Three Restriction Endonucleases from Anabaena flos-aquae

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Three site-specific endonucleases, AflI, AflII and AflIII, have been partially purified from the cyanobacterium Anabaena flos-aquae CCAP 1403/13f. Their recognition and cleavage specificities have been determined to be:

AflI  \[5'-G\downarrow G\downarrow (A)\downarrow -C-C-3']

AflII  \[5'-C\downarrow T\downarrow T\downarrow A-A-G-3']

AflIII  \[5'-A\downarrow C\downarrow Pu-Py-G-T-3']

AflII and AflIII are new specificities and may be useful in molecular cloning, as well as in the analysis of DNA. The distribution of type II restriction endonucleases in the cyanobacteria is briefly discussed.

INTRODUCTION

Type II restriction endonucleases are widely used in the analysis and restructuring of DNA molecules, and provide good systems for the study of DNA–protein interactions. The current compilation of restriction endonuclease activities (Roberts, 1984) contains 475 such activities, representing a minimum of 103 different specificities from 356 bacterial strains. Enzymes with new specificities are particularly useful in that they increase the range of DNA manipulations that can be performed.

The cyanobacteria are a rich source of type II restriction endonucleases. Both the filamentous cyanobacteria (Anabaena, Nostoc) and the unicellular cyanobacteria (Aphanotohece, Dactylcocopsis) contain these activities (Murray et al., 1976; Reaston et al., 1982; Whitehead & Brown, 1982; N. L. Brown, unpublished observations). In many cyanobacteria there are multiple enzymes in a single strain, and one strain of Nostoc contains five such activities (Reaston et al., 1982). Some of the enzymes are isoschizomers (i.e. enzymes with the same recognition specificity occurring in different strains), and these often occur in a variety of combinations with other endonucleases in different strains of cyanobacteria. For example, Nostoc sp. PCC 7524 (Reaston et al., 1982) contains enzymes with the recognition specificities of NspI, AvaI, AsuI, AsuII and SduI; Nostoc sp. PCC 7413 contains enzymes recognizing NspI and AvaII sites (M. G. C. Duyvesteyn, J. Reaston & A. deWaard, unpublished observations reported in Roberts, 1984); Anabaena variabilis ATCC 27892 contains AvaI, AvaII and AvaIII (Murray et al., 1976; Reaston et al., 1982); Anabaena subcyldindrica contains AsuI, AsuII and an enzyme recognizing the AcyI site (deWaard & Duyvesteyn, 1980); Anabaena cylindrica contains AcyI (deWaard et al., 1978).

We have screened a number of bacterial strains as part of a search for novel restriction endonuclease activities. In this paper we describe the partial purification and characterization of three type II restriction endonucleases in the strain Anabaena flos-aquae CCAP 1403/13f.

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Abbreviations: Pu, purine nucleoside; Py, pyrimidine nucleoside.

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METHODS

DNA and enzymes. Bacteriophage λcl857Sam7 DNA, bacteriophage φX174am3cs70 replicative form I (RFI) DNA, plasmid pAO3 DNA and pBR322 DNA were prepared as described previously (Brown et al., 1980). Adenovirus-2 DNA was a gift of Dr Janet Arrand, St Mary's Hospital Medical School, London, UK, and recombinant bacteriophage M13mp2 and M13mp7 DNAs containing fragments of Tn5Ol or pBR322 DNA were gifts of Dr D. C. Fritzinger and R. D. Pridmore. Restriction endonucleases, DNA polymerase and other DNA modifying enzymes were purchased from Boehringer, New England Biolabs or BRL.

Purification of restriction endonucleases from Anabaena flos-aquae. Packed cells of Anabaena flos-aquae CCAP 1403/13f were provided by Professor A. E. Walsby, Department of Botany, Bristol University, UK, and were stored at -70 °C. These had been grown in the medium described by Walsby & Booker (1980) at 25 °C and sparged with air under 500 lx illumination (rising to 1500 lx later in growth). Frozen packed cells (1-1.5 g) were sonicated in 20 ml extraction buffer (10 mM-2-mercaptoethanol, 10 mM-Tris/HCl, pH 7.5) for 20 × 30 s at 60 W, while maintaining the temperature below 4 °C. The cell suspension was centrifuged at 100000 g for 90 min. The supernatant was made 1-0 M with respect to NaCl and applied to a Sepharose 4B column (50 × 2 cm in 1.0 M-NaCl, 10 mM-mercaptoethanol, 10 mM-Tris/HCl, pH 7-5) in order to separate the endonucleases from the bacterial DNA. Fractions containing endonuclease activity were pooled and dialysed against PC buffer (10%, v/v, glycerol; 0-1 mM-EDTA; 10 mM-2-mercaptoethanol; 10 mM-potassium phosphate, pH 7-4) and applied to a phosphocellulose P-11 (15 × 2 cm) in PC buffer. The column was washed in PC buffer and developed with a gradient (200 ml) of 0-1.0 M-KCl in PC buffer. Endonuclease-containing fractions were identified and pooled according to their patterns of digestion on bacteriophage λ DNA, and dialysed against PC buffer. Fractions eluting at 0-1-0.4 M-NaCl contained the two enzymes AflI and AflII, and those eluting at 0-4-0.6 M-NaCl contained the two enzymes AflIII and AflIVIII. These enzymes were separated on DEAE-cellulose columns (15 × 1.5 cm) developed with gradients (100 ml) of 0-1.0 M-NaCl in PC buffer. The separate enzymes were dialysed against PC buffer and further purified by chromatography on heparin-Sepharose columns (5 × 1 cm) developed with a gradient of 0-1.0 M-NaCl in PC buffer. The salt concentrations at which the enzymes elute from the columns were measured using a conductivity meter, and are given in Table 1.

Endonuclease assays. Column fractions were assayed for site-specific endonuclease activity by incubating 1-5 μl with 1 μg bacteriophage λcl857Sam7 DNA in 25 μl 10 mM-mercaptoethanol, 10 mM-MgCl₂, 10 mM-Tris/HCl, pH 7.9, at 37 °C for 18 h. Each reaction was stopped by adding EDTA to 10 mM, and analysed by electrophoresis on 1% agarose slab gels in 90 mM-Tris/borate, pH 8.3, 2.5 mM-EDTA, 0.5 μg ethidium bromide ml⁻¹. The ethidium bromide-stained DNA was visualized on a long-wave UV transilluminator (Ultra-violet Products, San Gabriel, Calif., USA) and photographed on Polaroid type 667 film through a Schott–Jena KV470 filter.

The optimum conditions of pH, temperature and monovalent cation concentration for cleavage of DNA with AflI, AflII and AflIII were determined by altering these parameters singly from the standard conditions (37 °C in 50 mM-NaCl, 10 mM-mercaptoethanol, 10 mM-MgCl₂, 10 mM-Tris/HCl, pH 7.5). Assays were performed on 0.5 μg bacteriophage λcl857Sam7 DNA for a time which did not quite give complete digestion of the product under standard conditions.

A unit of endonuclease activity is defined as that required to digest to completion 1 μg of bacteriophage λcl857Sam7 DNA in 1 h in a 50 μl reaction volume under optimal buffer and temperature conditions.

Determination of the recognition and cleavage specificity of AflI, AflIII and AflIVIII. The recognition and cleavage specificities of the enzymes were determined by the method of Brown & Smith (1980). The numbers of cleavage sites for each enzyme on a variety of fully sequenced DNA molecules were determined in both single digests and in digests with another enzyme. These data were then compared with the computer-generated tables of Fuchs et al. (1980) in order to indicate possible recognition sites. Single-stranded recombinant M13mp2 and M13mp7 DNA derivatives (Gronenborn & Messing, 1978; Messing et al., 1981) containing a DNA fragment with a single site for the restriction enzyme were obtained from a DNA-sequencing project in this laboratory (Brown et al., 1983; Diver et al., 1983). The method for identifying the phosphodiester bonds cleaved by the restriction endonuclease is described in detail elsewhere (Brown & Smith, 1980; Whitehead & Brown, 1982).

RESULTS

Partial purification of the site-specific endonuclease activities

Three different site-specific endonuclease activities were isolated from Anabaena flos-aquae CCAP 1403/13f. The salt concentrations at which these activities eluted from the chromatographic media used are given in Table 1. The protocol is designed to remove contaminating nucleases activities, and not to maximize the specific activity of each endonuclease. The approximate final yields of each enzyme per g wet weight of cells were: AflI, 250 units; AflII,
Restriction enzymes from Anabaena flos-aquae

Table 1. Conditions for the elution of Afl restriction endonucleases from chromatographic media

<table>
<thead>
<tr>
<th>Restriction endonuclease</th>
<th>Phosphocellulose P-11</th>
<th>DEAE-cellulose</th>
<th>Heparin-Sepharose CL-6B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AflI</td>
<td>0-1-0-4</td>
<td>0-1-0-3</td>
<td>0-1-0-4</td>
</tr>
<tr>
<td>AflII</td>
<td>0-1-0-6</td>
<td>0-4-0-7</td>
<td>0-2-0-4</td>
</tr>
<tr>
<td>AflIII</td>
<td>0-5-0-6</td>
<td>0-2-0-4</td>
<td>0-4-0-5</td>
</tr>
</tbody>
</table>

* Molarity of NaCl in PC buffer (10%, v/v, glycerol; 0.1 mM-EDTA; 10 mM-2-mercaptoethanol; 10 mM-potassium phosphate, pH 7.4).

Table 2. Observed frequency of cleavage of sequenced DNA molecules by restriction endonucleases from A. flos-aquae

<table>
<thead>
<tr>
<th>DNA</th>
<th>Minimum number of sites for endonuclease*</th>
</tr>
</thead>
<tbody>
<tr>
<td>λE1857Sam7</td>
<td>&gt;25 (35)</td>
</tr>
<tr>
<td>Adenovirus-2</td>
<td>&gt;34 (73)</td>
</tr>
<tr>
<td>SV40</td>
<td>5 (6)</td>
</tr>
<tr>
<td>pBR322</td>
<td>6 (8)</td>
</tr>
<tr>
<td>φX174am3cs70</td>
<td>1 (1)</td>
</tr>
<tr>
<td>M13</td>
<td>0 (0)</td>
</tr>
<tr>
<td>pA03</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>

* These are the minimum numbers of sites, as two sites close together may not be distinguished from one another. Numbers in parentheses give the number of sites predicted by computer search routines, and are equal to or greater than the observed minimum number of sites, indicating that the enzymes are substantially free of contaminating specific endonucleases.

300 units; AflIII, 100 units. The absence of contaminating non-specific nucleases was demonstrated by digestion of bacteriophage DNA with a 10-fold excess of enzyme for 1 h, followed by gel electrophoresis of the products, which gave sharp bands on the gel; and also by the DNA sequence analysis of the cleavage sites (see below). This latter method did show a very small amount of contamination of one batch of AflII by a specific endonuclease (see Fig. 2b) but this was not seen in other preparations. Exonuclease contamination of the enzymes would give rise to a series of bands in channels I and II of the site location experiment due to the successive removal of nucleotides from the fragment termini; and this was not seen.

The three endonucleases showed maximal activity at 37°C in 50 mM-NaCl, 10 mM-2-mercaptoethanol, 10 mM-MgCl2, 10 mM Tris/HCl, pH 7-5.

Determination of the recognition and cleavage specificities

The observed minimum numbers of cleavage sites of the three endonucleases on a variety of DNAs are given in Table 2. These data are compiled from both single-enzyme digests and digests with a second enzyme. Fig. 1 shows the results of gel electrophoresis of the products of digestion of bacteriophage λ DNA or adenovirus-2 DNA with AflI, AflII or AflIII.

The logic for determining the recognition and cleavage sequences is different for each of the three enzymes, and will be considered separately.

The AflI site. The sizes and number of fragments produced by AflI digestion of bacteriophage λ DNA were similar to those produced by AvaII. Digestion of pBR322 DNA with AflI or with AvaII showed that the fragment patterns were identical, and a double digest of pBR322 with both enzymes together produced no additional fragments (data not shown). The restriction
Fig. 1. Products of cleavage of bacteriophage λc1857Sam7 DNA and adenovirus-2 DNA with (a) AflI, (b) AflII and (c) AflIII, separated by electrophoresis in 1% agarose in 90 mM-Tris/borate, pH 8.3, 0.25 mM-EDTA, 0.5 μg ethidium bromide ml⁻¹.

Fragment patterns produced by cleavage of fully sequenced DNAs with AflI agree with the patterns predicted for an enzyme recognizing the sequence

\[ 5' - G - G - \left( \text{T} \right) - C - C - 3'. \]

A recombinant DNA molecule containing a HgiAI fragment of pBR322 DNA cloned into bacteriophage M13mp2 was provided by Dr D. C. Fritzinger. This contained the AvaII site at position 1135 of pBR322 DNA (Sutcliffe, 1979). The single-stranded bacteriophage DNA was used as a template to determine the site of AflI cleavage in both DNA strands by the method of Brown & Smith (1980). The autoradiograph of the sequencing gel is shown in Fig. 2(a). The sites of cleavage of AflI within this sequence are:


demonstrating that AflI cleaves the same phosphodiester linkages in this sequence as does AvaII. Some radioactive fragment in channel II remains unprocessed by DNA polymerase; this is probably due to AflI nicking one strand of the DNA, and the nicked DNA not being a substrate for the DNA polymerase reaction (Carter et al., 1980).
Restriction enzymes from Anabaena flos-aquae

The AflII site. The two AflII sites on bacteriophage \( \phi X174 \alpha m3c70 \) RF1 DNA (Table 1) were mapped by standard double-digestion methods to position 4400 (±20) and either position 2420 (±20) or 2920 (±20) on the \( \phi X174 \) sequence (Sanger et al., 1978). Inspection of the sequence in these regions, and reference to computer-generated tables (Fuchs et al., 1980) revealed that the sequence 5'-C-T-T-A-A-G-3' is present at positions 4413 and 2914, and nowhere else in the \( \phi X174 \) sequence. The observed AflII cleavage patterns of other fully sequenced DNAs (Table 2) were those predicted for an enzyme cleaving at this hexanucleotide sequence.

A recombinant bacteriophage M13mp7 containing a TaqI fragment of transposon Tn501 was identified which contained the sequence postulated to be the AflII recognition site. This is at position 4764 of the complete Tn501 sequence (N. L. Brown, R. D. Pridmore & D. C. Fritzinger, unpublished data; position 535 in that part of the Tn501 sequence published in Diver et al., 1983). The autoradiograph from the cleavage site-location experiment (Brown & Smith, 1980) using this recombinant DNA is shown in Fig. 2(b). The site of AflII cleavage within this sequence is shown by the solid arrows:

\[
\begin{align*}
5' & - G - G - C - C - G - G^1 T - T - T - A - A - G - T - G - C - T^1 C - 3' \\
\end{align*}
\]

AflII cleaves the hexanucleotide sequence symmetrically, to give fragments with 5'-terminal extensions. On prolonged incubation faint secondary cleavage sites are observed (Fig. 2b) as shown by the dotted arrows. These secondary cleavage sites are probably due to a minor contaminating specific endonuclease activity. This contaminant was not reproducibly found, and we have been unable to further characterize the activity. It has not been seen on normal restriction digests, only in the very sensitive site-location analyses, and it does not interfere with the normal use of AflII.

The AflIII site. The AflIII cleavage sites on pBR322 DNA and on \( \phi X174 \) RF1 DNA were mapped by standard double-digestion methods to position 2480 (±20) on pBR322 and positions 220 (±20) and 2159 (±20) on \( \phi X174 \). The tables of Fuchs et al. (1980) suggested that the
sequence 5'-A-C-Pu-Py-G-T-3' was the best candidate for the \textit{AflIII} recognition sequence. Members of this family of related sequences are found in pBR322 DNA at position 2481 (5'-A-C-A-T-G-T-3') and in \phi X174 RFI DNA at positions 221 and 2146 (both 5'-A-C-G-C-G-T-3'). Comparison of the patterns of cleavage of fully-sequenced DNAs by \textit{AflIII} with the patterns predicted for an enzyme cleaving at this proposed site gave good correlation, indicating that this is indeed the recognition site of \textit{AflIII}.

A recombinant bacteriophage, M13mp2, containing the \textit{ThaI} fragment from positions 2179-2520 of pBR322 DNA was used as template DNA for the site-location experiment. The autoradiograph showing the results of this experiment is shown in Fig. 2(c). \textit{AflIII} cleaves in the sequence:

\[
5'-G-C-T-C-A-C-A-T-G-T-C-T-T-T-3' \\
\]

The cleavage sites are symmetrically placed within the hexanucleotide recognition site, and \textit{AflIII} cleaves to give 5'-tetranucleotide terminal extensions.

\textbf{DISCUSSION}

We have described the partial purification and characterization of three restriction endonucleases from \textit{Anabaenaflos-aquae} CCAP 1403/13f. A fourth activity was detected but not characterized. \textit{AflI} recognizes and cleaves the sequence:

\[
5' - G \downarrow T - C - C - 3' \\
A - T
\]

and has the same specificity as \textit{AvaII}. The remaining activities, \textit{AflII} and \textit{AflIII}, are novel and recognize the sequences:

\[
5' - A \downarrow C - T - A - A - G - 3' \\
5' - A \uparrow C - Pu - Py - G - T - 3'
\]

respectively. Both of these enzymes cleave to give 5'-tetranucleotide terminal extensions, and may be useful in molecular cloning, although the termini produced by \textit{AflIII} are degenerate and cannot be annealed to all other \textit{AflIII} fragments. Both \textit{AflII} and \textit{AflIII} are further additions to the range of enzymes available for DNA manipulation.

\textit{A.flos-aquae} CCAP 1403/13f contains at least three (and possibly more than four) site-specific endonuclease activities. One of these (\textit{AflI}) has the same cleavage specificity as another \textit{Anabaena} endonuclease, \textit{AvaII} (Hughes & Murray, 1980). Endonucleases with this specificity are also found in the cyanobacteria \textit{Fremyella} and \textit{Nostoc}, as well as in \textit{Bacillus}, \textit{Caryophanon}, \textit{Chloroflexus}, \textit{Escherichia}, \textit{Herpetosiphon} and \textit{Salmonella} (Roberts, 1984). There is no evidence to suggest that these endonucleases in the 'AvaII family' are proteins with a common evolutionary origin, but it is tempting to speculate that these enzymes are very closely related and that they and their corresponding modification methylases have been mobilized in some way. Such mobilization could occur by the genes being plasmid-borne or bacteriophage-borne functions. Other families of isoschizomeric restriction endonucleases exist, such as the \textit{PstI} family and the \textit{EcoRII} family (see Roberts, 1984), and these may also be the products of widely distributed genes.

Another notable feature of the distribution of restriction endonucleases in the cyanobacteria is that many strains contain multiple restriction endonuclease activities, and that these activities occur in different combinations in different strains. This is likely to be one of the factors causing difficulty in establishing a gene transfer system in the filamentous cyanobacteria (Wolk \textit{et al.}, 1984). Duyvesteyn \textit{et al.} (1983) have shown that the variation in restriction endonuclease content between strains may be important in the biological restriction of cyanophage DNA, and the multiple endonuclease activities may be required for adequate protection against the large number of cyanophages.
Lambert & Carr (1984) presented data which suggest that the filamentous and unicellular cyanobacteria differ in the extent to which their DNA is modified. DNAs from the filamentous genera (Anabaena, Nostoc, etc.) are extensively modified in a manner independent of the type II restriction/modification systems found in each strain, whereas DNAs from the unicellular cyanobacteria (Anacystis, Aphanothece, etc.) show variation in the extent of modification. The presence of other DNA-modifying enzymes in the cyanobacteria complicates the interpretation of the functional role of multiple type II restriction/modification systems in the cyanobacteria.

It may be that the presence of multiple type II restriction/modification systems, in which different specificities occur in different combinations in different strains, provides an 'index of relatedness' between cyanobacteria and permits gene transfer in vivo between certain strains, but not between others. Further characterization of the distribution of type II restriction/modification systems in the cyanobacteria is required to test this idea.

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REFERENCES


