The *Ecl*18kI restriction-modification system: cloning, expression, properties of the purified enzymes

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Abstract ***Ecl*18kI is a type II restriction-modification system isolated from *Enterobacter cloaceae* 18kI strain. Genes encoding *Ecl*18kI methyltransferase (M.*Ecl*18kI) and *Ecl*18kI restriction endonuclease (R.*Ecl*18kI) have been cloned and expressed in *Escherichia coli*. These enzymes recognize the 5′...↓CCNGG...3′ sequence in DNA; M.*Ecl*18kI methylates the C5 carbon atom of the inner dC residue and R.*Ecl*18kI cuts DNA as shown by the arrow. The restriction endonuclease and the methyltransferase were purified from *E. coli* B834 [p18Ap1] cells to near homogeneity. The restriction endonuclease is present in the solution as a tetramer, while the methyltransferase is a monomer. The interactions of M.*Ecl*18kI and R.*Ecl*18kI with 1,2-dideoxy-D-ribofuranose containing DNA duplexes were investigated. The target base flipping-out mechanism is applicable in the case of M.*Ecl*18kI. Correct cleavage of the abasic substrates by R.*Ecl*18kI is accompanied by non-canonical hydrolysis of the modified strand.**

Key words: *Ecl*18kI DNA methyltransferase; *Ecl*18kI restriction endonuclease; Purification; Abasic substrate analog

1. Introduction

Type II restriction-modification (R-M) systems including restriction endonucleases and cognate DNA methyltransferases which cleave and modify DNA with extreme specificity are widely distributed among different prokaryotes. The R-M enzymes are attractive models for studies of structural aspects of DNA-protein recognition.

Recently, *Ecl*18kI type II R-M system from *Enterobacter cloaceae* 18kI strain was described [1]. It was shown that *Ecl*18kI enzymes recognize the 5′...↓CCNGG...3′ sequence in DNA; *Ecl*18kI DNA methyltransferase (M.*Ecl*18kI) methylates the C5 carbon atom of the inner dC residue and *Ecl*18kI restriction endonuclease (R.*Ecl*18kI) cleaves DNA as shown by the arrow [1]. Genes for *Ecl*18kI and *Sso*II R-M systems have essentially the same sequence [1,2]. The nucleotide sequence data for *Ecl*18kI R-M system reported in [1] have been deposited in the GenBank nucleotide sequence database with accession number J16897. One of the ways to obtain knowledge on how *Ecl*18kI enzymes recognize the DNA substrates is to study the interaction of the endonuclease and the methylase with a number of suitably modified DNA substrates which carry the modification in only one position of the recognition sequence. The success of this study depends on the availability of highly purified enzymes.

In this paper, we report the general approach to simultaneous purification of *Ecl*18kI restriction endonuclease and DNA methyltransferase and examine the effect of non-nucleoside inserts within the recognition site on the interaction of the enzymes with DNA. Some properties of these proteins are described as well.

2. Materials and methods

Plasmids pUC129 [3] and pECL18 [1] were used. Isolation of plasmid and phage DNA, transformation, restriction analysis, and other genetic engineering manipulations were carried out as described [4]. DNA fragments with different 5′-protruding ends were cloned after filling in the ends using *Escherichia coli* DNA polymerase I Klenow fragment. Transformants with recombinant plasmid DNA were selected by phage restriction as described earlier [5].

Restriction endonucleases were from Fermentas (Lithuania). The recombinant T4 DNA ligase was isolated in our laboratory (IBPM). To prepare cell-free extracts, the biomass was collected by centrifugation at 6000 rpm in JA-10 rotor (Beckman), suspended in buffer A (12.5 mM potassium phosphate, pH 7.2, 1 mM EDTA, 7 mM 2-mercaptoethanol) containing 200 mM NaCl, disrupted on an MSE ultrasonic disintegrator for 10 min at 0°C, and the debris was removed by centrifugation for 1 h at 20000 rpm on a JA-20 rotor. All steps of enzyme isolation were carried out at 4°C.

The molecular mass of the proteins was estimated by electrophoresis in a 9–20% gradient of sodium dodecyl sulfate polyacrylamide gel [6].

1,2-Dideoxy-D-ribofuranose (ddR)-containing oligodeoxyribonucleotides were synthesized by Ya.I. Alexeev. Oligodeoxyribonucleotides with C5-methylcytosine (m^5C) residues were commercial preparations. ^{32}P-Phosphorylation of the oligonucleotides was carried out using T4 polynucleotide kinase and [γ-^{32}P]ATP. Endonuclease activity of R.*Ecl*18kI was determined in 20 μl of 20 mM Tris-HCl buffer, pH 8.5, 7 mM 2-mercaptoethanol, 1 mM EDTA, 100 mM KCl, 10 mM $MgCl_2$ containing 1 μg of λcI857 DNA and 2 μl of the enzyme. DNA fragments were separated by electrophoresis in 0.8% agarose gel.

Cleavage assay. ^{32}P-Labelled DNA duplexes (0.35 μM) I, V–VIII (Table 1) were incubated with 2 units of R.*Ecl*18kI in 20 μl of 10 mM Tris-HCl buffer, pH 7.5 containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol at 10°C for 30 min. The products of cleavage of ^{32}P-labelled duplexes were analyzed in 20% polyacrylamide gel containing 7 M urea. Radioactivity of gel slices was determined by Cerenkov counting. Cleavage percent was defined as a quotient of product radioactivity to total radioactivity of the product plus that of the uncleaved substrate.

Methylation assay. The efficiency of methylation was monitored by incorporation of radioactivity ($C[^3H]_3$) into DNA duplexes I–VIIIm. Methylation reactions were carried out at 10°C for 15 min in 10 μl reaction mixtures containing 50 mM Tris-HCl, pH 7.6, 5 mM DTT, 10 mM EDTA, 1 μCi of [methyl-^{3}H]AdoMet (Amersham, 15 Ci/mmol) and 0.35 μM of DNA duplexes. Samples (8 μl) were spotted on DE81 filters (2.5 cm, Whatman), washed with 50 mM KH_2PO_4 (5×150 ml), dried and counted in a liquid scintillation spectrometer. For each series blank values (without enzyme) were treated like the

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samples (with complete washing procedure). To determine methylation of each strand of DNA duplex, the ^3H incorporation into non-methylated (duplexes I, V, VI, VII and VIII, Table 1) (cpmT) and hemimethylated DNA duplexes II, III, Vm, VIm, VIIm and VIIIm (cpm1) was calculated. The cpm1 and cpm2 (cpm2=cpmT−cpm1) values correspond to the ^3H incorporation into each DNA strand. The relative methylation percent was calculated as the ratio of the ^3H incorporation into each strand (or both strands) of the modified duplexes to ^3H incorporation into the corresponding strand of the canonical duplex (I) (Table 1). The sequence of the plasmid DNA was determined according to [7].

3. Results and discussion

3.1. Construction of plasmids

Genes encoding M.*Ecl*18kI and R.*Ecl*18kI are located on a 5.5 kb plasmid (pECL18) isolated from *E. cloaceae* 18kI strain [1]. They have been cloned in *E. coli* [1].

In order to increase a copy number of the genes for the endonuclease and the methylase the gel purified large *Pvu*II fragment of pECL18 DNA (3.7 kb) was ligated to pUC129 cleaved with R.*Eco*RV. The recombinant plasmid called p18Ap1 was used to obtain the cell-free extracts for the purification of *Ecl*18kI R-M enzymes. The level of the R-M enzymes synthesis in strains containing recombinant plasmid was found to be about 5 times higher than that of the parent strain.

3.2. Purification of R.Ecl18kI and M.Ecl18kI

Homogeneous R.*Ecl*18kI was purified from 8 g of *E. coli* B834 [p18Ap1] cells in 2 l overnight culture of LB medium. Cells were resuspended in 40 ml of cold buffer A (12.5 mM K-phosphate buffer, pH 7.2, 7 mM 2-mercaptoethanol, 1 mM EDTA) with 200 mM NaCl. The suspension was sonicated, centrifuged and the enzyme was purified by subsequent chromatography on DEAE cellulose (DE-52, Whatman, bed volume 100 ml) and on phosphocellulose P11 (Whatman, bed volume 50 ml). After washing both connected columns by 500 ml of buffer A containing 200 mM NaCl, the enzyme was eluted from the P11 column with a 300–800 mM NaCl gradient (at 430 mM) in buffer A. Active fractions were collected and applied to a K-9 column (20 ml) with hydroxyapatite HA-Ultragel and washed with buffer B (20 mM Tris-HCl, pH 7.2, 7 mM 2-mercaptoethanol, 1 mM EDTA) and the enzyme was eluted at 12.5–250 mM K-phosphate buffer containing 7 mM 2-mercaptoethanol (at 175 mM). Active fractions were pooled, dialyzed against buffer B, and loaded onto a heparin-Sepharose column (10 ml). The enzyme was eluted with a 200–800 mM NaCl gradient in buffer B. The endonuclease activity (20 unit/μl or higher) was detected in the eluent at 320 mM NaCl. Rechromatography on the DE-52 column of the active fractions gave nearly homogeneous enzyme. The preparation of R.*Ecl*18kI (6.8 mg; 1.7 mg/ml) was practically electrophoretically homogeneous (95% purity) and free from detectable non-specific nucleases (Fig. 1A).

M.*Ecl*18kI was found to be eluted at 540 mM NaCl on P11 column after chromatography of R.*Ecl*18kI. The enzyme was dialyzed against buffer B containing 200 mM NaCl and applied to the K-9 column with heparin-Ultragel A4R (10 ml). After washing the column with buffer B containing 200 mM NaCl, the enzyme was eluted with a 200–1000 mM NaCl gradient (at 500 mM NaCl) in buffer B. Active fractions were collected and dialyzed against buffer B containing 50% of glycerol and 100 mM NaCl. The preparation of M.*Ecl*18kI (2.0 mg; 0.5 mg/ml) was almost homogeneous (about 95% purity) and was the major component of the proteins after analysis by SDS-PAGE (Fig. 1B).

3.3. Molecular properties of R.Ecl18kI and M.Ecl18kI

We have estimated the M_r of the R.*Ecl*18kI polypeptide under denaturing conditions (SDS-PAGE) to be 36 kDa (Fig. 1C). The subunit structure of the enzyme under non-denaturing conditions was estimated by gel filtration of homogeneous R.*Ecl*18kI. The partition coefficient (K_{av}) for

Table 1
Interaction of M.*Ecl*18kI with modified substrates

No.	DNA duplex	X	Y	Total relative methylation (%)[a]	Each strand relative methylation (%)[a]
I	5′-GCCAA**CCTGG**CTCT			100	100
	3′-CGGTT**GGACC**GAGA				100
II	5′-GCCAA**CXTGG**CTCT	m^5C		30	0
	3′-CGGTT**GGACC**GAGA				60
III	5′-GCCAA**CCTGG**CTCT	m^5C		130	260
	3′-CGGTT**GGAXC**GAGA				0
IV	5′-GCCAA**CXTGG**CTCT	m^5C		0	0
	3′-CGGTT**GGAXC**GAGA				0
V	5′-GCCAA**CCYGG**CTCT		ddR	40	0
	3′-CGGTT**GGACC**GAGA				80
Vm	5′-GCCAA**CCYGG**CTCT	m^5C	ddR	0	0
	3′-CGGTT**GGAXC**GAGA				0
VI	5′-GCCAA**CCTYG**CTCT		ddR	110	0
	3′-CGGTT**GGACC**GAGA				220
VIm	5′-GCCAA**CCTYG**CTCT	m^5C	ddR	0	0
	3′-CGGTT**GGAXC**GAGA				0
VII	5′-GCCAA**CCTGY**CTCT		ddR	0	0
	3′-CGGTT**GGACC**GAGA				0
VIIm	5′-GCCAA**CCTGY**CTCT	m^5C	ddR	0	0
	3′-CGGTT**GGAXC**GAGA				0
VIII	5′-GCCAA**CCTGG**YTCT		ddR	70	60
	3′-CGGTT**GGACC**GAGA				80
VIIIm	5′-GCCAA**CCTGG**YTCT	m^5C	ddR	30	60
	3′-CGGTT**GGAXC**GAGA				0

[a]The determination of the total and relative methylation % is described in Section 2.

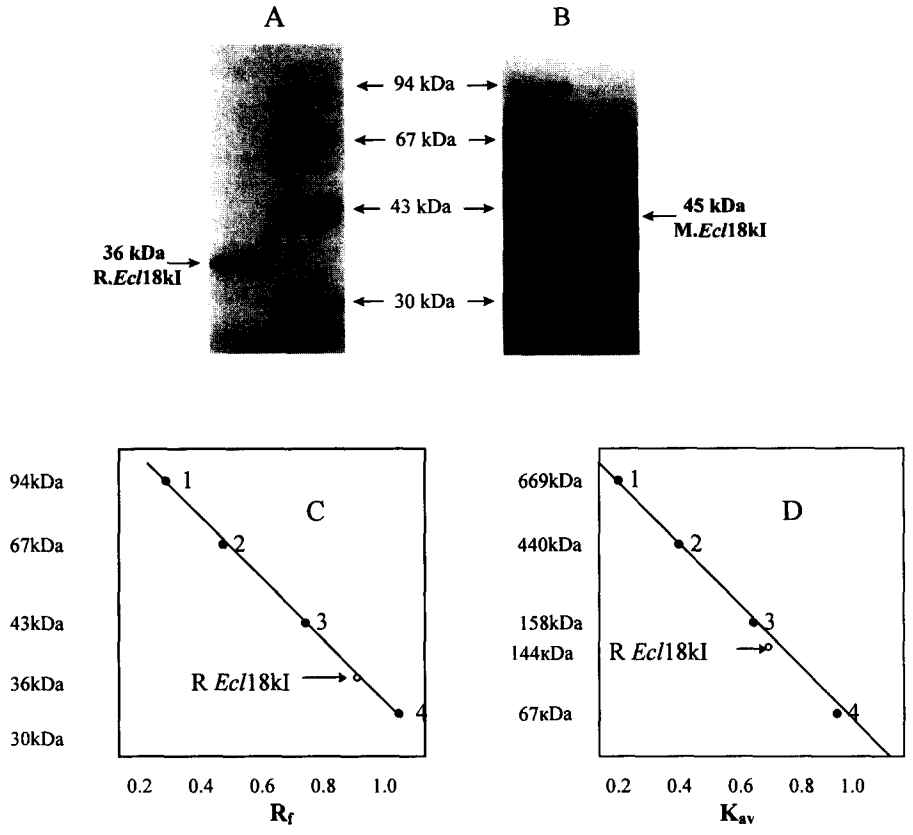

Fig. 1. A, B: Denaturing SDS-PAGE of the R.*Ecl*18kI (A) and M.*Ecl*18kI (B). Standards (phosphorylase B, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa) and purified proteins are shown. C, D: Determination of the R.*Ecl*18kI molecular mass: (C) under denaturing conditions (SDS-PAGE) and (D) with HPLC size exclusion column Superose 12; calibration standards are (1) thyroglobulin (669 kDa), (2) ferritin (440 kDa), (3) aldolase (158 kDa), (4) BSA (67 kDa).

R.*Ecl*18kI was determined using calibration standards (Fig. 1D). Based on data of gel filtration, the M_r of native R.*Ecl*18kI was estimated to be 144 kDa, i.e. it was eluted as a molecular species significantly larger than 36 kDa. The M_r of R.*Ecl*18kI determined by the first method was in good agreement with the sequencing data (305 aa, 35937 Da). From these data we suppose that R.*Ecl*18kI in 100 mM NaCl at a concentration of 1.7 mg/ml exists as a tetramer. This is similar, for instance, to R.*Bam*HI which also exists under the same conditions as a tetramer [8]. Under similar conditions M.*Ecl*18kI exists as a monomeric form (data not shown).

3.4. *Interaction of Ecl18kI R-M enzymes with modified substrates*

To determine the peculiarities of the mechanism of the *Ecl*18kI R-M enzymes functioning, such as possible contacts with heterocyclic bases of the recognition sequence and substrate distortion within the enzyme-DNA complex, methylation and cleavage of modified 14-mer DNA duplexes by these enzymes have been studied. 1,2-Dideoxy-D-ribofuranose residues have been introduced as single substituents into the 5'...CCNGG...3' recognition site or into the flanking nucleotide sequence to create abasic sites (Table 1).

3.5. *Ecl18kI methyltransferase*

To study methylation efficiency of each strand of DNA duplexes by M.*Ecl*18kI hemimethylated substrates and substrate analogs were used (Table 1). The strong dependence of methylation efficiency on abasic site location in the recognition sequence was demonstrated for the canonical strand (Table 1, duplexes V–VIII). M.*Ecl*18kI methylates much better the substrate analog (VI) containing an abasic site in place of the guanine paired to the target cytosine. Hence, destabilization of this G-C pair favors the methylation. This can be interpreted as evidence for the operation of the target base flipping-out mechanism in the case of *Ecl*18kI methylase similar to that described for M.*Hha*I [9].

No methylation occurred in the case of substrate analog containing an abasic site instead of the outer guanine residue of the recognition sequence (duplex VII). Comparison of the methylation efficiency of the modified substrates containing ddR residues instead of either inner or outer guanine residue (duplexes VI and VII) revealed importance of the discrimination contacts of M.*Ecl*18kI with the outer guanine. On the other hand, the contact of *Ecl*18kI methylase with the inner guanine (which is opposite the target cytosine) seems to be not important for recognition of the substrate. The introduction of a ddR residue in place of the central dT residue of the recognition sequence (duplex V) does not influence on the efficiency of methylation of the opposite strand of the DNA duplex; the ddR-containing strand is not methylated. Thus, in all cases (duplexes V–VIII) the introduction of a non-nucleoside insert in the recognition site results in loss of methylation of the modified strand. The location of the abasic site in the flanking nucleotide sequence (duplex VIII) does not affect the methylation of the DNA duplex.

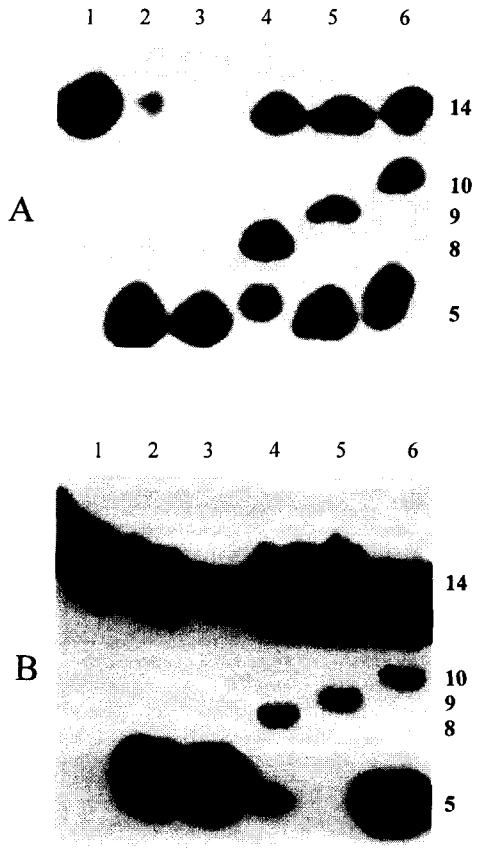

Fig. 2. Cleavage of DNA duplexes with non-nucleoside inserts by R.*Ecl*18kI, the enzyme concentration 100 nM (A) and 10 nM (B). Lanes 2–6 correspond to cleavage of DNA duplexes I, V, VI, VII and VIII respectively; lane 1 is control (duplex I); ^{32}P label is in the upper strand of the duplexes (Table 1). Numbers on the right indicate the length of oligonucleotides (products of hydrolysis).

3.6. *Ecl18kI endonuclease*

Introduction of a non-nucleotide insert into the *Ecl*18kI recognition site instead of dG residues (DNA duplexes VI and VII) produces non-canonical breaks in the modified strand of DNA in addition to specific ones (Fig. 2, lanes 4, 5). Cleavage experiments were performed at two enzyme concentrations (Fig. 2). In both cases new cleavage points were observed in modified strands at the position of abasic site. At the same time the correct cleavage was observed in canonical strands (data not shown). Previously we observed similar cleavage pattern for DNA containing non-nucleoside inserts in the case of *Sso*II endonuclease which is closely related to R.*Ecl*18kI [10]. The most interesting property we found for *Ecl*18kI endonuclease is its change in cleavage specificity even if the recognition site is preserved as canonical and a non-nucleotide insert is placed next to the *Ecl*18kI recognition site (DNA duplex VIII). In this case, endonuclease *Ecl*18kI also cleaves the modified strand at the position of the modification (Fig. 2, lane 6), while the non-modified strand is cleaved at the canonical position (data not shown). Thus, endonuclease *Ecl*18kI cleaves DNA duplex VIII producing blunt ends. Non-canonical breaks were provoked by DNA double helix distortion at the site of modification due to removal of the heterocyclic base. The data suggest that DNA substrate complexed with R.*Ecl*18kI undergoes considerable conformational disturbance. DNA 'open' complex formation seems to occur upon binding of substrate to R.*Ecl*18kI at a step preceding catalysis.

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