

Targeted Mutagenesis in *E. coli*: A Powerful Tool for the Generation of Random Mutant Libraries

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Mutagenesis is widely used in the fields of genetics, enzyme catalysis, ligand-binding recognition, metabolic regulation, control of gene expression, and to study mechanisms of DNA repair. Mutations are also introduced to optimize enzyme performance. This area has attracted renewed interest with the increased use of biocatalysts in chemical and pharmaceutical synthesis, in bioremediation, and in biotechnology.

Individual mutations are introduced, based on detailed structural and functional information, to generate enzymes or other proteins with novel properties and to change the properties of regulatory sequences such as promoters and origins of replication (site-directed mutagenesis). Alternatively, variants of interest can be identified in large libraries harboring random substitutions (random mutagenesis). Unlike site-directed mutagenesis, random mutagenesis requires little or no previous information of the targeted genes. This approach, however, typically allows only a small fraction of all possible mutants to be analyzed. This is due to a number of factors including the inordinate numbers of possible mutations, the need to identify individual mutants of interest from a large pool, the generation of

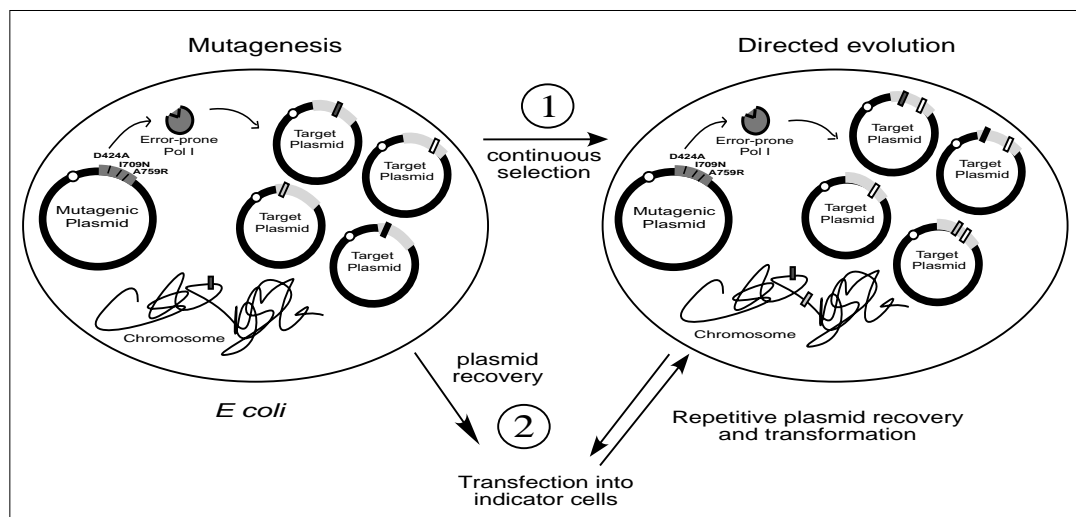
non-functional mutants, and the nature of the genetic code.

Libraries containing random mutations may be generated in vitro by treatment of DNA with mutagens, by mutagenic PCR, or by substituting portions of plasmid-encoded genes with oligonucleotides containing random substitutions. These protocols have few limitations on the number of mutations that may be introduced per gene. Random mutant libraries may also be generated in vivo by replicating the DNA encoding the sequence of interest in a mutagenic cellular environment. Libraries generated in "mutator strains" tend to exhibit a wider spectrum of mutants given that no cloning steps are involved. However, the complexity of these libraries (i.e., the number of mutants containing multiple substitutions) is restricted by the fact that high levels of mutagenesis normally interfere with the survival of the host. To overcome this limitation, complex combinations of mutations may be built up by allowing the accumulation of mutations with a positive effect on the desired phenotype through iterative mutagenesis and selection, a strategy known as "directed evolution."

A system of random mutagenesis specifically designed to facilitate the directed evolution of proteins or of regulatory sequences has recently been developed (Camps et al., 2003). The system is delineated in the figure below. Mutagenesis is performed in *E. coli* cells transformed with 2-plasmids: one plasmid encodes an error-prone Pol I protein ("mutagenic plasmid") and the second plasmid encodes the sequence to be mutagenized ("target plasmid"). The mutagenic plasmid is Pol I-independent. Errors in the replication of the target plasmid by error-prone Pol I result in mutations. Provided that a complementation or drug selection can be established in the strain used for mutagenesis, a continuous positive selection should lead to the directed evolution of the target sequence.

Alternatively, the libraries thus generated may be retrieved and retransformed into the appropriate indicator strain for selection or screening. In this case, directed evolution may be accomplished by iterating mutagenesis with selection/screening of the recovered library.

The 2-plasmid system was first described by Fabret et al. (2000), which expressed a mutant of Pol I deficient in proofreading (*exo⁻*) in a mismatch-deficient strain. In an advanced version of this system, we generated a mutant of Pol I containing



three point mutations in determinants of fidelity (Camps et al., 2003). This highly error-prone Pol I was expressed in a Pol I-deficient strain. The frequency of mutagenesis in the target sequence is in the same range as that exhibited by the most efficient mutator strain described to date, XL1-Red (Greener et al., 1997).

However, this advanced 2-plasmid system offers a number of critical advantages, all of which ultimately derive from the fact that mutagenesis is driven exclusively by error-prone Pol I replication. No inactivation of major pathways of DNA repair in the host is required, with the consequent improvement in growth and in transformation efficiency. Further, given that Pol I only plays a limited role in chromosomal replication, mutations of the target plasmid are 400 times more frequent than mutations in chromosomal genes. Thus, saturation of mismatch repair, which typically results from widespread chromosomal mutagenesis, is avoided. As a consequence, our 2-plasmid system yields a more random distribution of mutations and a more balanced mutation spectrum in the target gene. Another advantage of the 2-plasmid system is that mutagenesis by Pol I (which is conveniently encoded in a transferable element) should be substantially independent from the genetic background of the host. Thus, in principle the 2-plasmid system may not be restricted to Pol I-deficient strains and should be amenable for use in different genetic backgrounds. Preliminary work in our laboratory confirmed that expression of error-prone Pol I is mutagenic in cells encoding wild-type Pol I. It appears, however, that efficient mutagenesis requires adjusting culture conditions to each particular strain.

In order to verify the efficacy of our 2-plasmid system, we evolved TEM-1 β -lactamase in the presence of a third generation antibiotic, aztreonam. Cells transformed with mutagenic and target plasmid were grown in the presence of increasing concentrations of aztreonam. In two independent selections, combinations of up to three mutations were identified. Individually, each of the mutations enhanced antibiotic resistance, with the triple mutant conferring the greatest resistance. Strikingly, selected clones harbored no neutral or detrimental non-synonymous mutations. Only one silent mutation was found in one of the selections. This high density of relevant mutations is in contrast to what would have been expected if multiple substitutions had been obtained in a single round of mutagenesis. These observations confirmed that the multiple substitutions in β -lactamase occurred by a process of directed evolution.

In summary, the 2-plasmid system of random mutagenesis in *E. coli* offers a simple approach for the random mutagenesis of proteins and regulatory sequences. Extensive mutagenesis can be achieved by growing a plasmid containing the sequence of interest in cells expressing a highly

error-prone Pol I. Mutagenesis and selection can be either performed simultaneously or they can be easily iterized. It is our hope that this system or others similarly derived will accelerate progress in fields that use mutagenesis to probe structure-function relationships or to overcome rate-limiting steps for industrial and chemical applications.

Ultimately, this technology should enable improvements in complex biosynthetic or degradative pathways and should accelerate the generation of improved enzymes for pharmaceutical or industrial applications.

References

Targeted gene evolution in *Escherichia coli* using a highly error-prone DNA polymerase I.

Camps M, Naukkarinen J, Johnson BP, Loeb LA. University of Washington, Seattle, WA, USA. *PNAS* 100:9727-9732, Aug. 19, 2003.

Summary: The authors describe the generation of a highly error-prone Pol I containing three point mutations at critical determinants of fidelity. Expression of this polymerase in a 2-plasmid system results in a dramatic increase in the frequency of mutations in a target plasmid, with a preference for the plasmid over the chromosomal sequence. β -lactamase is successfully evolved using the advanced 2-plasmid system. Strong evidence of sequential mutagenesis is presented.

Efficient gene targeted random mutagenesis in genetically stable *Escherichia coli* strains.

Fabret C, Poncet S, Danielsen S*, Borchert TV*, Ehrlich SD, Janniere L.

INRA, 78352 Jouy-en-Josas, France and Novo Nordisk (*), Bagsvaerd, Denmark. *Nucl Acids Res* 28:e95, 2000.

Summary: The 2-plasmid system approach is described for the first time. A Pol I mutant with inactivated proofreading function is used as error-prone Pol I. Additional inactivation of mismatch repair is required to enhance (Pol I) mutagenesis. Efficient mutagenesis is reported using the LacI and the lipase-based forward mutation assays, and is shown to be cumulative with prolonged passage in culture.

An efficient random mutagenesis technique using an *E. coli* mutator strain.

Greener A, Callahan M, Jerpseth B. Stratagene Inc., La Jolla, CA, USA. *Mol Biotechnol* 7:189-195, 1997.

Summary: The authors present protocols for use of the mutator strain XL-1 Red, which expresses a DNA polymerase III deficient in proofreading activity (MutD) in combination with inactivation of mismatch repair (Mut T) and of the oxo-dGTP repair pathway (MutS). A high frequency of mutations is achieved, but with some associated problems.