*Eae*I: a restriction endonuclease from *Enterobacter aerogenes*

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We describe the isolation and characterization of a type II restriction endonuclease from *Enterobacter aerogenes*. This recognises and cleaves the family of related sequences:

↓
5′-Py–G–G–C–C–Pu-3′

to generate DNA fragments with 5′-tetranucleotide extensions. *Eae*I may be useful in molecular cloning experiments, especially in conjunction with other enzymes which generate the same terminal extensions.

Potential problems in the methods used to determine the cleavage specificity are discussed.

Site-specific endonuclease *DNA sequencing* *Molecular cloning* *DNA methylation* *DNA–protein recognition* *Restriction mapping*

1. INTRODUCTION

Type II restriction endonucleases are widely used in the analysis and restructuring of DNA molecules, and are being increasingly studied as model systems to investigate the mechanisms of DNA–protein interactions [1]. New specificities are continually required in order to increase the number of ways in which DNA can be manipulated in vitro and to provide new insights into the mechanisms whereby proteins recognise nucleotide sequences in DNA.

This paper describes the enzyme *Eae*I which recognises a family of related sequences, but cleaves at least one member of this family more slowly than the others. Restriction endonucleases of related specificity are known, and *Eae*I may be useful in molecular cloning and in the study of DNA–protein interactions.

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2. EXPERIMENTAL

2.1. *DNA and reagent*

Plasmid and bacteriophage DNA substrates were prepared as in [2]. Enzymes and chemicals were obtained from the suppliers in [2,3].

2.2. *Preparation of* Eae*I*

Enterobacter aerogenes PW201 was isolated by Dr K. Goverd, and was maintained in nutrient agar stab cultures. Cells were grown in nutrient broth with shaking at 37°C, harvested in stationary phase and stored as a cell paste at −20°C.

The cell paste (11.5 g) was resuspended in 20 ml 10 mM 2-mercaptoethanol, 10 mM Tris (pH 7.5), sonicated at 60 W (20 × 30 s with intermittent cooling), and centrifuged at 100000 × *g* for 90 min. The supernatant was applied directly to a column of phosphocellulose P11 (20 × 2 cm, equilibrated in PC buffer: 10% glycerol, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM potassium phosphate, pH 7.4) and was eluted with a gradient of 0–1.0 M KCl in PC buffer. Fractions were assayed for endonuclease activity as in [3] and active fractions (0.25–0.45 M KCl) were pooled and

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dialysed against PC buffer. The enzyme was further purified on DEAE-cellulose and eluted at 0.4–0.5 M KCl in PC buffer, immediately after a non-specific nuclease activity. Active fractions judged free of non-specific nuclease in prolonged assays were concentrated by chromatography on heparin–Sepharose. The enzyme eluted at 0.5–0.75 M KCl in PC buffer, and was dialysed against 50% glycerol in PC buffer prior to storage at −20°C. The yield of enzyme was about 500 units/g wet wt of cells, where a unit of enzyme is that required to fully digest 1 μg DNA in 60 min at 30°C in 50 μl buffer (section 3.1). This yield was difficult to determine due to the presence of persistent partial digestion products (sections 3 and 4).

2.3. *Determination of the cleavage specificity of* EaeI

The methods for mapping *Eae*I cleavage and for the identification of the phosphodiester bonds cleaved in the DNA sequence are essentially those in [2,4]. The specific problems encountered in determining the cleavage specificity of *Eae*I are described in sections 3 and 4.

3. RESULTS

3.1. *Conditions for* EaeI *digestion*

Assays were performed to determine the salt concentration and temperature optima for *Eae*I. The enzyme was found to be rapidly inactivated in the absence of DNA at 37°C or 30°C, and gradually lost activity in the presence of DNA. This inactivation was minimised by the inclusion of gelatin in the reaction mixture. Bovine serum albumin was not as efficient in minimising inhibition as was gelatin. Suitable conditions for *Eae*I digestion were found to be at 30°C in a buffer containing 50 mM NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μg gelatin/ml (autoclaved as a 2 mg/ml stock prior to addition) and 10 mM Tris–HCl (pH 7.5).

3.2. *Mapping* EaeI *cleavage sites*

Plasmid pBR322 DNA was digested with *Hae*III and *Eae*I both singly and in a double digestion experiment, and the products were analysed by slab gel electrophoresis (fig.1, channels a–c). *Eae*I cleaved at more than 5 sites on pBR322 DNA, but there was no apparent difference between the *Hae*III and *Hae*III + *Eae*I digest patterns. This indicates that the *Hae*III site constitutes part of the *Eae*I site.

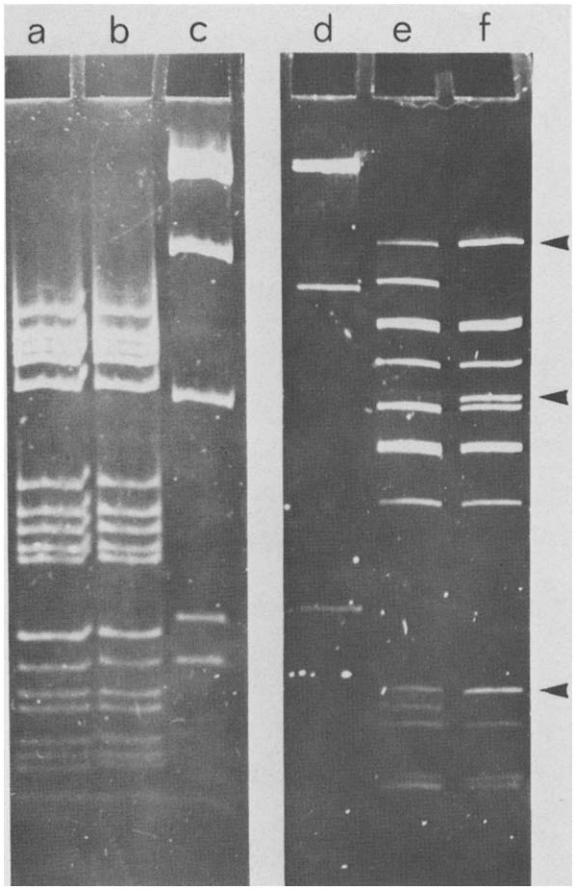

Fig.1. Gel electrophoretic separation of the products of digestion of pBR322 DNA with: (a) *Hae*III; (b) *Hae*III and *Eae*I; (c) *Eae*I. Of M13mp7 RFI DNA with: (d) *Eae*I; (e) *Eae*I and *Hpa*II; (f) *Hpa*II, on 5% acrylamide slab gels which were run, stained and photographed by standard methods [3]. The arrows indicate the M13mp7 *Hpa*II fragments cleaved by *Eae*I; the smallest such fragment is one of a doublet.

Other experiments (not shown) show that bacteriophage λ DNA contained about 25 *Eae*I cleavage sites, bacteriophage φX174 RFI DNA contained 2 *Eae*I sites and pA03 DNA and SV40 DNA contained no sites. Bacteriophage M13 RFI DNA contained 1 *Eae*I site whereas M13mp7 RFI DNA contained 3 sites, indicating that two of the sites were in the *lac* region of M13mp7 used for DNA sequence analysis. In order to map these 3 sites, M13mp7 was digested with *Hpa*II and *Eae*I

in single and double digests (fig.1, channels d–f).

*Eae*I generates DNA fragments of 6040, 950 and 245 nucleotide pairs on M13mp7 RFI DNA (fig.1, channel d), and these sites map in *Hpa*II fragments 1, 5 and 10b (fig.1, channels e,f). As *Eae*I sites coincide with *Hae*III sites, and from the calculated sizes of the fragments generated by double-digestion with *Hpa*II and *Eae*I, *Eae*I sites were mapped at positions 5080, 6036 and 6281 in the M13mp7 DNA sequence [5]. These *Hae*III sites, and only these *Hae*III sites in M13mp7, are members of the family of related sequences:

5′-Py–G–G–C–C–Pu-3′
3′-Pu–C–C–G–G–Py-5′

and this is proposed to be the recognition sequence of *Eae*I. The only sequence in this family of related sequences that is not represented in M13mp7 RFI DNA is 5′-C–G–G–C–C–G-3′, but this is present in pBR322 DNA [6] and is cleaved by *Eae*I (not shown).

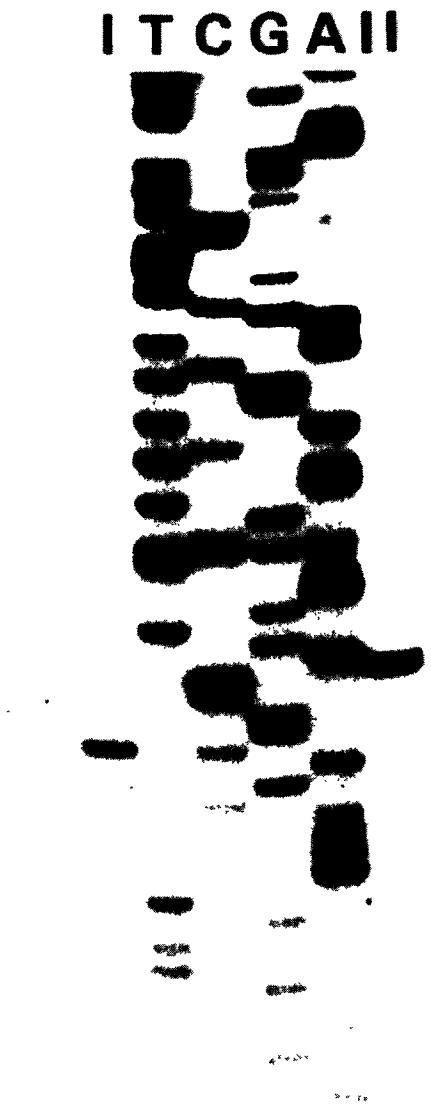

Fig.2. Autoradiograph of a sequencing gel locating the *Eae*I cleavage site in the 'universal primer' region of M13mp2 DNA. Channel I locates the phosphodiester bond cleaved in the sequenced strand; channel II locates that in the template strand. (The autoradiograph is slightly overexposed to show the smaller bands. Bands with the same mobility in channels T, C, G and A are due to end-repair of the primer DNA.)

3.3. *Determination of the cleavage site of* EaeI

One of the *Eae*I sites mapped in M13mp7 DNA is in the region corresponding to the 3′-end of the 96 basepair 'universal' primer used for DNA sequencing [7]. The 96 basepair primer was digested with exonuclease III as in [8] and used as primer on M13mp2(+) strand DNA as template for sequence analysis through the *Eae*I site by the chain-termination method [9]. The sites of cleavage of *Eae*I in both DNA strands were determined as in [4]. The results of the site location experiment are shown in fig.2. The bands in channels I and II show the positions of the phosphodiester bonds cleaved in the newly-synthesized DNA strand, and in the complementary (template) strand, respectively. The sites of cleavage in the sequence are:

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          ↓
5′-C–G–A–C–G–G–C–C–A–G–T–G-3′
3′-G–C–T–G–C–C–G–G–T–C–A–C-5′
                  ↑
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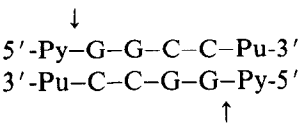
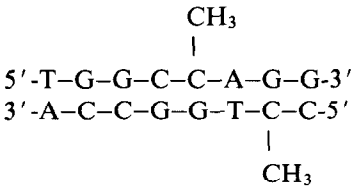
where the top strand is that read from the autoradiograph in fig.2. We suggest that the other members of the family of *Eae*I recognition sites are cleaved in an identical manner.

4. DISCUSSION

The recognition and cleavage site of *Eae*I was predicted to be:



Computer analysis [2,10] of the predicted number of cleavage sites on the fully sequenced DNA molecules [5,6,11–14] from plasmids pA03 and pBR322, from bacteriophage ϕX174, M13 and M13mp7 and from SV40 shows good agreement with the observed number of cleavage sites. The only discrepancy is the sequence at position 1446 in the pBR322 sequence, where the sequence 5′-T–G–G–C–C–A-3′ is not cleaved by *Eae*I. This sequence is cleaved, albeit slowly, in M13mp7 RFI DNA. The sequence in pBR322 DNA overlaps with the recognition sequence for the *mec* methylase of *Escherichia coli* (5′-C–C–A–G–G-3′), and in the C600 (pBR322) strain used as a source of pBR322 DNA, this sequence would be methylated to give 5′-methylcytosine residues as follows:



This methylation appears to prevent *Eae*I cleavage. Such problems have been noted previously [15]. We have shown (not given) that chromosomal DNA from *E. aerogenes* is modified against cleavage by *Eae*I, but the nature and site of this modification is unknown.

The method used to determine the cleavage specificity of *Eae*I is general [4] and offers several advantages over computer methods (see [2]). However, the method does require that the ^{32}P-labelled DNA fragments used for the site-location (channels I and II) share a common 5′-end with the fragments generated in the chain-termination sequencing reactions. This only occurs with the cleavage site proximal to the common end of the fragments. If the primer DNA contains a site for the restriction enzyme, as with *Eae*I, this precludes sequence analysis of sites in adjacent cloned fragments [2,16] except under carefully controlled conditions. In order to minimize problems of this sort we use small synthetic primers of known DNA sequence whenever possible, and we check novel restriction endonucleases for cleavage sites within the primer-complementary region and the cloning site of M13mp7 RFI DNA and related DNAs.

The enzyme *Gdi*III from *Gluconobacter dioxyacetonicus* has been reported to have a recognition sequence 5′-Py–G–G–C–C–G-3′ (or 5′-C–G–G–C–C–Pu-3′) and to cut to give 5′ tetranucleotide extensions (M. van Montagu, unpublished observations reported in [17]). *Gdi*II recognises and cleaves the same sequences as *Eae*I, except the sequence 5′-T–G–G–C–C–A-3′. In all our experiments this sequence appears to be cleaved much more slowly than other members of the family of sequence recognised by *Eae*I. This is noticeable in fig.1 (channels d–f) in which *Hpa*II fragment 1, which contains the *Eae*I site 5′-T–G–G–C–C–A-3′, is only partially cleaved whereas *Hpa*II fragment 5, containing 5′-T–G–G–C–C–G-3′, is completely cleaved by *Eae*I. Under normal digestion conditions *Eae*I gives about 25 fragments from bacteriophage λ DNA, suggesting that the sequence 5′-T–G–G–C–C–A-3′ (18 sites out of 39 *Eae*I recognition sites; [18]) is cleaved only infrequently. From the DNA sequence [18] this result cannot be explained by modification of *Eae*I sites by known methylases. It is possible that *Eae*I and *Gdi*II have the same specificity, but that the 5′-T–G–G–C–C–A-3′ cleavage is more readily detected with *Eae*I.

Another enzyme (*Cfr*I) with the same specificity as *Eae*I has been isolated from *Citrobacter freundii* [19]. No data on the relative rate of cleavage of different sites are given.

The enzyme *Xma*III [20] recognises one of the family of sequences recognised by *Eae*I (5′-C–G–G–C–C–G-3′) and cleaves it at the same position. This sequence occurs once on the plasmid pBR322 in the tetracycline resistance gene (position 941). *Xma*III provides a single target on pBR322 into which *Eae*I fragments (or *Gdi*II or *Cfr*I fragments) could be cloned and recombinants could be screened by insertional inactivation of tetracycline resistance. This may be of use in molecular cloning experiments.

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