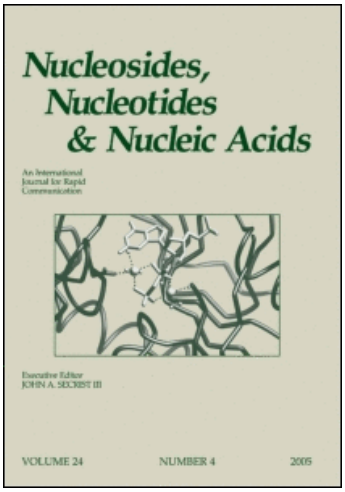

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Site Directed Mutagenesis on the Restriction Endonuclease Eco RI Using Mixed Oligonucleotides

Andreas Dusterhöft[a]; Manfred Kroger[a]
[a] Institut für Mikrobiologie und Molekularbiologie der Justus-Liebig-Universitat Gießen, Gießen, West-Germany

To cite this Article Dusterhöft, Andreas and Kroger, Manfred(1988) 'Site Directed Mutagenesis on the Restriction Endonuclease Eco RI Using Mixed Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 7: 5, 737 — 740
To link to this Article: DOI: 10.1080/07328318808056321
URL: http://dx.doi.org/10.1080/07328318808056321

NUCLEOSIDES & NUCLEOTIDES, 7(5&6), 737-740 (1988)

SITE DIRECTED MUTAGENESIS ON THE RESTRICTION ENDONUCLEASE *Eco*RI USING MIXED OLIGONUCLEOTIDES

*Andreas Düsterhöft and Manfred Kröger**
Institut für Mikrobiologie und Molekularbiologie der
Justus-Liebig-Universität Gießen
Frankfurter Straße 107, D-6300 Gießen, West-Germany

ABSTRACT The restriction endonuclease *Eco*RI could be modified via site directed mutagenesis at position Arg200. Using the thiophosphate system we introduced either Lys, Glu or Gly in a one pot procedure. Although G recognition should be affected, Lys200 showed wildtype specificity.

Restriction enzymes are the well known basic tools in gene technology. In order to enlarge the number of available specificities, numerous microorganisms have been screened throughout the world. However, it seems that only a limited number of specificities are available from natural sources, while certain identical specificities are repeatingly found. Thus we started a program, to achieve new specificities via protein design.

Some basic prerequisites have to be considered for the realization of such a program: i) Artificially obtained restriction enzymes need to be expressed in the absence of any natural protection system; thus they must be obtainable in the absence of methylation activity. ii) Complete structural information must be available in order to change amino acid residues in a controlled manner. iii) A simple and quick methodology must be available to both create and test mutant restriction enzymes. We present data, that we fulfilled these prerequisites on the model enzyme *Eco*RI, which recognizes the GAATTC sequence and on which recently an X-ray Structure was published.[1]

Expression vector system. We used the *E.coli* p_{tac}-*lac*I promoter-repressor system[2], within a derivative of the plasmid pJF118[3], which contains the *lac*I gene and additionally a more effective origin of replication, a defined translation start sequence, several multicloning sites and two more operator sequences in defined distance. In contrast

to our previous constructions, the promoter was now only active in the presence of the inductor isopropylthiogalactoside (IPTG). No leakage was observed, when we cloned the genes for the restriction endonucleases *Eco*RI or *Pst*I in the absence of their cognate methylase. In some cases we had to use *E.coli* cells with preformed repressor on a separate plasmid; see below. We present an example for our expression vector in Fig.1.

Structural information upon Eco*RI*. The published X-ray structure of *Eco*RI[1] provides evidence, that only three amino acid residues contribute to the specific recognition of the GAATTC DNA-sequence: Glu144, Arg145 and Arg200. The contact of these three residues is towards five different nucleic acid bases, due to a most astonishing bridging character of the Glu144 and Arg145 residues. However, some of the original conclusions seem to be already reevaluated by the same authors[4]. The Arg200 residue is described, to be solely responsable for the recognition of Guanosine within the GAATTC sequence. Since *Eco*RI possesses a so called star activity[5], we proposed, that in the absence of salt the contact between Arg200 and the Guanosine is missing. Any enzyme with such a star activity would be a 16 times more aggressive poison to the *E.coli* DNA than the wildtype enzyme. We wanted to learn, whether our system still provides enough protection for the cell.

Construction of mutants in position Arg200. We isolated from the plasmid pHK514 (see Fig.1) a *Bgl*II-*Pst*I fragment and cloned it into the single strand phage M13. As given in Figure 2 we used a mixture of oligonucleotides with twelve identical nucleotides each flanking the variations at two directly adjacent central positions. Preparation was performed on an Applied Biosystems A370 synthesizer with the mixed oligonucleotide option. Using a mixture of four oligonucleotides we were able to find simultaneously in a single mutagenesis procedure using the α-thiophosphate method[6] both single exchange mutants within twelve phages. The double exchange mutant and the revertant were obtained, when the experiment was repeated on the basis of the Lys mutant, again within only twelve phages analyzed. In a second step, the mutated fragments were reimplantated into pHK514, yielding the plasmids named in Fig.2.

*Properties of the mutated Eco*RI *restriction enzymes*. We constructed for each mutant two plasmids, either with or without the cognate methylase. As control the unmodified and the revertant enzyme were tested identically. The cells were killed almost immediately after induction

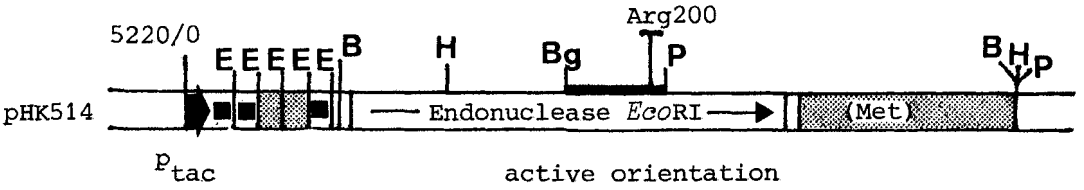

FIGURE 1. Partial map of the typical expression vector pHK514 containing three *lac*-operator sequences (■). The methylase (Met) is desactivated by truncation. The position of the mutated Arg200 is indicated above the reimplantated *Bgl*II-*Pst*I fragment drawn in a bold line. E = *Eco*RI, B = *Bam*HI, H = *Hin*dIII, Bg = *Bgl*II, P = *Pst*I.

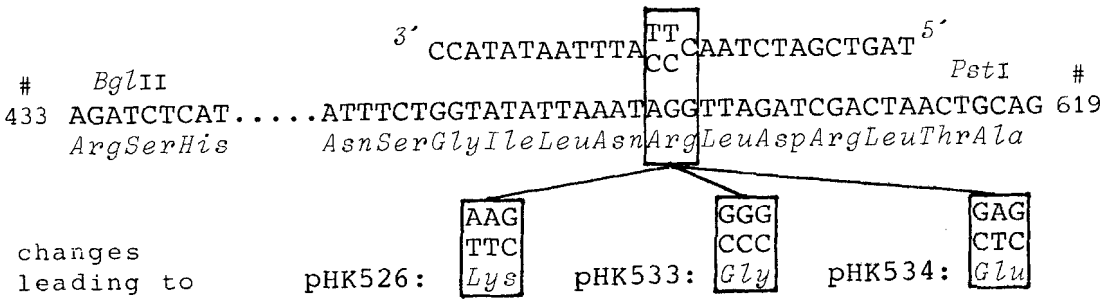

FIGURE 2. Position and sequence of the mixed oligonucleotide used for simultaneous introduction of three mutations at the Arginine 200. The given numbers refer to the ATG startcodon of *Eco*RI gene.

with IPTG, when the wildtype enzyme, the revertant or the Lys200 mutant were present in the correct orientation. The Gly200 and the Glu200 variants were, however, inactive even after induction.

One might have expected, that the acidic Glu200 residue leads to a severe change within the surface of the recognition pocket. One may also attribute a change in the overall structure of the enzyme, when the Gly residue is introduced. Thus it is comprehensible, that the enzyme lost all activity by introduction of these two modifications. However, introduction of the lysine instead of arginine is a minimal change, since the positive charge is still retained and the surrounding of the α-carbon is not changed directly. Thus we were pleased to find us unable, to introduce the Lys200 mutant into normal HB101 *E.coli* cells. Only, when we used an HB101 strain containing already another plasmid for high level expression of *lacI* repressor - pHK278 with tetracycline resistance

gene - we could clone the Lys200 mutant pHK526. Comparing the kinetics of the decrease in surviving cell number, we observed a more effective killing rate compared to the wildtype. However, the unmodified methylase gave full protection to both the Lys200 mutant and the wildtype enzyme. One may interpretate this result in the way, that we did not yet change the specificity, but the kinetic parameters of the enzyme.

In contrast to the observed increased activity in our *in vivo* experiments, the purified Lys200 mutant rather showed a thousandfold decreased activity, compared to the wildtype; leading to a clearly observable nicking activity at the GAATTC sequence. These data are obtained in cooperation with Dr. A. Pingoud (Hannover; to be published elsewhere), and can not be attributed to any uncontrolled change in the primary structure of the enzyme, since the Lys200 mutant is resequenced completely in Hannover.

Further studies will be performed, regarding the influence of physiological rather than optimal salt conditions, and must await a refined X-ray structure.

REFERENCES

1) J.A.McClarin, C.A.Frederick, B.C.Wang, P.Greene, H.Boyer, J.Grable and J.Rosenberg (1986) *Science 234*, 1526 - 1541.
2) E.Amann, J.Brosius and M.Ptashne (1983) *Gene 25*, 167 - 178.
3) J.P.Fürste, W.Pansegau, R.Frank, H.Blöcker, P.Scholz, M.Bagdasarian and E.Lanka (1986) *Gene 48*, 119 - 131.
4) S.D.Yanofsky, R.Love, J.A.McClarin, J.M.Rosenberg, H.W.Boyer and P. Greene (1987) *PROTEINS: Structure, Function and Genetics 2*, 273 - 282.
5) C.Kessler and H.-J.Höltke (1986) *Gene 47*, 1 - 153.
6) J.W.Taylor, J.Ott and F.Eckstein (1985) *Nucleic Acids Research 13*, 8765 - 8785.