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Synthetic Point Mutagenesis

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1. Introduction

Over the last 5 decades, because of the revolution in recombinant DNA technology, site-directed mutagenesis became one of the most powerful tools in molecular life sciences. Its power lies in the ability to manipulate a specific DNA sequence in a definable and often predetermined way. Subsequently, genetic changes are correlated with a change in phenotype. Hence, this method offers the possibility to test protein functions down to the individual amino acid level and to characterize single nucleotides within regulatory RNA and DNA elements. In addition, synthetic point mutagenesis makes it possible to test whether specific mutations in a gene are benign or detrimental and thus lead to disease or not, in a model organism (Smith, 1985).

2. Point mutagenesis, a historical perspective

During the 1920's, Hermann Joseph Muller, was the first scientist to report the effects of mutagens on DNA. He demonstrated that there was a quantitative relation between the exposure to x-ray radiation and lethal mutations in *Drosophila*. He used this technique later on to create *Drosophila* mutants for his genetic research. Furthermore, he noted that x-rays could not only mutate genes in fruit flies but also have effects on the human genome (Crow, 2005).

In 1941, Charlotte Auberach demonstrated similar results as Muller using mustard gas as a mutagen in microbes and *Drosophila*. Within her studies several biological effects of mutations were described such as mosaicism and the heritability of mutations when occurring in the gametes (Sobels, 1975).

In 1978, Hutchison *et al.* (Hutchison et al., 1978) were among the first to describe site-directed mutagenesis using synthetic oligonucleotides. They demonstrated that a chemically synthesized oligonucleotide annealed to a phage genome could produce a specific mutation when incorporated in a closed circular phage genome by *in vitro* enzymatic reactions. The basic procedure was to use a mutation-bearing oligonucleotide as a primer for DNA polymerase to synthesize a mutant single-stranded genome. A ligase was included so that the newly elongated DNA strand was ligated into the circular vector (Smith, 1985; Hutchison et al., 1971; Hutchison et al., 1978). Later on, several other techniques were described, including site-directed mutagenesis of double-strand plasmid DNA vectors and targeted random mutagenesis using chemical reagents (Botstein et al., 1985). A major

downside of these site directed point mutagenesis techniques was that they were time-consuming. In particular the introduction of the mutated target sequence into the appropriate plasmid vector systems required multiple time-consuming steps. Furthermore, designed mutations were often obtained at low frequencies (Ho et al., 1989).

The development of the Polymerase Chain Reaction (PCR) technique by Mullis in 1983 provided opportunities to resolve these drawbacks. PCR uses two synthetic oligonucleotides as primers to exponentially amplify a specific DNA sequence. The primers are complementary to the ends of the amplified DNA and are oriented in opposite directions. Exponential amplification of target DNA occurs in multiple cycles of DNA duplex denaturation, primer annealing to single strand DNA and annealed primer extension by DNA polymerase. PCR is familiar as a method for the detection of a specific DNA sequence (Bartlett et al., 2003). As exemplified below, this method is also commonly applied to modify DNA sequences by design, using protocols that are collectively known as PCR-mediated site-directed mutagenesis.

3. Site-directed mutagenesis I: PCR synthesis of bacterial plasmid vectors

Site-directed mutagenesis is an important method to investigate the relationship between a given gene and its function. The technique is based on hybridization of a synthetic DNA primer containing the desired point mutation(s) with single-stranded target DNA encompassing the gene of interest. The hybridized DNA primer is extended by a DNA polymerase creating double-stranded DNA that can eventually be transformed into a host cell and cloned (Carter, 1986).

Over the years optimization has taken place, creating a rapid and simple to execute protocol to alter plasmid-borne genes with high fidelity in a specific and precise fashion *in vitro*.

One of the first methods used mutagenic primers which, after PCR, incorporate point mutations close to the PCR fragment's ends. After PCR amplification the PCR products had to be cleaved with restriction enzymes and inserted in place of the wild-type sequence in a bacterial plasmid vector. However, the above site directed point mutagenesis protocol was limited by the (non)availability of restriction sites in the close vicinity of the DNA sequence to be mutated. Furthermore, wild-type clones were recovered at a high frequency, making it tedious to isolate the desired mutant clones (Kadowaki et al., 1989). Hemsley *et al.* (Hemsley et al., 1989) improved the protocol by using an adaptation of inverse PCR, amplifying the whole circular plasmid to incorporate mutations at any site of the plasmid without relying on restriction sites in the close vicinity. This approach was fast and simple, and only a small amount of plasmid template DNA was required. Still, after PCR, linear instead of circular mutated plasmid DNA was obtained and this was physically separated from the original plasmid and circularized by ligation before transformation in *E. coli*. Despite these improvements the frequency of transformed bacteria harboring the desired mutant plasmid was still relatively low (Hemsley et al., 1989). Furthermore, the rates of unwanted random base substitution in these PCR reactions varied according to the reaction conditions, the precise DNA sequence and the DNA polymerase that had been used. Optimization of DNA polymerase fidelity in several PCR applications reduced the error-rate and thereby increased efficiency. The 3'→5' exonuclease activity of proofreading DNA polymerases is one of the most important replication error correction mechanisms *in vivo* (Eckert et al.,

1991). Where former studies used thermostable DNA polymerases without 3'→5' exonuclease activity, nowadays thermostable DNA polymerases with proofreading activity are included to reduce the error-rate. For PCR controlled mutagenesis a balance between proofreading and mutagenesis is sought so as to allow introduction of desired point mutations into the target DNA sequence but not other mismatches. Combining two different DNA polymerases not only reduced the random error-rate but also allowed efficient amplification of considerably longer PCR fragments. This led to the invention of 'long-distance PCR' where fragments up to 40kb in length are amplified, solving some of the technical limitations ascribed to gene and plasmid size (Barnes, 1994).

Current site-directed mutagenesis of plasmid-borne DNA sequences (eg. Figure 1) use two complementary mutagenic overlapping primers that bind to the two complementary sequences at the site of the desired mutation and amplify the entire plasmid. Mutated plasmids are created through heteroduplex formation between the wild type template and the mutation-bearing primers during the first PCR cycles, normally using a mixture of DNA polymerases with and without proofreading activity, where consecutive cycles realize amplification. Where former mutagenesis protocols had trouble separating the mutated plasmid from the original template, newly marketed techniques resolved this problem. Restriction enzymes, like *DpnI* (target sequence, 5'-G^{me}ATC-3') are specific for methylated and hemimethylated *E. coli* DNA and will degrade the original plasmid by enzymatic digestion (Figure 1) (Fisher et al., 1997; Li et al., 2002). The mutant PCR products, however, will be preserved from cleavage because they are enzymatically synthesized *in vitro* using unmethylated adenosine, contrary to the original plasmid templates produced by *E. coli* which are methylated at every GATC palindromes. Although after amplification by PCR the mutant plasmid DNA is not a pristine closed double stranded DNA plasmid, the presence of single strand nicks is not an obstacle for transformation in bacteria, indicating that endogenous bacterial enzymes repair the PCR products (Figure 1). Overall, this technology offers the possibility to easily engineer and obtain mutated cloned plasmids in a highly efficient fashion in less than one week (Aslam 2010).

4. Site-directed mutagenesis II: Vector engineering via homologous recombination in bacteria

A second modern point mutagenesis protocol relies on homologous recombination of PCR products in bacteria and was pioneered by the Stewart laboratory at the EMBL in the late 1990s.

Homologous recombination is a class of genetic recombination in which genetic information is exchanged between two identical or nearly identical DNA molecules. In eukaryotes homologous recombination is used to repair several types of DNA damage and it plays a vital role during meiosis, creating genetic diversity in sexually reproducing populations. In bacteria, the main function of homologous recombination is to rescue DNA replication forks that collapsed due to template strand DNA damage. Bacteriophages also code for homologous recombination enzymes, indicating that they employ homologous recombination to maintain themselves at some yet undetermined point of their life cycle. Furthermore, bacterial homologous recombination also occurs during conjugation when plasmid and/or chromosomal DNA is transferred between bacterial cells in a process called horizontal gene transfer.

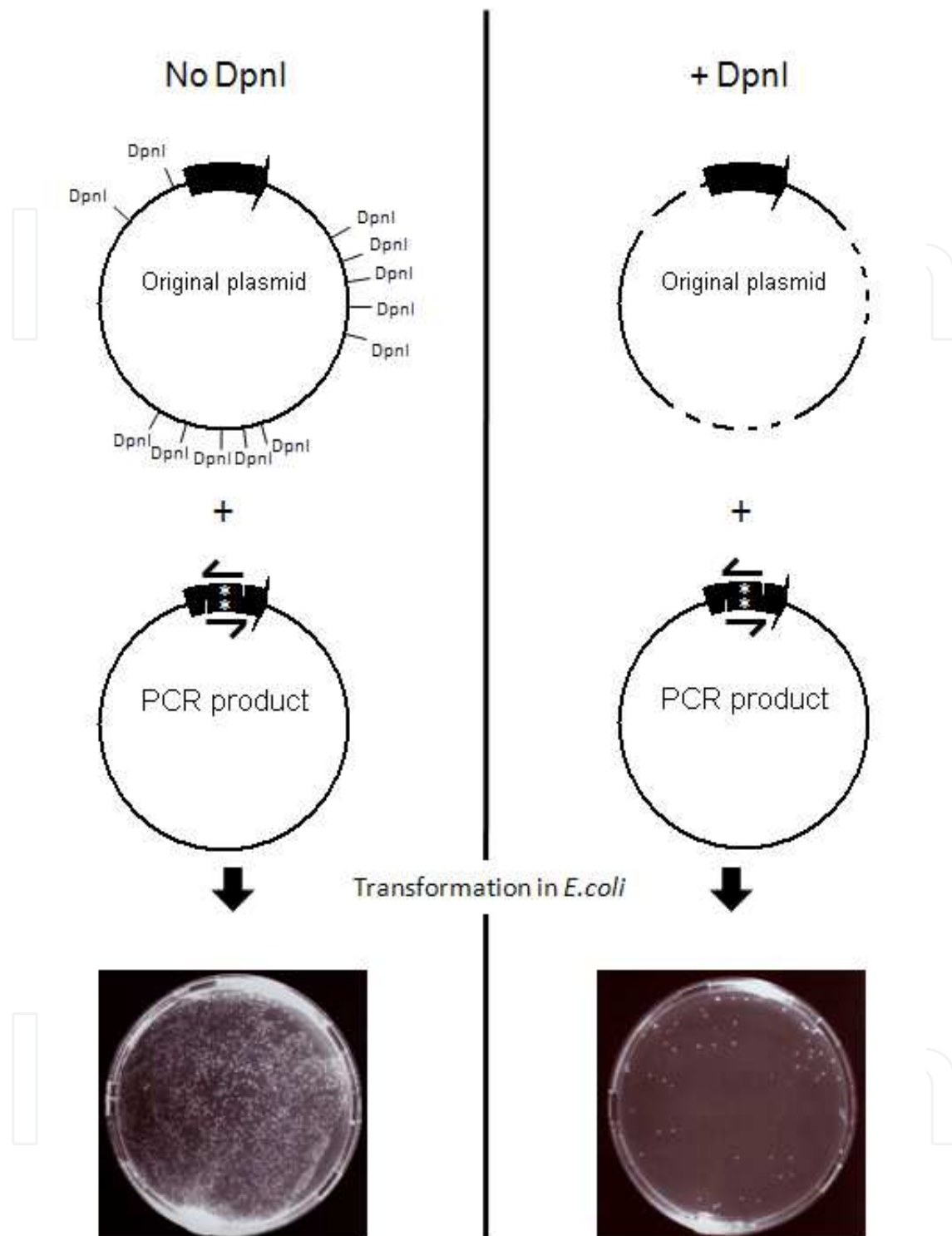


Fig. 1. Bacterial plasmid vector mutagenesis by PCR. A purified bacterial plasmid with the sequence to be mutated is amplified by two complementary PCR primers (black arrows) bearing the desired point mutation (indicated by a white star). The *DpnI* methylated-DNA-specific restriction enzyme is then used to destroy the original bacterial DNA vector, leaving only the mutant PCR version of the plasmid intact (right hand side). Destruction of the original template greatly reduces the number of *E. coli* clones, most of which now harbour the desired mutant plasmid.

Recombinogenic engineering, also known as 'recombineering' exploits homologous recombination to modify a DNA molecule inside bacteria in a precise and specific manner (Figure 2). Modification of a DNA molecule is accomplished by generating a synthetic targeting DNA construct (e.g. by PCR amplification) containing two regions that are homologous to regions flanking the target site. The targeting construct is introduced into an appropriate bacterial host cell that already bears the locus to be modified. Homologous recombination factors first resect the double strand DNA ends of the targeting construct into 3' single strand DNAs that align the construct with the double strand target locus and then insert it there via DNA polymerization at the invading 3' end, followed by resolution of the resulting Holliday junctions. This is the case for simple modifications, such as inserting or deleting a DNA sequence. In many cases a second recombination step is performed to remove the selectable and counter selectable gene (Figure 2). One major advantage of this technique is that the sequences of homology regions can be selected freely, so long as they are unique, which makes it possible to specifically alter DNA sequences in bacteria at almost any position. The second advantage of two step recombineering is that it is possible to design strategies whereby the resultant mutant DNA bears no sequence alterations other than the desired mutation (Muyrers et al., 2000a; 2001).

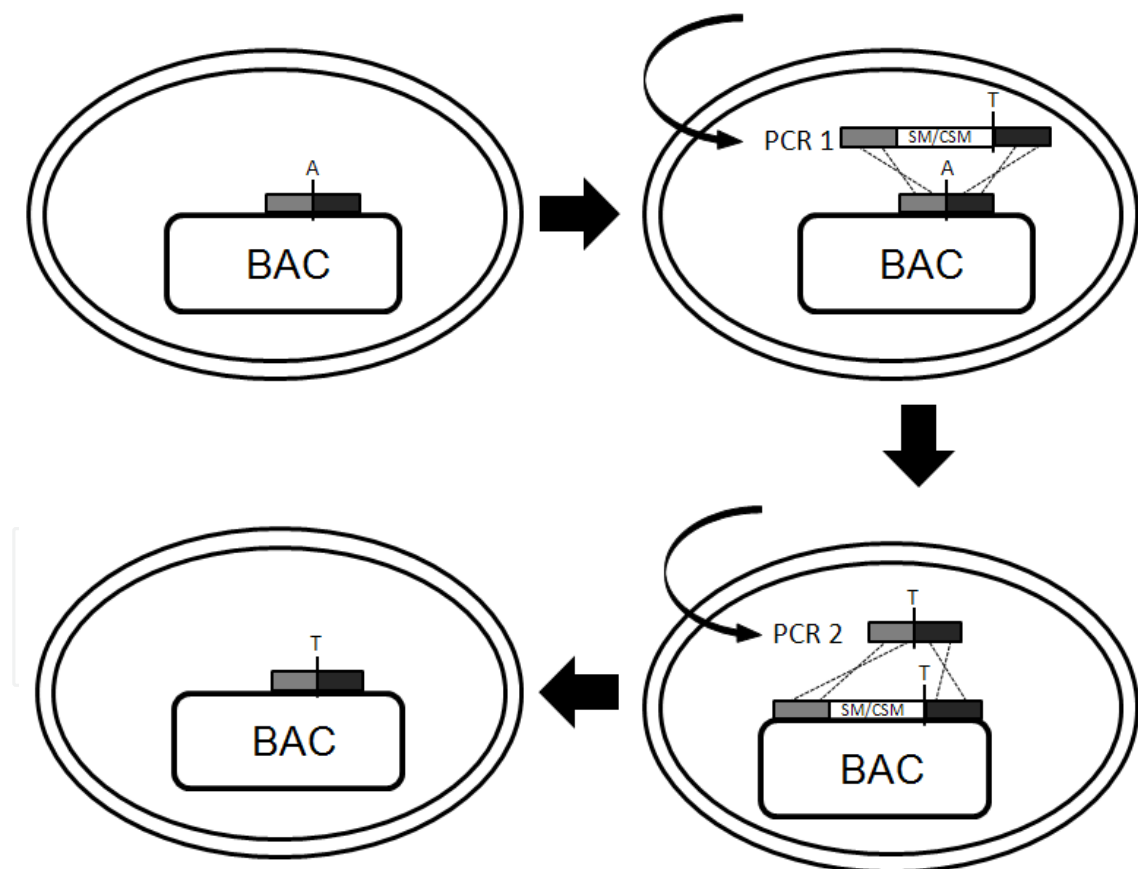


Fig. 2. Bacterial artificial chromosome point mutagenesis Two PCR products, bearing ~ 50 bp homology arms (gray and black boxes) are used in sequence to select bacteria harbouring, firstly a chromosome with the desired point mutation together with a selectable/counter selectable gene and secondly to remove the selectable/counter selectable marker, leaving only the desired point mutation.

Bacterial artificial chromosomes (BAC) are usually more than 100 kb large, rendering their ex-vivo manipulation exceedingly difficult, mainly because these giant DNA molecules tend to fragment through exposure to shearing forces typically associated with liquid solution manipulation. Because BACs are vectors that can carry very large inserts, they do offer the opportunity to manipulate complete mammalian genes because the genomic DNA inserts include gene promoters, exons, introns and regulatory regions. BACs thus provide a milieu to modify higher eukaryote chromosomal segments. Recombineering can be applied to introduce precise changes in the nucleotide sequence of BACs in bacteria via homologous recombination factors and to then transfer the engineered DNA back into a mammal. The first part of this procedure has been named ET recombination, as it precisely engineers BACs using the homologous recombination factors RecE and RecT enzymes of the λ prophage or the equivalent Red α and Red β proteins of Lambda phage. RecT and Red β are DNA strand annealing factors (Erler et al., 2009) whilst RecE and Red α are their partner 5'→3' exonucleases (Muyrers et al., 2000b). ET recombination relies on synthetic linear DNA fragments that contain at least 50 nucleotide long homology arms, which are normally generated by PCR, to mediate homologous recombination at the desired region on the target (BAC) DNA. The use of linear targeting DNA fragments increases the efficiency of gene modification significantly because double strand DNA ends are the actual substrates for the RecE and Red α exonucleases.

A two-step use of ET recombination was developed to create subtle changes in DNA in bacteria, such as point mutations. The two-step protocol is based on a fusion protein that permits selection as well as counter selection. Hence, mutagenesis is divided in a selection and a counter selection step. In the first step, PCR is applied to create a linear DNA fragment containing the (counter)selectable fusion gene, the desired point mutation and 50 nucleotide homology arms identical to the target DNA (Figure 2). Once that is successfully introduced by ET recombination, a second PCR fragment containing the original sequence of the BAC region and the desired point mutation is introduced into competent bacteria. This second PCR product enables a homologous recombination event that precisely deletes the counterselectable marker. The bacteria with the desired mutation are then selected by killing all the bacteria that still harbor the counterselectable gene. This two-step ET recombination can be applied to introduce single nucleotide substitutions as well as kilobase-sized insertions or deletions at any position in a desired locus encompassed by a BAC. Site-directed mutagenesis can also be performed on the actual bacterial chromosome, on BACs as exemplified here, or on any other double stranded DNA that is stably propagated in bacteria (Muyrers et al., 2001).

5. Conclusion

Over the years site-directed mutagenesis became an important tool to specifically mutate DNA molecules by design. Multiple studies contributed to the development of several techniques that can incorporate different kinds of mutations in rapid, simple and efficient ways. PCR fragment-mediated mutagenesis is one of these techniques. It permits efficient point mutagenesis of genes borne on plasmid vectors. On the other hand, combining PCR with recombineering in bacteria results in methodologies that can be applied to modify megabase-sized DNA fragments borne on artificial bacterial chromosomes, relying on the high fidelity associated with in vivo DNA replication. Subsequent transfer of such modified

BAC DNA into mice makes it possible to study the consequences of subtle chromosomal alterations, down to single nucleotide changes, within the native chromosomal environment. Application of these techniques on a large scale is leading to a better fundamental understanding of biology and disease aetiology (Skarnes et al., 2011) as well as providing opportunities to improve on what nature has offered to date.

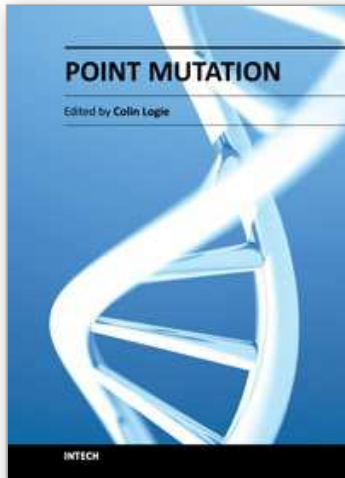
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This book concerns the signatures left behind in chromosomes by the forces that drive DNA code evolution in the form of DNA nucleotide substitutions. Since the genetic code predetermines the molecular basis of life, it could have been about any aspect of biology. As it happens, it is largely about recent adaptation of pathogens and their human host. Nine chapters are medically oriented, two are bioinformatics-oriented and one is technological, describing the state of the art in synthetic point mutagenesis. What stands out in this book is the increasing rate at which DNA data has been amassed in the course of the past decade and how knowledge in this vibrant research field is currently being translated in the medical world.

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