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Investigation of Restriction Endonuclease EcoRII Complex with DNA in Solution by FTIR Spectroscopy

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Abstract—The X-ray structure of type IIE EcoRII restriction endonuclease has been solved but the structure of the R.EcoRII–DNA complex is still unknown. We report here on the structure of the pre-reactive R.EcoRII–DNA–Ca^{2+} complex in solution examined by FTIR spectroscopy. The secondary structure of R.EcoRII as well as the structure of the target DNA in the R.EcoRII–DNA–Ca^{2+} complex was characterized. It was shown that the R.EcoRII–DNA–Ca^{2+} complex formation is accompanied by changes in the spectrum of both DNA bases and DNA sugar-phosphate backbone that suggest contacts of the enzyme with different groups of atoms in DNA. The change of the R.EcoRII secondary structure in the R.EcoRII–DNA–Ca^{2+} complex is also observed.

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INTRODUCTION

Resriction endonucleases (RE) operate in all procariotic organisms, in Archean and viruses of some unicellular algae as components of restriction-modification (R–M) protection systems [1, 2]. RE of type II recognize specific nucleotides sequences in DNA and hydrolyze phosphodiester bonds in the recognized sequence or near it; therewith 5'-phosphate and 3'-hydroxy groups are formed [3]. Commonly RE operation requires the presence of Mg^{2+} ions. Recently several RE subtypes of type II were characterized [4–9]. Among these subtypes restriction endonucleases of IIE type have a unique feature: To perform efficient DNA splitting they should simultaneously bind two copies of their palindromic recognition sites of DNA; whereas one copy serves as a target of splitting, the second one is an allosteric effector [3]. RE of IIE type are attractive models for investigating specific DNA–protein interactions in complex genetic processes where the enzymes react simultaneously with several sites in the DNA.

Homodimer protein R.EcoRII was the first example of an enzyme of IIE type [10]. R.EcoRII cleaves in DNA ↓CCA/TGG in both chains (the splitting point is indicated by an arrow). Low efficiency of splitting of R.EcoRII DNA-substrates with one or several recognition sites divided by 1000 n.p. was overcome by adding short DNA-duplexes with a single recognition site of EcoRII (owing to *trans*-interactions) [11]. The study of splitting by R.EcoRII of two separate recognition sites on a single DNA molecule showed that the cooperation between EcoRII-sites was achieved by the formation of a bend or a loop of DNA between these sites (*cis*-interactions) [12]. It was demonstrated by transmission electron microscopy that R.EcoRII served as a linkage in the loop formation [13]. Further studies revealed that the active enzyme-substrate complex was formed by two subunits of R.EcoRII interacting with two recognition sites in DNA [12, 14]. Recently based on the study of splitting kinetics by R.EcoRII of plasmids containing up to

three recognition sites it was suggested that R.EcoRII binded simultaneously three and not two recognition sites in order to perform concerted splitting of two DNA strands in one recognition site [15]. An X-ray diffraction analysis of R.EcoRII was carried out [16]. R.EcoRII monomer consists of an *N*-terminal effector-binding domain and *C*-terminal endonuclease-like domain [16, 17]. The presumed catalytic site of R.EcoRII is situated in the *C*-terminal domain and it is sterically blocked by the *N*-terminal domain. The removal of the *N*-terminal effector-binding domain of R.EcoRII resulted in transformation of the enzyme of IIE type into an active enzyme of IIP type [17]. The study of DNA binding showed that the isolated C-terminal domain was a dimer that bound one recognition site, and the *N*-terminal domain was a monomer which bound one recognition site [18]. According to this model the full-size R.EcoRII contains three DNA-binding cavities: one cavity formed of two *C*-terminal domains and two cavities of *N*-terminal domains [18].

It is known from the crystal structures of II type RE with DNA that the DNA-target is subjected to strong distortions, like bending, untwisting, and even to deviation of a base from the composition of the DNA double helix [1, 19–21]. Besides conformational transformations in the protein were observed on formation of the complex with DNA, namely, structured regions arose that were previously random [1]. The X-ray diffraction analysis of a complex of II type RE, R.NaeI, with DNA revealed considerable changes in the tertiary structure of R.NaeI on binding with DNA with simultaneous partial ordering of its secondary structure [10, 16]. The structural changes in DNA and enzyme on formation of a complex R.EcoRII–DNA were not investigated.

FTIR spectroscopy is a well-known method for the study of the secondary structure of proteins [22, 23]. The elements of the protein secondary structure and their content in the overall secondary structure can be established from the analysis of absorption in the region of the stretching vibrations of the carbonyl of the peptide bond (band amide I) using methods of resolution refining and spectrum expansion [24–26]. Besides IR spectroscopy was extensively applied to the study of the variation in the formation of the complementary pairs of bases and of conformational transitions in the carbohydrate-phosphate DNA backbone [27]. The DNA–protein complexes were relatively poorly studied by the IR spectroscopy. The study of complexes between DNA and proteins of chromatin HMGB1 and histone H^1 revealed the changes in the DNA structure indicating the interaction of the proteins with the bases and phosphate groups of DNA [28]. The analysis of characteristic absorption bands of the phosphodiester DNA backbone (the region 900–800 cm^{-1}) revealed the changes in the double helix geometry in DNA complexes with histones H^{2A}, H^{2B}, H^3, or H^4 [29, 30]. In the complex DNA-protein the changes in the secondary structure of H^1 were observed [31, 32].

The goal of the present study was the analysis of the structure of a "preactive" complex R.EcoRII–DNA–Ca^{2+} by means of FTIR spectroscopy. The secondary structure of R.EcoRII and also the structure of DNA-substrate in the complex R.EcoRII-DNA were investigated.

MATERIALS AND METHODS

Reagents and enzymes. R.EcoRII containing six histidine residues in the *N*-terminal part was purified by chromatography on Ni–NTA-agarose as described in [33]. The concentration of dimer R.EcoRII (2.3 mM) was estimated by Bradford procedure. Buffer A: 40 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM $CaCl_2$, 7 mM DTT, water Milli-Q.

Concentrations of oligodeoxyribonucleotides 5'-GAG**CCAGG**TTGG (**12**$^{\mathbf{A}}$) and 5'-CCAA**CCTGG**CTC (**12**$^{\mathbf{T}}$) (Sintol, Russia) were measured by spectrophotometry. Extinction factors at 260 nm (ε_{260}) of unmodified oligodeoxyribonucleotides were calculated in keeping with [34].

IR-spectroscopy. DNA-duplex **12**$^{\mathbf{A}}$**/12**$^{\mathbf{T}}$ was purified from salt on a concentrator MICROCON (3500 Da, MILLIPORE). R.EcoRII was concentrated on concentrators MICROCON (5000 Da, MILLIPORE). Complex R.EcoRII–DNA was prepared by mixing 17 nmol of R.EcoRII with 34 nmol of duplex **12**$^{\mathbf{A}}$**/12**$^{\mathbf{T}}$ in buffer A with subsequent incubation at 37°C for 10 min. The samples were lyophilized at 5°C and dissolved in 1–5 ml of D_2O. IR spectra were measured in the range from 1800 to 700 cm^{-1} on a spectrophotometer Perkin Elmer 2000 at 20°C. The spectra were averaged for 10 scans with a resolution of 1 cm^{-1}. The data were treated using programs P.E. Spectrum (two-point base line, Fourier transform, second derivative) and Grams (galactic software). The preparation of the complex R.EcoRII–DNA and

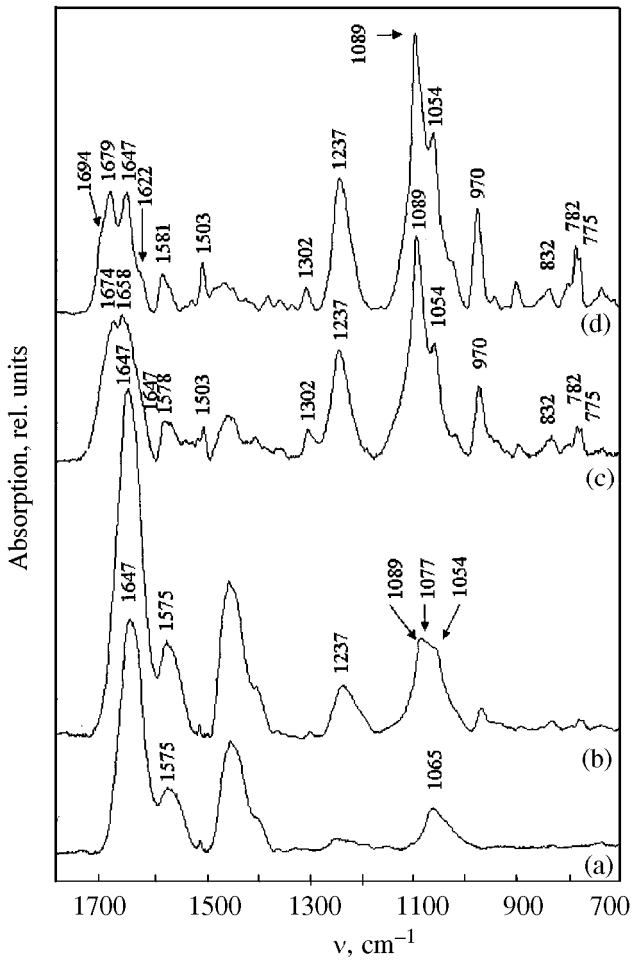

Fig. 1. IR spectra in D_2O solution: (a) R.EcoRII (buffer A, 1×); (b) complex R.EcoRII/(**12^A/12^T**) (buffer A, 1×); (c) DNA duplex in the complex; (d) free DNA-duplex **12^A/12^T**. The spectra are corrected with respect to D_2O absorption.

recording of the spectra of the complex, R.EcoRII, and DNA was repeated thrice.

RESULTS

In order to reveal presumable structural alterations in DNA and R.EcoRII due to complex formation the 12-unit DNA-duplex **12^A/12^T** containing one recognition site EcoRII, R.EcoRII, and the complex R.EcoRII (**12^A/12^T**) were investigated by means of IR spectroscopy in the presence of an analog of cofactor, ions Ca^{2+}. Short DNA-duplexes are optimal for investigation of local structural changes in DNA by IR spectroscopic experiments. DNA-duplex **12^A/12^T** was efficiently split by R.EcoRII in the presence of cofactor, Mg^{2+} ions. On Fig. 1a IR spectrum of R.EcoRII is presented; on Figs. 1b and 1d, those respectively of the complex R.EcoRII/(**12^A/12^T**) and DNA-duplex **12^A/12^T** [35]. The curve Fig. 1*c* is the spectrum of **12^A/12^T** in the complex obtained by subtraction of the contribution of enzyme into the spectrum of the complex, (b)–(a). The spectra were recorded in the region 1800–700 cm^{-1} in D_2O solution. The curves are presented after subtraction of the D_2O absorption.

The region 1750–1550 cm^{-1} of the spectrum of R.EcoRII in 1×-buffer A contains a broad absorption band amide I with a maximum in the region of 1647 cm^{-1} (Fig. 1a). We expected to observe several bands corresponding to various secondary structures in the enzyme. The overlapping of these bands results in a broad band that may be expanded into components in keeping with the procedure of spectrum expansion. The first estimation of the number of various components can be obtained by calculation of the second derivative of the spectrum or by Fourier transform of the original spectrum (the data are not reported) [25]. Both procedures were applied to estimate the first set of parameters permitting proceeding to the curve expansion.

The position of bands corresponding to the secondary structure were selected according to published data [22–26]: α-helix, 1650–1660 cm^{-1}; antiparallel β-layer, 1630–1636 cm^{-1} and 1680–1690 cm^{-1}; β-turns, 1669–1677 cm^{-1}; random glome, 1639–1648 cm^{-1}; associated β-layer, 1615–1628 cm^{-1}. A series of successive iterations was performed to obtain the minimum route-mean-square deviation (RMS). Then the wave numbers were fixed, and the intensities were varied. Six bands have been identified that are shown on Fig. 2b together with the experimental spectrum (full line) and the one calculated as the sum of all six bands (dotted line). The absorption bands are enumerated in the table, and the content of the elements of secondary structure of R.EcoRII in solution is estimated. The fractions of structures of α-helix, β-layer, β-turns, and random glome are similar in 1×- and 50×-buffer A (the latter spectra are not reported).

To estimate the secondary structure of R.EcoRII in the complex R.EcoRII/(**12^A/12^T**) we excluded from the spectrum of the complex the DNA contribution into the region of amide I by subtraction of the spectrum of duplex **12^A/12^T**. The spectrum obtained by subtraction was processed as described above (Fig. 2a). In Fig. 2a the six components obtained are shown (in 1×-buffer

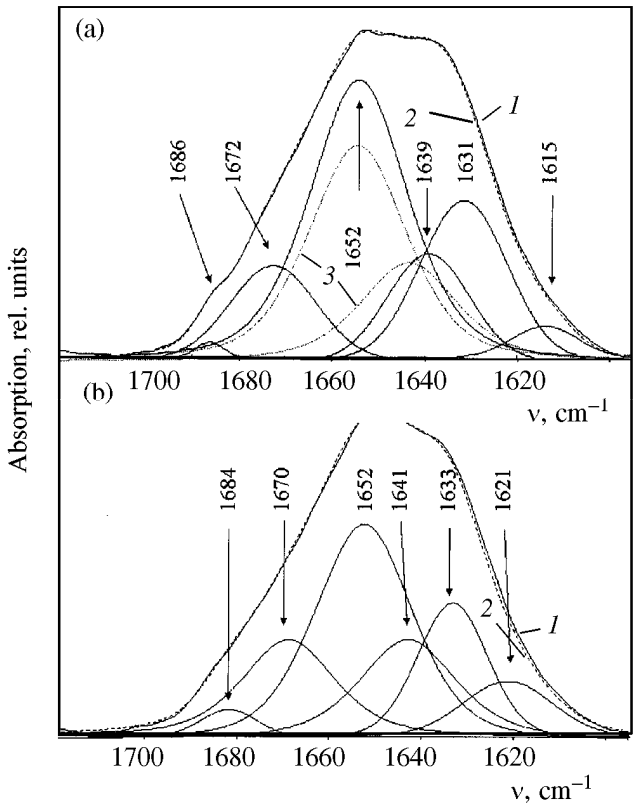

Fig. 2. Amide I region of IR spectra in D_2O solution: (a) R.EcoRII in the complex R.EcoRII/(**12^A/12^T**), spectrum of subtractiong DNA contribution from the spectrum of the complex R.EcoRII/(**12^A/12^T**) (buffer A, 1×); (b) R.EcoRII (buffer A, 1×). (*1*) experimental spectra; (*2*) theoretical spectra summing up the individual contributions; (*3*) contribution of a-helices and random glomes for free R.EcoRII taken from B.

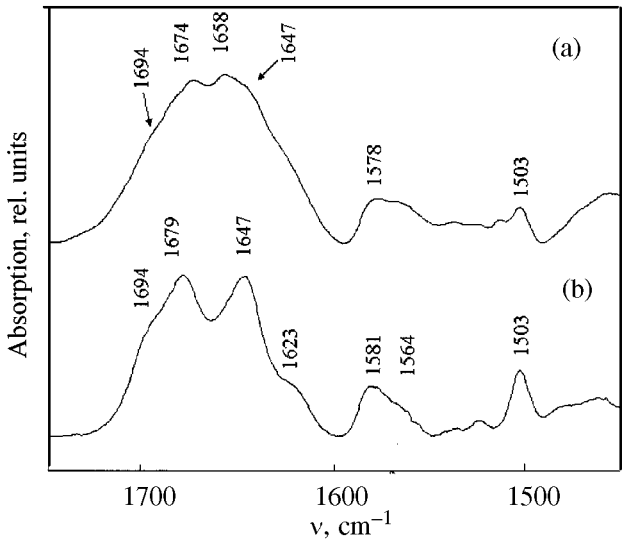

Fig. 3. Region of IR spectra corresponding to in-plane vibration of double bond in DNA bases: (a) spectrum of DNA duplexes **12^A/12^T** in the complex R.EcoRII/(**12^A/12^T**) obtained by subtracting from the spectrum of the complex the enzyme contribution; (b) spectrum of free DNA duplexes **12^A/12^T**.

A; analogous spectra have been recorded also in 50×-buffer A) alongside the experimental (full line) and calculated summary (dotted line) spectra. We also present for comparison the spectrum expansion of free R.EcoRII (Fig. 2b). The secondary structure R.EcoRII in the complex R.EcoRII/(**12^A/12^T**) is enriched with a-helices whereas the content of random structures decreases (cf. the table).

The spectrum of the complex R.EcoRII/(**12^A/12^T**) (Fig. 1b) contains absorption bands of both components of the complex. Informative region for the variations in the DNA structure due to the complex formation is the region 1700–1500 cm^{-1}. Here the in-plain stretching vibrations of the double bonds of the heterocyclic bases appear. They are sensitive to the hydrogen bonds formation and stacking of the bases. The absorption bands of duplex **12^A/12^T** observed in this spectral range may be ascribed to the following groups of atoms (Fig. 1d and 3b): 1694 cm^{-1}, stretching vibrations of the $C^2{=}O^2$ group of thymine; 1679 cm^{-1}, stretching vibrations of the $C^6{=}O^6$ group of guanine; 1647 cm^{-1}, overlapping stretching vibrations of $C^4{=}O^4$ of thymine and $C^2{=}O^2$ of cytosine; 1623 cm^{-1}, bending vibrations of ND_2 of adenine related to the ring vibrations of the heterocycle; 1581 and 1564 cm^{-1}, vibrations of guanine ring, and 1503 cm^{-1}, vibrations of cytosine ring [27].

On the formation of the complex of R.EcoRII with duplex **12^A/12^T** the DNA spectrum suffers changes in the region under consideration (Figs. 1c and 3a). We observed a small shift of the stretching vibrations of $C^6{=}O^6$ of guanine to smaller wave numbers (Δn = –5 cm^{-1}) and splitting of the band at 1647 cm^{-1} in two components, one in the same position, the other shifted to 1658 cm^{-1} (Δn = +11 cm^{-1}).

In the region 1500–700 cm^{-1} in the spectra of the free duplex **12^A/12^T** (Fig. 1d) or **12^A/12^T** in the complex (Fig. 1c) absorption bands appeared characteristic of the B-form of DNA. The bands at 1237 and 1089 cm^{-1} were commonly assigned to the antisymmetric and symmetric stretching vibrations of phosphate groups respectively. The bands of 1054 and

Secondary structure of R.EcoRII and R.EcoRII in a complex with DNA-duplex $\mathbf{12^A/12^T}$ obtained by expansion of IR spectra in the region 1600–1700 cm^{-1}

Secondary structure	% of structure (ν, cm^{-1})			
	50× buffer A, D_2O		1× buffer A, D_2O	
	R.EcoRII	R.EcoRII/$\mathbf{12^A/12^T}$	R.EcoRII	R.EcoRII/$\mathbf{12^A/12^T}$
Antiparallel b-layer (1680–1690)	7 (1683)	7 (1688)	2 (1684)	1 (1686)
β-turn (1669–1677)	16 (1670)	28 (1674)	20 (1670)	13 (1672)
α-helix (1650–1660)	31 (1654)	38 (1652)	36 (1652)	55 (1653)
Random glome (1639–1648)	18 (1641)	8 (1642)	19 (1641)	9 (1640)
Antiparallel β-layer (1630–1636)	20 (1630)	12 (1629)	16 (1633)	17 (1631)
Associated β-layer (1615–1628)	8 (1615)	7 (1618)	7 (1621)	5 (1617)

970 cm^{-1} correspond to the vibrations of the carbohydrate residue [28]. An absorption band observed at 832 cm^{-1} is characteristic of *S*-conformation of the carbohydrate residue [27]. All these facts show that the phosphate groups and carbohydrate residues in the DNA-duplex $\mathbf{12^A/12^T}$ included into the DNA-protein complex possess the geometry of the B-form of double helix.

DISCUSSION

The possible changes in R.EcoRII and DNA occurring on formation of the complex R.EcoRII-DNA were studied by means of IR spectroscopy. The analysis of amide I region in the IR spectrum of R.EcoRII made it possible to calculate the relative content of the secondary structure components of R.EcoRII at various buffer concentrations (see the table). The content of α-helices and β-turns was consistent with the composition estimated from the analysis of the crystal structure of R.EcoRII (34.5% α-helices and 19.7% β-turns). The content of b-layers determined from IR spectra (see the table) and by crystallography (14.5% β-layers) was different [16].

The formation of the complex R.EcoRII–DNA is accompanied by increase in the content of α-helices and decrease in the amount of random structures (see the table). The binding model in R.EcoRII DNA suggested by Zhou et al. [16] involved considerable alterations in the tertiary structure of R.EcoRII on binding DNA. We believe that the changes in the tertiary structure of R.EcoRII are accompanied with the alteration of the secondary structure.

The changes in DNA absorption on the formation of the complex R.EcoRII–DNA were observed in the region 1750–1500 cm^{-1} (Fig. 3) of the stretching vibrations of the C=O groups of bases [36]. According to biochemical findings a number of contacts is presumed to exist between heterocyclic bases of the site CCA/TGG and amino acids residues of R.EcoRII [37, 38]. These contacts may produce the observed DNA absorption changes on binding with R.EcoRII. The band at 1679 cm^{-1} in the DNA spectrum belongs to the stretching vibrations of $C^6{=}O^6$ of guanine [28]. 6-Oxo group of guanine is accessible from the side of the big groove of DNA. The shift of this band in the spectrum of the complex R.EcoRII/($\mathbf{12^A/12^T}$) may be due to the interaction between R.EcoRII and O^6 in the big groove of DNA [37]. This is in agreement with the simultaneous change in the relative intensity of the guanine band at 1564 cm^{-1} belonging to the stretching vibrations of the $C^6{=}O^6$ group of guanine [39]. The absorption at 1647 cm^{-1} in the DNA spectrum corresponds to the overlapping of the stretching vibrations of $C^2{=}O^2$ of cytosine and $C^4{=}O^4$ of thymine [36]. The shift of a component of this complex band from 1647 to 1658 cm^{-1} in the spectrum of the complex R.EcoRII/($\mathbf{12^A/12^T}$) may be due to the interactions of R.EcoRII with residues of cytosine or thymine of the site CCA/TGG that have been formerly predicted [37, 40]. The band at 1622 cm^{-1} in the DNA spectrum is due to the in-plane vibrations of adenine ring with bending vibrations of ND_2 [36]. The decrease in the relative intensity of this band may be caused by the interaction of R.EcoRII with this base in the site CCA/TGG. The interaction of R.EcoRII with the central pair A/T of the recognition site of EcoRII was previously predicted in the study of splitting of substrate analogs [37] and by affine modification of the enzyme by photoactivated substrates [40].

We expected from the general mechanism of DNA splitting with restriction endonucleases and from the studies of R.EcoRII interaction with modified substrates [35, 41] that R.EcoRII would interact at least with two phosphodiester bonds capable to be split by the enzyme and with two adjacent phosphate groups within the recognition site of EcoRII. However the formation of the complex R.EcoRII–DNA did not lead to considerable changes in position and relative intensity of the bands at 1237 and 1089 cm^{-1} corresponding to antisymmetric and symmetric vibrations of O=P=O groups. This is probably due to the small number of phosphate groups interacting with the enzyme compared to their overall number (twenty two phosphate groups) in DNA.

Yet small changes were observed in the conformation of the carbohydrate–phosphate backbone. In the region of carbohydrate residues vibrations [27] a decrease in relative intensity of the bands at 1054 and 970 cm^{-1} was observed presumably because of the change in the conformation of the carbohydrate-phosphate backbone at the interaction of R.EcoRII with the phosphate groups of the site CCA/TGG (Figs. 1c, 1d).

In the spectrum of the complex R.EcoRII–DNA a band was present at 832 cm^{-1} characteristic of the S-conformation of sugar in the B-form of the double helix [27]. No absorption at 858 cm^{-1} was observed corresponding to the A-form of DNA (Figs. 1c, 1d). Hence, the formation of the complex with enzyme did not involve transition from the B- to A-form of DNA double helix in agreement with the fact that R.EcoRII could not split DNA–RNA-duplexes [28].

Therefore on formation of the complex R.EcoRII–DNA a change occurred in the secondary structure of R.EcoRII and in the spectrum in the region of heterocyclic bases and the carbohydrate-phosphate backbone of DNA suggesting the contact of the enzyme with these DNA components. The observed structural changes in DNA and R.EcoRII correspond to DNA–protein interactions both in the *N*-terminal effector-binding and *C*-terminal endonuclease-like domains of R.EcoRII. It would be interesting to study further individually interactions of each domain with DNA.

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