-cyclopentene were determined by ESI-MS. n.d. = not determined.

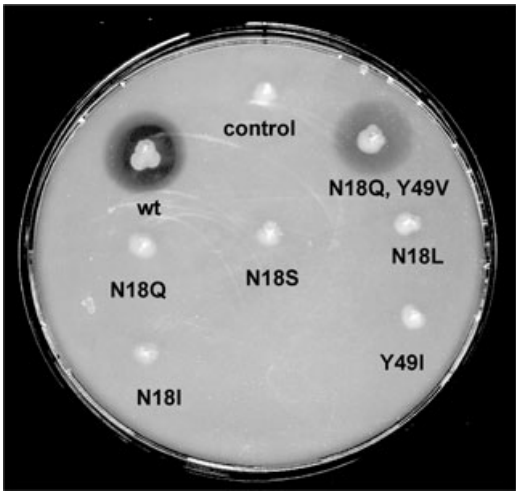


Figure 4. Lipolytic activities of wild-type BSLA and its enantioselective variants. *E. coli* clones expressing BSLA variants were plated on tributyrin-indicator plates and incubated for 24 h at 37 °C. Variant 37-01-G5, which contains amino-acid substitutions N18Q and Y49V, shows wild-type (wt) activity indicated by the size of the clear halo around the colony.

combinants that showed lipase activity. All randomly chosen clones had acquired up to six different point mutations by recombination (Table 2); this indicates a high diversity of the library (Table 3). In total, 39 sequences of randomly chosen recombinants have been sequenced; this gives an estimated error rate of 1×10^{-4} for the MUPREC method, which is comparable to the rate observed for *Taq*-DNA-polymerases. Our results clearly show that MUPREC allows the directed recombination of previously identified point mutations without introducing a significant number of novel and possibly unwanted mutations, in contrast to other homology-dependent recombination protocols like DNA-shuffling (seven additional point mutations per kb) or StEP (0.6 additional point mutations per kb). Therefore, the application of MUPREC could help to significantly facilitate screening efforts. Furthermore, MUPREC did not produce any wild-type genes, as determined by screening for enantioselectivity or by DNA-sequencing.

Conclusion

We have described here a novel in vitro recombination method for application in directed-evolution experiments. The MUPREC process can be used to recombine single point mutations previously generated in directed-evolution experiments. This method avoids the amplification of wild-type genes and effectively prevents the formation of novel base substitutions. Therefore, the size of a recombination library is minimized, thereby enabling a complete library screen. The method does not require DNase I digestion for gene fragmentation and can therefore be easily carried out in a single PCR-step. Thus, MUPREC should prove useful in optimizing directed-evolution protocols based on libraries created by epPCR or complete saturation mutagenesis.

Experimental Section

Bacterial strains and growth conditions: Plasmids were constructed and transformed into *E. coli* strains XL1-blue or DH5α. *E. coli* cells were grown overnight in Luria–Bertani (LB) medium (5 mL) in glass tubes at 37 °C and in the presence of appropriate amounts of ampicillin (100 μg mL^{-1}). The heterologous expression of BSLA and its variants was performed with *E. coli* BL21(DE3) in the presence of carbenicillin (100 μg mL^{-1}; Serva, Heidelberg, Germany).

General DNA techniques and plasmids: Plasmid DNA was prepared by using the plasmid purification midi-kit from QIAGEN (Hilden, Germany). Genomic DNA from *B. subtilis* 168 (obtained from the *Bacillus* Genetic Stock Center, Ohio, USA) was prepared by using the DNeasy Tissue Kit (QIAGEN, Hilden, Germany). Recombinant DNA techniques were performed as described by Sambrook et al.[30] Restriction digestion reactions and ligations were performed with enzymes from Fermentas (St. Leon-Rot, Germany) under conditions recommended by the manufacturer.

The plasmids pET22 lipA or pUlipA were used as templates in PCR reactions. Both plasmids contain the BSLA gene with different up and downstream regions. The construction of the plasmid pET22 lipA has been described elsewere.[26] A BSLA-gene-containing plasmid with up- and downstream regions different from pET22 lipA was constructed by amplification of the lipase gene by using a standard PCR reaction with the 30 bp upper primer BSLA1 5′-ATATGATATCGCTGAACACAATCCAGTCGT-3′ and the 29 bp lower primer BSLA2 5′-TATAGAGCTCTCATTAATTCGTATTCTGG-3′. Genomic DNA from *B. subtilis* 168 (10 ng) was used as the template. The resulting 557 bp PCR product was cloned, blunt-end, into a *Hin*cII-digested pUC18 vector (Stratagene, Heidelberg, Germany) to result in plasmid pUlipA.

Standard-PCR conditions: Amplification of DNA fragments was performed in a 50 μL reaction mixture with plasmid (1 ng) or genomic DNA (10 ng) as template, primers (each 25 pmol), dNTPs (0.2 mM), *Taq* (2.5 U, Eurogentec, Seraing, Belgium) or *Pfu* polymerase (2.5 U, Stratagene, Heidelberg, Germany). Buffers containing $MgCl_2$ or $MgSO_4$ were used as recommended by the manufacturers. Conditions for PCR reactions were: 1× (3 min at 98 °C); 35× (1 min at 95 °C; 2 min at 58 °C, 1 min at 72 °C) and 1× (7 min at 72 °C). The PCR reactions were performed by using a Mastercycler Gradient (Eppendorf, Hamburg, Germany).

Multiplex-PCR conditions: Multiplex-PCR reactions were performed in 50 μL reaction mixtures, as described above for standard PCR, by using *Pfu* polymerase. The primers used for mutagenesis in this study are summarized in Table 1. To meet optimal annealing temperatures for every primer within the sample, the PCR conditions used were as follows: 1× (3 min at 98 °C); 35× (1 min at 95 °C; 2 min gradient from 70°–50°; 1 min at 72 °C) and 1× (7 min at 72 °C). The multiplex-PCR reactions were also performed by using a Mastercycler Gradient (Eppendorf, Hamburg, Germany). After identifying 65 °C to be the most efficient annealing temperature, we used this temperature in all following multiplex-PCR reactions: 1× (3 min at 98 °C); 35× (1 min at 95 °C; 2 min at 65 °C, 1 min at 72 °C) and 1× (7 min at 72 °C).

High-throughput screening for enantioselectivity: The recombined BSLA genes were cloned into the expression vector pET22b (Novagen, Madison, USA) as in-frame fusions to the *pelB*-signal sequence; this enables Sec-dependent protein secretion. The resulting plasmids were used to transform *E. coli* BL21(DE3) (Novagen, Madison, USA). The clones were cultured at 37 °C in 96-deep-well microtiter plates that were filled with LB/M9 medium (1 mL; 10 $g\,L^{-1}$ tryptone, 5 $g\,L^{-1}$ yeast extract, 5.5 $g\,L^{-1}$ NaCl, 4 $g\,L^{-1}$ glucose, 0.25 $g\,L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.02 $g\,L^{-1}$ $CaCl_2$, 7 $g\,L^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 3 $g\,L^{-1}$ KH_2PO_4, 1 $g\,L^{-1}$ NH_4Cl) supplemented with carbenicillin (100 $\mu g\,mL^{-1}$). After 6 h of shaking at 37 °C ($OD_{580} = 0.5–0.7$), lipase expression was induced by adding isopropyl-β-D-thio-galactopyranoside (final concentration 0.3 mM). The induced culture was grown at 37 °C, and the cells were separated from the medium by centrifugation at 5000 *g* for 10 min. An aliquot of 100 μL from the culture supernatant was taken from each well and pipetted into another 96-deep-well microtiter plate that contained Na_2HPO_4/KH_2PO_4 buffer (800 μL; 10 mM, pH 7.5) and the substrate (100 μL) dissolved in dimethylsulfoxide (100 mM). After 24 h shaking at RT, the reaction solution was extracted with ethyl acetate and screened by electrospray ionization mass spectroscopy (ESI-MS).[29]

DNA sequence analysis: DNA sequence analysis of the mutant genes was performed by SequiServe (Vaterstetten, Germany) by using standard T7-promoter and T7-terminator primers.

Acknowledgements

This work was partly funded by the European Commission within the framework of the Biotechnology program (project no. QLK3-CT-2001-00519). S.A.F. is a recipient of a scholarship from the graduate college "Molekulare Physiologie: Stoff- und Energieumwandlung" funded by the Deutsche Forschungsgemeinschaft (DFG).

Keywords: directed evolution · enantioselectivity · lipases · polymerase chain reaction · protein engineering

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Received: November 23, 2004
Published online on May 6, 2005

