# Three sequence-specific endonucleases from *Escherichia coli* RFL47

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The characterization of the new restriction enzyme *Eco*47III recognizing a hexanucleotide palindromic sequence 5′AGC↓GCT and cleaving, as indicated by the arrow, is reported. It was isolated from *Escherichia coli* strain RFL47. Another two specific endonuclease *Eco*47I (isoschizomer of *Ava*II) and *Eco*47II (isoschizomer of *Asu*I) were also found in this strain. There are two *Eco*47III recognition sites on λ DNA at 20997 and 37060 basepairs. The central *Eco*47III fragment can be replaced by a cloned fragment in λ vector mutant in $t_{R2}$ gene; i.e., λ *gt*

E. coli *site-specific endonuclease* *molecular cloning*

## 1. INTRODUCTION

Although a relatively large number of site-specific endonucleases has been described [1] the search for new restrictases remains urgent due to the outstanding role these enzymes play in the structural analysis and cloning of DNA.

The characterization of the restriction enzyme *Eco*47III recognizing a hexanucleotide palindromic sequence $5'\overset{AGC\downarrow GCT}{TCG\uparrow CGA}$ and cleaving, as indicated by arrows, is reported. *Eco*47III was isolated from *Escherichia coli* RFL47. Another two restriction enzymes *Eco*47I (isoschizomer of *Ava*II) and *Eco*47II (isoschizomer of *Asu*I) were also found in this strain.

## 2. MATERIALS AND METHODS

### 2.1. *Strain and culture*

*E. coli* RFL47 was cultivated at 37°C as in [2].

### 2.2. *Restriction enzymes*

*Eco*47I, *Eco*47II and *Eco*47III were separated and isolated until essentially free of contaminating non-specific nuclease activities by chromatography on phosphocellulose P11, DEAE-cellulose and heparin–Sepharose. The complete purification procedure will be published elsewhere. Restriction enzymes *Eco*RI, *Bam*HI, *Sal*GI, *Asu*I, *Pvu*II and polynucleotide kinase were isolated in our laboratory. Alkaline phosphatase and pancreatic DNAse were obtained from Sigma and VPDE from Merck.

### 2.3. *DNA and reagents*

DNA of phages λC1857S7, fd and φX174 and plasmid pBR322 were kindly provided by K. Sasnauskas; pBR322 $dcm^+$ and pBR322 $dcm^-$ DNAs were used ($dcm^+$ indicates the presence, and $dcm^-$ the absence of modification at the dcm methylase site [3,4]). [$\gamma$-$^{32}$P]ATP was obtained from Isotope (Tashkent), agarose from Sigma and Sephadex G-50 (fine) from Pharmacia. All other reagents were analytical grade commercial products.

### 2.4. *Determination of the activity of* Eco*47I,* Eco*47II and* Eco*47III*

Endonuclease activity was assayed by adding 2–10 μl enzyme solution to 40 μl reaction mixture: 10 mM Tris–HCl (pH 7.8), 5 mM $MgCl_2$, 20 mM NaCl, 10 mM 2-mercaptoethanol, 100 μg/ml albumin and 2 μg DNA. Incubations were performed at 37°C for 1 h. Restriction fragments were

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Table 1

Comparison of computer-generated (C) and experimentally determined (E) *Eco*47I and double digest patterns of pBR322 DNA[a] (fragment sizes in bp)

| *Eco*47I | | *Eco*47I + *Eco*RI | | *Eco*47I + *Bam*HI | | *Eco*47I + *Pvu*II | |
|---|---|---|---|---|---|---|---|
| C | E | C | E | C | E | C | E |
| 1746 | 1800 | 1746 | 1800 | 1746 | 1800 | 1439 | 1440 |
| 1433 | 1430 | 799 | 800 | 1011 | 1010 | 1433 | 1430 |
| 303 | 300 | 634 | 625 | 422 | 420 | 307 | 300 |
| 279 | 280 | 303 | 300 | 303 | 300 | 303 | 300 |
| 249 | 250 | 279 | 280 | 279 | 280 | 279 | 280 |
| 222 | 220 | 249 | 250 | 249 | 250 | 249 | 250 |
| 88 | 90 | 222 | 220 | 222 | 220 | 222 | 220 |
| 42 | –[b] | 88 | 90 | 88 | 90 | 88 | 90 |
| | | 42 | – | 42 | – | 42 | – |

[a]dcm[−] DNA was used; [b]– fragments missed on gel

separated by electrophoresis in 0.7% or 1% agarose gels prepared in 0.1 M sodium borate buffer (pH 8.2), 2 mM EDTA. To determine fragment sizes 2% agarose gels were used.

Double digests were performed by simultaneous addition of enzymes to the above buffer solution.

### 2.5. *Determination of enzyme specificity*

A comparision drawn between results from cleavage of some plasmid and phage DNAs with known nucleotide sequences and tabulated data [5] was used to predict the nucleotide sequence. To confirm the predicted sequence pBR322 single and double digests were performed and fragment number and sizes were determined. The experimental data were compared with computer-predicted values based on the known pBR322 sequence [6].

To determine *Eco*47III cleavage site pBR322 DNA was cleaved with the enzyme. The resulting fragments were dephosphorylated with alkaline phosphatase and labeled at the 5′-termini using T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. Labeled DNA was isolated on Sephadex G-50, precipitated with ethanol and digested with pancreatic DNAse to yield 5′-end-labeled oligonucleotides representing part of the cleavage site. An aliquot of partially hydrolyzed DNA was digested using VPDE [7] and the 5′-terminal mononucleotide was identified by paper electrophoresis on Whatman 1 (pH 3.5).

To determine the 5′-end structure of restriction fragments the mixture of partial and exhaustive [5′-$^{32}$P]DNA hydrolysis products were subjected to sequence analysis by mapping on two-dimensional homochromatography [8].

## 3. RESULTS AND DISCUSSION

It was determined that *Eco*47I makes 8 cuts in pBR322, 1 in $\phi$X174 and no cuts in fd DNAs. These results, when compared with tabulated data given in [5], suggested 5′GG(A/T)CC as the

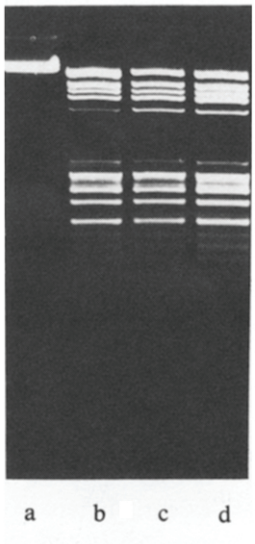


Fig.1. Comparison of cleavage patterns on agarose gels: (a) λDNA; (b) *Eco*47II + λDNA; (c) *Eco*47II + *Asu*I + λDNA; (d) *Asu*I + λDNA.

Table 2

Comparison of computer-generated (C) and experimentally determined (E) *Eco*47III and double digest patterns of pBR322 DNA (fragment sizes in bp)

| *Eco*47III | | *Eco*47III + *Bam*HI | | *Eco*47III + *Sal*GI | | *Eco*47III + *Eco*RI | | *Eco*47III + *Pvu*II | | *Eco*47III + *Pst*I | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C | E | C | E | C | E | C | E | C | E | C | E |
| 2868 | 2842[a] | 2868 | 2842[a] | 2868 | 2842[a] | 2634 | 2612[a] | 2529 | 2512[a] | 1882 | 1900 |
| 952 | 970 | 952 | 970 | 952 | 970 | 952 | 970 | 952 | 970 | 986 | 1000 |
| 280 | 290 | 280 | 290 | 262 | 260 | 280 | 290 | 339 | 330 | 952 | 970 |
| 262 | 260 | 143 | 140 | 156 | 160 | 262 | 260 | 280 | 290 | 280 | 290 |
| | | 119 | 120 | 124 | 130 | 234 | 230 | 262 | 260 | 262 | 260 |

[a] Fragment size obtained by subtracting from the total molecule length the sum of the lengths of all other fragments, deduced from electrophoresis experiments

recognition sequence of *Eco*47I. To confirm this sequence the number and sizes of restriction fragments generated by pBR322, when digested with *Eco*47I alone or with *Eco*47I and some other enzymes, were determined and compared with computer-predicted ones for the 5′GG(A/T)CC sequence (table 1). It is obvious that the calculated sizes of fragments are in good agreement with the experimentally determined ones. The obtained data confirm that *Eco*47I recognizes 5′CC(A/T)CC and is an isoschizomer of the known restriction enzyme *Ava*II recognizing the given sequence [9].

In the above mentioned experiment partial resistance of one cleavage site located at 1439 bp from *Eco*RI site to the action of *Eco*47I was observed when pBR322 dcm$^-$ was substituted for pBR322 dcm$^+$ DNA (not shown). This is the site in which *Eco*47I and dcm methylase recognition sequences overlap: 5′$\overset{GGTC^mCT}{CCAG\ GA^mCC}$5′. Cytosines modified by dcm methylase [4] are indicated as $^m$C. From these results it can be inferred that methylation in only one strand of the left marginal cytosine in *Eco*47I recognition sequence renders DNA resistant to *Eco*47I cleavage.

### 3.1. *Characterization of* Eco*47II*

The cleavage pattern of *Eco*47II examined by agarose gel electrophoresis was identical to that of *Asu*I (fig.1) which is known to recognize the 5′GGNCC sequence.

### 3.2. *Characterization of* Eco*47III*

It was observed that *Eco*47III cuts pBR322 DNA into 4 fragments and does not cleave $\phi$X174 DNA and fd DNA. According to the tabulated data in [15] 5′AGCGCT and 5′GCCGGC were found to be the only nucleotide sequences giving the same fragmentation frequency of the above mentioned substrates, such as *Eco*47III. Experimentally determined sizes of *Eco*47III restriction fragments generated by pBR322 were in good agreement with computer-predicted ones (fig.2, table 2) for the 5′AGCGCT sequence. Double digests of pBR322 DNA with *Eco*47III and some other known restriction enzymes also confirm the suggested sequence (fig.2, table 2).

5′-Termini analysis was performed to determine the cleavage site. Not less than 90% of radioactivi-

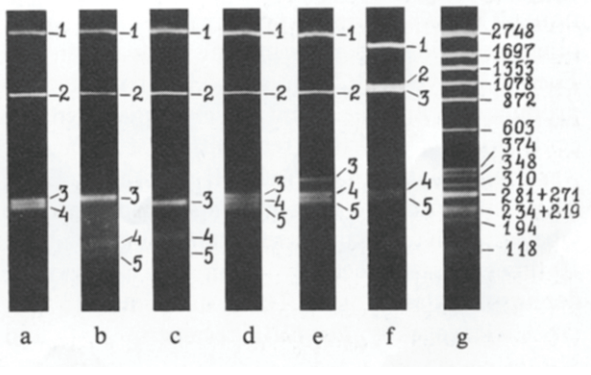


Fig.2. Gel electrophoresis of pBR322 digests. Samples were analyzed in 2% agarose gels (a) *Eco*47III; (b) *Eco*47III + *Bam*HI; (c) *Eco*47III + *Sal*GI; (d) *Eco*47III + *Eco*RI; (e) *Eco*47III + *Pvu*II; (f) *Eco*47III + *Pst*I; (g) $\phi$X174 + *Msp*I and $\phi$X174 + *Bsp*I. Sizes of the markers in (g) are indicated on the right in base pairs (bp). The sizes of *Eco*47III fragments in (a–f) are given in table 2.

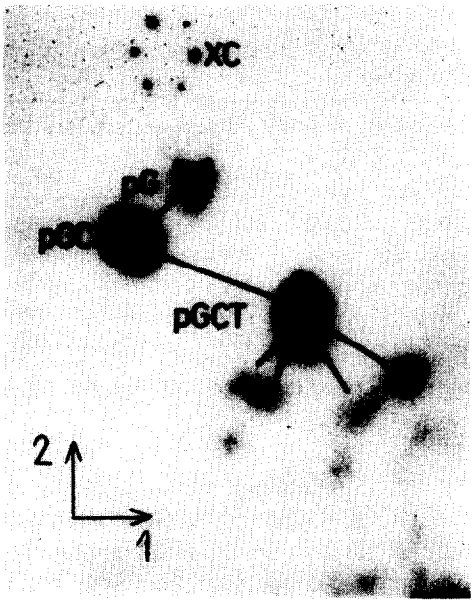


Fig.3. Two-dimensional map of the partial pancreatic DNAse and venom phosphodiesterase digestion products of 5' terminally labeled pBR322 *Eco*47III restriction fragments (1st dimension). Electrophoresis on cellulose acetate strip in pyridine acetate at pH 3.5; (2nd dimension) homochromatography on a DEAE-cellulose thin-layer plate in homomixture VI [8]. XC-xylene cyanol FF.

ty applied was found in the pdG spot after [5'-$^{32}$P]mononucleotide electrophoretic separation. The wandering spot analysis (fig.3) of oligonucleotides neighboring the cleavage site of *Eco*47III gave 5'GCT as a specific trinucleotide product so that the point of cleavage is in the center of $5'\overset{AGC\downarrow GCT}{TCG\uparrow CGA}5'$ recognition sequence. It was determined that *Eco*47III-generated DNA fragments untreated with alkaline phosphatase are labeled with T4 polynucleotide kinase about 10-times less effectively than the previously dephosphorylated ones. These data indicate that DNA cleavage by *Eco*47III results in 5'-P and 3'-OH ends.

As far as we know, *Eco*47III is a new addition to the list of known restriction enzymes. It was determined that *Eco*47III cuts phage λ DNA into 3 fragments (not shown). The nucleotide sequence of phage λ [12] was inspected manually and two *Eco*47III recognition sites at 20997 and 37060 bp were found. According to the genetic map of λ [13] the central λ·*Eco*47III fragment contains genes that are dispensable for lytic growth and a part of gene *N*, so that the central fragment of *Eco*47III can be replaced by a cloned fragment in a vector mutant in $t_{R2}$ gene, i.e., λgt [14].

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