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Saturation mutagenesis of Thr862, the amino acid essential for substrate specificity of Eco57I restriction endonuclease

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Type IIG restriction endonuclease (RE) Eco57I cleaves DNA 16/14 nucleotides away from the asymmetric recognition sequence 5'-CTGAAG. The enzyme also possesses methyltransferase activity that modifies the second A base within the 5'-CTGAAG strand of the target duplex (underlined). In previous studies, Eco57I mutants with altered substrate specificity 5'-CTGRAG were isolated. These mutant enzymes have Asn or Ser instead of Thr in the 862th position of the protein. In order to evaluate the impact of T862 on the substrate specificity, it was changed to the other 17 amino acids. The *in vivo* cleavage activity and substrate specificity of the resulting mutant enzymes was examined (i) by testing lethality of the mutants to the host cells in the absence or presence of Eco57I (specificity 5'-CTGAAG) and GsuI (specificity 5'-CTGGAG) methyltransferases, and (ii) by testing the ability of the mutants to induce SOS DNA repair response in the absence or presence of protecting methyltransferases. The results indicate that mutants T862G, T862C and, probably, T862A and T862D could display altered substrate specificity. The recognition sequence of T862F, H, K, L, Q, M and Y mutants was the same as that of the wild type enzyme. The remaining substitutions rendered the enzyme catalytically inactive.

Key words: Eco57I restriction endonuclease, altered specificity, saturated mutagenesis, SOS induction

INTRODUCTION

Type II restriction endonucleases are components of bacterial restriction-modification (RM) systems. Their principal biological function is protection of the host genome against foreign DNA, in particular bacteriophage DNA [1]. These enzymes recognize DNA sequences of 4–8 bp and, in the presence of Mg^{2+}, cleave DNA within or in close proximity to the recognition sequence [2]. In the bacterial cell, a corresponding DNA methyltransferase modifies the recognition sites of the host DNA, thereby protecting it from cleavage. As cleavage by restriction endonucleases at non-canonical DNA sequences would be highly toxic for the host, restriction enzymes have evolved to possess a remarkable degree of specificity. They cleave their recognition sequences by 5–6 orders of magnitude more quickly than sequences differing by only one base pair from the canonical sequence [3, 4].

Owing to their extreme specificity, restriction endonucleases are a paradigm for proteins that specifically interact with DNA, and are a challenging target for protein engineering. They have become indispensable tools in molecular biology and diagnostics. Despite the fact that more than 240 enzymes of different sequence specificity have been isolated from various bacterial strains, many specificities are still unavailable (www.rebase.neb.com). An increasing demand for the wider selection of restriction enzymes with varying recognition sequences stimulated efforts to generate new specificities by rational, *i.e.* structure-guided, design and random mutagenesis. However, engineering of new endonuclease substrate specificities has shown only limited success [5–8]. Evidence is accumulating that the specificity of REs is achieved by forming numerous contacts between an endonuclease and each base pair, as well as with the phosphodiester backbone of its recognition sequence and by coupling recognition and catalysis [2]. On the other hand, the absence of powerful and stringent selection methods was probably the main reason why experiments designed to select mutant REs possessing altered specificity after the random mutagenesis have failed.

Recently, we have developed a new procedure, called Methylation Activity Based Selection (MABS), for generating REs with a new specificity. MABS uses a unique property of bifunctional type II REs to methylate DNA targets they recognize. The effi-

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ciency of the MABS technique was demonstrated by altering the sequence specificity of the bifunctional RE Eco57I [9]. This enzyme recognizes and cleaves the DNA sequence 5'-CTGAAG$(N)_{16/14\downarrow}$, and methylates the second adenine (underlined) within its recognition sequence in the indicated DNA strand of the target duplex [10]. After MABS procedure, Eco57I mutants exhibiting an altered substrate specificity, 5'-CTGRAG, were isolated. It was the first successful example of experimental generation of a type II RE with a new specificity. All sequenced mutants had substitutions of Asn or Ser for Thr862 in the Eco57I protein sequence. Here, to understand the impact of 862th amino acid on the substrate specificity, we subjected T862 to saturation mutagenesis. The activity and substrate specificity of the resulting mutant enzymes was studied *in vivo*.

MATERIALS AND METHODS

The *E. coli* strain ER1727 [(*mcr*BC-*hsd*RMS-*mrr*)2::Tn*10*, *mcr*A1272::Tn*10*(Tet^r), F'*lac pro*AB *lac*I^q (*lac*Z)-M15] was used in cloning procedures and for expression of mutant genes. The indicator strain ER1992 [*dinD1* :: *lacZ* (Kan^R) F$^-$ *e14*$^-$ *(McrA*$^-$*) endA1 supE44 thi-1 relA1? rbfD1? spoT1? (mcrC-mrr)114 : IS10*] [11] was the host for SOS induction experiments. The strains were grown in Luria–Bertani (LB) broth in the presence of the following antibiotics when necessary: ampicillin (Ap), 60 μg/ml; kanamycin (Kan), 50 μg/ml; chloramphenicol (Cm), 30 μg/ml. Most of recombinant DNA experiments were performed as described in [12]. All enzymes were obtained from UAB Fermentas (Vilnius, Lithuania) and used according to the manufacturer's recommendations. Oligonucleotides used in site-directed PCR mutagenesis were synthesized at the facilities of UAB Fermentas. All desired mutations were confirmed by DNA sequencing.

RESULTS AND DISCUSSION

The recombinant plasmid pZA-Eco57I (Cm^R) contains the Eco57I endonuclease gene [13] under the control of $P_{lac/ara}$ promoter [14]. It was used for construction and expression of the mutants. A unique restriction endonuclease BpiI site near the T862 codon was created by introducing silent mutation and was used for the subcloning of DNA fragments obtained after mutagenesis. Recombinant plasmids bearing T862N and T862S mutant genes were constructed by substitution of DNA fragments carrying corresponding mutations from pET-Eco57IR$^{D78K/T862N}$ and pET-Eco57IR$^{D78K/T862S}$, respectively, [9] for the appropriate fragment in the pZA-Eco57I and were used as positive controls. T862 was changed to the other 17 remaining amino acids via site-directed PCR mutagenesis using mutagenic primers. The resulting mutant proteins were analyzed for their activity and specificity *in vivo*. The results are summarized in Table. The inactive mutants are not shown in the table.

Table. **Properties of Eco57I endonuclease mutants *in vivo***

Variant[a]	Colony growth[b]		SOS induction[c]		
	ER1727	ER1727 (M.Eco57I)	ER1992 (M.Eco57I)	ER1992	ER1992 (M.Eco57I, M.GsuI)
T (wild type)	–	+	db	LB	n.d.
N	–	–/+	db	db	MB
S	–	–/+	db	DB	LB
G	–	–/+	db	MB	MB
C	–	–/+	db	MB	MB
A	–	+	db	MB	LB
D	–	+	DB	MB	LB
F	–	+	DB	LB	n.d.
H	–	+	DB	LB	n.d.
K	–/+	+	DB	LB	n.d.
L	–/+	+	DB	LB	n.d.
Q	–/+	+	DB	LB	n.d.
M	–/+	+	DB	LB	n.d.
Y	–/+	+	DB	LB	n.d.

[a] The mutations were made in 862 position of Eco57I protein. Inactive mutants T862R, E, I, W, V, P are not shown in the table.

[b] The colonies were grown on LB agar plates containing IPTG (1 mM) and arabinose (0.05%); +, normal colony growth; –/+, small, poorly growing colonies; –, no growth. Three independent experiments were carried out.

[c] Transformants were plated on LB agar containing X-Gal (35 μg/ml) and arabinose (0.05%). Colony colour: db, dark blue, reduced in size; DB, dark blue, normal colony size; MB, medium blue; LB, light blue; n.d., not determined. Control transformants with pZA vector plasmid formed light blue colonies. Three independent transformation experiments were carried out.

Under noninducing conditions, the basal level of Eco57I expression from pZA-Eco57I is very low and not toxic for *E. coli* cells even in the absence of protecting methyltransferase (Mtase). Full induction of the Eco57I endonuclease gene with 1 mM IPTG and 0.05% arabinose kills host cells if the Eco57I recognition sites are not protected by methylation. However, cells survive in the presence of Eco57I Mtase encoded by the compatible plasmid pKpnORI-Km-Eco57IM (Kan^R) [9]. Recombinant plasmids carrying mutant Eco57I genes were introduced into *E.coli* ER1727 both in absence and in presence of a plasmid with the Eco57I Mtase gene. A few transformants were replicated in parallel onto LB agar plates containing IPTG and arabinose or without inducers. As expected, cells harboring all mutants could survive without Eco57I methylase protection if the expression of the mutant genes had been not induced. Overexpression of mutants T862N, S, G, C, A, D, F, H in the ER1727 strain was lethal for the host cells, while T862K, L, Q, M, Y mutants were only moderately toxic and formed small, poorly growing colonies. The results suggest that these mutants could exhibit unchanged or slightly reduced endonuclease activity. The remaining mutants T862R, E, I, W, V and P had no influence on cell growth, indicating that their endonucleolytic activity was reduced to the level undetectable *in vivo*. It is quite possible that the mutants could be inactive due to the loss of their native structure and insolubility (not determined). The effect of T862A, D, F, H, K, L, Q, M and Y mutants was blocked by Eco57I methyltransferase in the strain ER1727 (M. Eco57I) containing the plasmid pKpnORI-Km-Eco57IM, suggesting that all of them retained the wild type specificity. In contrast, four mutants (T862N, S, G and C) were toxic even in the presence of Eco57I Mtase, suggesting that these variants display a specificity differing from that of Eco57I. We have shown in our earlier experiments described in [9] that the specificity of the T862N mutant is 5'-CTGRAG. The same recognition sequence may be expected for mutants T862S, G and C, however, other specificities could not be excluded, either. The *E. coli* strain ER1727 (M. Eco57I, M. GsuI) containing Eco57I Mtase (recognition sequence 5'-CTGAAG) on pKpnORI-Km-Eco57IM and GsuI Mtase (recognition sequence 5'-CTGGAG) on pBR322-GsuIM (Ap^R) was cotransformed with plasmids encoding T862N, S, G and C mutants. Under conditions of full induction no one mutant had a toxic effect on cell growth, suggesting that their specificity was 5'-CTGRAG (not show).

Wild type Eco57I and mutants were also characterized for their ability to induce SOS repair response in the absence of Eco57I Mtase, in the presence of M. Eco57I and both M. Eco57I and M. GsuI. The DNA damage indicator strain ER1992 [11] carrying the *dinD :: lacZ* fusion was transformed with plasmids containing either the wild type or mutant Eco57I genes. The resulting transformants were plated on LB medium containing X-Gal indicator (35 μg/ml) and arabinose (0.05%) and were scored for colony colour (under these conditions the expression of *eco57IR* from $P_{lac/ara}$ promoter is only partially induced). Wild type Eco57I and mutants T862N, S, G, C, A induced SOS response in a strain lacking Eco57I methyltransferase, forming dark blue colonies of reduced size. Transformants carrying mutants T862D, F, H, K, L, Q, M and Y formed dark blue colonies of normal size, suggesting that their cleavage activity was partially reduced. The color of transformants containing mutants R, E, I, W, V and P was light blue like of negative control transformants carrying a vector plasmid. When plasmids with wild type or mutant genes were introduced into a strain carrying a plasmid expressing Eco57I Mtase, the mutants T862N, S, C, G, A and D damaged the DNA of their host cells and induced SOS response, indicating that the specificity could differ from the wild type Eco57I. Since the mutants T862A and T862D in our previous experiment were not toxic for cells in the presence of M. Eco57I, DNA cleavage at noncanonical sites by these mutants seems to be very week. Alternatively, the SOS response could be not due to their altered specificity. The T862N mutant induced SOS response even in the presence of both of M. Eco57I and M. GsuI. A possible explanation for such phenotype could be that the methylation carried out by M. Eco57I and M. GsuI was not complete. The same phenotype has been observed with mutants T862C and T862G, suggesting that their specificity is the same as that of T862N. However, other specificities are possible too.

These *in vivo* observations suggest that, in addition to previously described [9] mutants T862N and T862S which possess an altered substrate specificity, the Eco57I mutants with substitutions T862C, T862G and probably T862A and T862D gained the ability to cleave DNA sites differing from the canonical Eco57I recognition sequence. The cleavage specificity and activity of these mutants will be determined after the protein purification.

Based on our previously published results [9] and on results described in this study, we propose that Thr862 is essential for the substrate specificity of Eco57I RE. The mutations of T862 could affect substrate recognition by altering or perturbing one of the features of the DNA–protein complex known to be involved in the specific interaction of restriction endonucleases with a target site: direct DNA base – protein contacts, phosphate–protein interaction or the allosteric machinery that couples DNA cleavage and substrate recognition. However, a detailed analysis of the structural-functional implications of the

T862 substitutions must wait for the 3D structure of Eco57I.

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THR862 AMINORÛGÐTIES, BÛTINOS RESTRIKCIJOS ENDONUKLEAZËS ECO571 SUBSTRATINIAM SPECIFIÐKUMUI, SATURAVIMO MUTAGENEZË

Santrauka

Restrikcijos endonuklеazë Eco57I atpaþásta asimetrinæ sekà 5'-CTGAAG ir kerpa dvigrandæ DNR uþ 16/14 baziø porø 5' kryptimi nuo atpaþinimo sekos bei metilina antràjá adeninà virðutinëje DNR grandinëje. Ankstesniuose mûsø eksperimentuose ið mutantø kolekcijos, gautos po atsitiktinës mutagenezës, pritaikius unikalià technologijà, buvo atrinkti ir apibûdinti Eco57I mutantai, atpaþástantys naujà sekà 5'-CTGRAG. Pirmà kartà pasaulyje eksperimentiniu bûdu buvo sukurtas iki tol gamtoje nerasto substratinio specifiðkumo fermentas. Visø apibûdintø mutantiniø baltymø specifiðkumo pasikeitimà lëmë aminorûgðties Thr862 mutacija á Asn arba Ser. Ðiame darbe vykdant kryptingà mutagenezæ buvo sukonstruoti mutantai, kuriuose Thr862 buvo pakeista á likusias 17 aminorûgðèiø. Mutantø aktyvumas ir specifiðkumas buvo ávertintas *in vivo*. Rezultatø duomenimis, mutantai T862C, T862G ir galbût T862A bei T862D pasiþymëjo substratiniu specifiðkumu, besiskirianèiu nuo laukinio tipo Eco57I restrikcijos endonukleazës specifiðkumo.