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The "Bringer" Strategy

A Very Fast and Highly Efficient Method for Construction of Mutant Libraries by Error-Prone Polymerase Chain Reaction of Ring-Closed Plasmids

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Abstract

Random mutagenesis constitutes a keystone in many strategies of directed evolution of biocatalysts and is often done by error-prone polymerase chain reaction (epPCR). Traditionally, the epPCR-generated DNA fragments are then subcloned into an expression vector to obtain a mutant library, which in turn is transformed into a suited host and screened for mutants that display the desired property. However, the vast majority of epPCR-generated fragments generally are lost during the subcloning step, making it the bottleneck in the mutant library construction procedure. Here we report a rapid and convenient strategy based on the epPCR amplification of a ring-closed expression plasmid that contains the gene of interest; after a *Dpn*I digest the product of the epPCR reaction constitutes the mutant library and can be used directly for screening procedures. Primers binding to the β-lactamase gene were chosen to allow application of the strategy to as broad a range of target plasmids as possible. The functionality of this approach was demonstrated by mutating the α-peptide coding region of the *lac*Z gene.

Index Entries: Error-prone polymerase chain reaction; α-peptide; ring-closed plasmids; *Escherichia coli*; Bringer strategy.

Introduction

While over the past two decades protein engineering based on site-directed mutagenesis has contributed to our understanding of enzyme

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catalysis, biologic design capabilities have been greatly enhanced recently by the development of "evolutionary" protein design methods that use random mutagenesis and gene recombination followed by screening or selection for a desired trait *(1,2)*. Using this approach, the properties of very different types of enzymes were remarkably improved, e.g., the enantioselectivity of lipases *(3)*, the hydroxylation activity of cytochrome P450 enzymes *(4)*, and the substrate specificity of aldolases *(5)*. The aim of this work was to establish a random mutagenesis technique for whole plasmids that can save time and material in comparison to established techniques, and thus facilitate the process of directed evolution of enzymes. Polymerase chain reaction (PCR) amplification of ring-closed plasmids using complementary primers with desired mutations is one of several possibilities to achieve site-directed mutagenesis. This technique (e.g., QuikChange site-directed mutagenesis kit; Stratagene, La Jolla, CA) starts with a double-stranded DNA vector including an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during a PCR of ring-closed plasmids. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. After PCR the product is treated with *Dpn*I, which is specific for methylated and hemimethylated DNA, to digest the parental DNA template and select for the synthesized DNA containing mutations. The nicked vector DNA incorporating the desired mutation is then transformed into *Escherichia coli* and can be recovered by plasmid preparation.

In the present study, plasmid amplification was used with rather the opposite intention, since the primers contained wild-type sequences and random mutagenesis was accomplished by error-prone PCR (epPCR), a technique that takes advantage of an increased error rate of DNA polymerases under certain conditions to introduce random mutations into a gene of interest *(6,7)*. The mutation frequency obtained in this process depends on the used polymerase and reaction conditions *(8,9)* and can, in many cases, be adjusted as desired. Primers binding to the β-lactamase gene were chosen for the "Bringer" strategy to allow its application to a broad range of target plasmids; however, other pairs of primers may also be used.

Materials and Methods

Plasmids and Chemicals

Plasmids pMOSblue (Amersham Pharmacia Biotech, Freiburg, Germany) and pWhitescript (Stratagene) are commercially available. Oligonucleotides were synthesized by BioTez (Berlin, Germany). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Roth (Karlsruhe, Germany), and isopropyl-1-thio-β-D-galactopyranoside (IPTG) was from BTS (St. Leon-Rot, Germany).

Oligonucleotide Sequences

The *bla* binding primers were Amp4 (5'-acg ata cgg gag ggc tta cca tct ggc cc-3') and Amprev (5'-ggg cca gat ggt aag ccc tcc cgt atc gt-3').

Error-Prone PCR

Basically, the Bringer protocol is a derivative of the Quik Change site-directed mutagenesis kit using PfuTurbo DNA polymerase (Stratagene). The reaction mix contained 5 µL of 10X reaction buffer (Stratagene), 25 ng of plasmid template, 125 ng each of oligonucleotide Amp4 and Amprev, 2 µL of dNTPs (5 m*M* each), 1 µL of TurboPfu, and ddH_2O to a final volume of 50 µL. The amplification program was as follows: (1) 95°C for 30 s, (2) 95°C for 30 s, (3) 55°C for 1 min, (4) 72°C for 9 min (2 min/kb of plasmid length), (5) repeat steps 2–4 29 times, (6) 10°C. The mutation rates observed in our experiments are in the range that would be expected according to the information from the polymerase manufacturer.

Restriction Digest and Transformation of E. coli

After epPCR, 1 µL of *Dpn*I was added and the reaction mix incubated for 3 h at 37°C. Five liters of *Dpn*I-treated DNA were used for transformation of supercompetent TOP10 cells (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions.

Results

Initial confirmation of the Bringer strategy was done by mutation of the α-peptide coding region of the enzyme β-galactosidase contained in plasmid pMOSblue (2.9 kb; Amersham). The aim of this experiment was to demonstrate that by epPCR of a ring-closed plasmid, sufficient numbers of mutations in the target gene (or cDNA) may be achieved. Thus, the plasmid was subjected to epPCR; digested with restrictase *Dpn*I to destroy the parental DNA; transformed into chemically competent *E. coli* cells; and plated onto media plates containing ampicillin, IPTG, and X-Gal. In this experiment, we obtained a total number of 1348 colonies, with 776 (= 57.6%) blue and 572 (= 42.4%) white. From these data, the overall mutation rate was calculated to be 3.7 mutations/plasmid or 12.8/10 kb. These results demonstrate that the very simple mutagenesis approach under study yielded considerable numbers of mutant lacZ clones.

The next step of our evaluation and a much more stringent test of the Bringer strategy was an experiment in which a specific codon had to be mutated. For this purpose, plasmid pWhitescript (4.5 kb; Stratagene) was chosen because it contains a stop codon (TAA) instead of a glutamine codon (CAA) at amino acid position 9 of the α-peptide coding region of lacZ. As before, the plasmid was amplified by epPCR; digested with *Dpn*I; transformed into competent *E. coli* cells; and plated onto media plates with ampicillin, IPTG, and X-Gal (Fig. 1). Three independent experiments yielded a

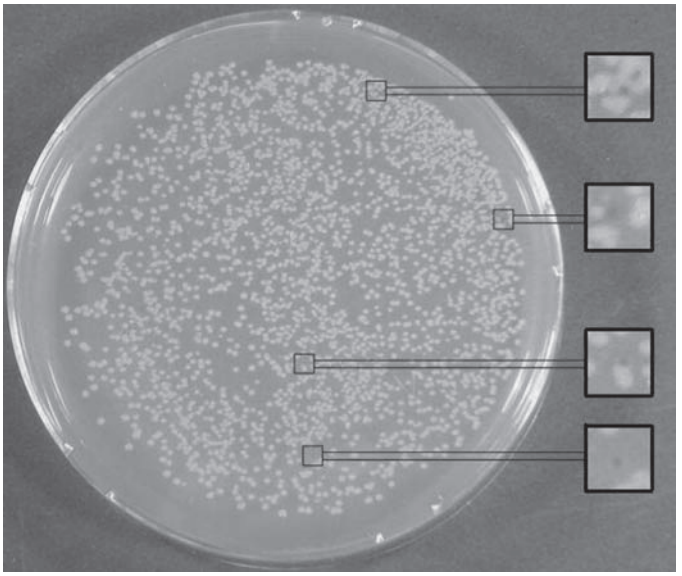

Fig. 1. Example of random mutagenesis of pWhitescript, which contains a point mutation at codon 9 of lacZ α-peptide sequence that introduces a stop codon, using Bringer strategy. The plasmid was subjected to epPCR and a *Dpn*I digest followed by bacterial transformation and growth on media plates with ampicillin, IPTG, and X-Gal. After overnight incubation, close to 2000 clones were obtained with four of them blue (boxes), which demonstrates the expression of a functional α-peptide and, thus, a successful reversion of the stop codon.

total colony number of about 5000, with 10 blue colonies (=0.2%). Sequencing of eight mutated plasmids (two were lost during isolation) confirmed that the TAA stop codon had been mutated to CAA in all cases, whereas the other Gln codon (CAG) was never observed. This preference would be expected since CAA is obtained from TAA by a single nucleotide exchange, whereas generation of CAG requires two mutation events. In addition, a bias of the polymerase might have played a role as well. We do not know whether substitution of Gln-9 by another side chain leads to a functional α-peptide but consider it unlikely since among all sequenced clones we did not find any triplet for one of the other five amino acids (Glu, Leu, Lys, Ser, Tyr) that could be created by a single mutation. Under the assumption that no other mutations had occurred, the mutation rate observed in this experiment was approx $0.002 \times 4500 = 9.0$/plasmid or 20/10 kb.

The setup of this experiment can be compared to a screen for gain-of-function mutations in a specific region of a protein (e.g., its active center or a binding region for another protein), and its outcome proves the applicability of the Bringer strategy to this type of experiment, because in one round of mutagenesis an average of about three hits of a single base pair was obtained. The mutation frequencies presented in this work are not among the highest ones reported to date, but this was not the aim of our study, because many publications on this topic are available *(10,11)*.

Discussion

Random mutagenesis of cloned genes is of fundamental importance for directed improvement or modification of well-characterized genes or gene products. Although many other creative approaches have been described, epPCR has emerged as the method of choice for most applications. The construction of random mutagenesis libraries using so-called megaprimers has been described very recently *(12)*, but this method, among other obstacles, suffers from unfavored PCR conditions owing to the utilization of very long and complementary cDNAs in the place of primers. In the present work, we present a versatile and convenient epPCR-based strategy coined Bringer that facilitates the construction of all kinds of mutant libraries. Although this system was established using oligonucleotides that bind to the *bla* gene, primers that bind to other selection markers (or to other components of plasmids) can be expected to perform similarly. The process of directed evolution by epPCR as a whole usually must cope with two bottlenecks *(13)*: (1) the subcloning step during the creation of the mutant library, and (2) the screening step once the desired library has been established. Which of these steps constitutes the more important obstacle depends on the particular circumstances of the task. In general, a distinction can be made between cases in which the screening can be done in bacteria (Fig. 2A) and those cases in which the library must be transferred into other cells (e.g., mammalian cell lines), usually after its amplification in *E. coli* (Fig. 2B).

Creation of mutant libraries using the Bringer procedure should in many cases avoid the subcloning bottleneck, and, accordingly, its application will bring most benefit where the creation of a useful mutant library constitutes the main impediment. This applies to many cases in which the screening procedure cannot be performed in bacteria, because library amplification in *E. coli* leads to a bias against sequences whose expression compromises or inhibits growth of the bacteria, thus leading to an overrepresentation of some sequences and to an underrepresentation or even to a loss of others. With the Bringer strategy this problem can be overcome, because every single mutation created by the epPCR should basically be represented in the library. Since this procedure creates mutations in all parts of the plasmid, unfavorable mutations might occur in the promotor region or in selection marker sequences; however, many promotor mutations can be expected to have only minor consequences, as will mutations in the *bla* gene if the screening system (such as a yeast two-hybrid screen) is not based on *E. coli*. In addition, the conditions of epPCR can be set to aiming for one mutation per plasmid, so that the simultaneous creation of desirable and undesirable mutations in the same plasmid becomes unlikely.

Point mutations of proteins are usually more harmful than beneficial, and, consequently, the probability that an enzyme will be improved with respect to its parent sequence decreases rapidly with increasing mutation

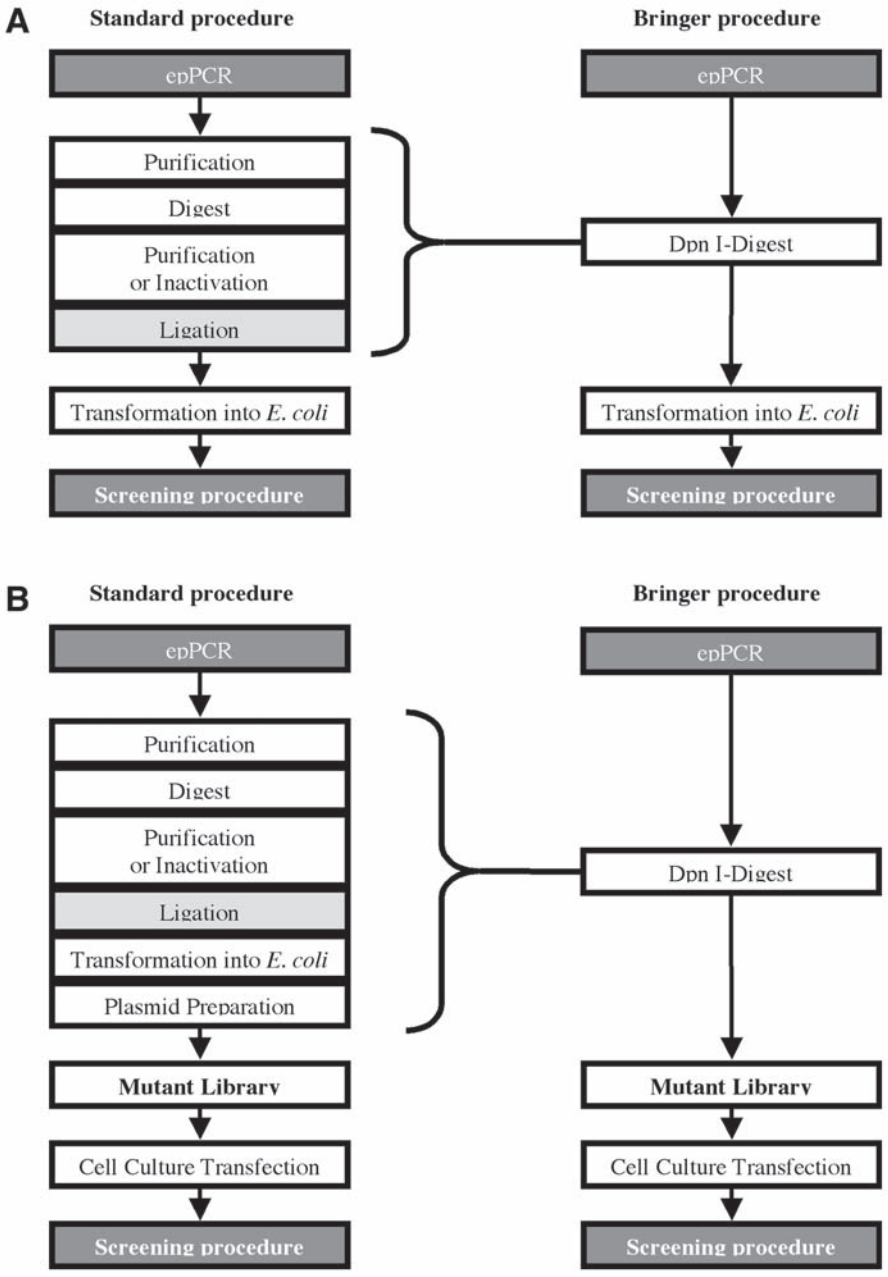

Fig. 2. Schematic comparison of creation of mutant libraries by random mutagenesis according to current strategy (*left*) and new Bringer strategy (*right*). **(A)** Procedures leading to mutant libraries that can be screened directly in *E. coli*. Since subcloning of the epPCR products is unnecessary with the new strategy, several manipulations (including the comparatively inefficient ligation and transformation steps) are omitted. Instead, the single necessary procedure is a simple restriction digest with *Dpn*I that eliminates the parental DNA. **(B)** Procedures leading to mutant libraries that are to be screened in other organisms, such as mammalian cells. In this case, the Bringer strategy allows omission of two additional steps (transformation into *E. coli* and plasmid preparation) in comparison to the first scenario, because after digestion of the parental plasmid by *Dpn*I, the epPCR product can be used directly for transformation of other cells.

rate. A very successful strategy of directed evolution therefore consists of the accumulation of many small improvements during multiple rounds of epPCR *(14)*. However, repeated cycles of any mutagenesis strategy lead to an accumulation of neutral mutations, which, e.g., may make a protein immunogenic *(15)*. Moreover, computer simulations *(16)* have suggested that point mutagenesis alone may often be too gradual to allow the block changes that are required for significant changes in selectivity or specificity, and the number of different sequences that can be created by mutating a protein grows exponentially with the number of mutations. Thus, the generation of very large libraries may be desired when selection can be employed to screen them, as in the case of antibiotica *(17)*, and application of the Bringer procedure in these instances should yield an important advantage.

In summary, if mutant libraries of rather normal size (i.e., containing about 10^4–10^6 variants *[14]*) are to be created by epPCR, and if the screening of these mutants can be done in bacteria, the main advantage of applying the Bringer strategy lies in its easy handling and in saving time. In those cases in which the same expression vectors are used for mutagenesis of different genes, which is often the case, mutant libraries of several genes can be constructed in parallel. Furthermore, after the preparation of an expression plasmid containing the wild-type gene that is to be mutated, a corresponding mutant library can be created within a few hours (in the time needed for the epPCR and the *Dpn*I digest), which constitutes a great reduction in the expenditure of time. For example, we created a yeast two-hybrid library of more than 40,000 independent clones overnight (data not shown). Further assets of this procedure emerge in those instances in which significantly larger mutant libraries are desired, screening cannot be done in bacteria, or the subcloning step faces specific difficulties (e.g., when working with nucleic acids or proteins that are toxic to bacteria). Accordingly, a significant improvement in directed evolution tasks by the use of the Bringer strategy can be expected if several of these conditions apply.

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