TWO SEQUENCE-SPECIFIC ENDONUCLEASES FROM *ANABAENA OSCILLARIOIDES*

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1. Introduction

There has been an increasing number of reports on sequence-specific endodeoxyribonucleases (endo R.) in cyanobacteria (blue–green algae) [1–4]. As their cleavage specificities have proven to be different from those of the many bacterial restriction enzymes already characterized, these new enzymes have been useful additions to the ever expanding endo R. catalog. We report here the isolation of two such endonucleases from one strain of *Anabaena oscillarioides* (*Aos*I and II) which cleave the nucleotide sequences 5′ TGC↓GCA 3′ and 5′ GPu↓CGPyC 3′, respectively.

2. Materials and methods

The strain of *A. oscillarioides* (family Nostocaceae) used (CCAP 1403/11) was obtained from the culture centre of Algae and Protozoa (Downing St, Cambridge). It was grown in an aqueous medium containing an autoclaved extract from garden soil (final conc. 2% (w/v)) and the following salts (mg/l): KNO_3, 1000; $MgSO_4 \cdot 7\ H_2O$, 250; $CaCl_2$, 50; K_2HPO_4, 250; Fe^{3+} (as EDTA complex) 5 (a 1:1 mixture of $FeSO_4 \cdot 7\ H_2O$ and EDTA aerated by shaking overnight); H_3BO_3, 3; $MnCl_2 \cdot 4\ H_2O$, 2; traces (0.2–0.5 mg/l) of Zn^{2+}, Cu^{2+}, Ti^{4+}, Cr^{3+}, Va^{5+}, Co^{2+}, Ni^{3+}, WO_4^{2-} and MO_4^{2-} at pH ~7.5. Sterile air/CO_2 (5%) was bubbled from below; illumination was from two 55 cm fluorescent lighting tubes placed at 40 cm distance. A 100 ml inoculum yielded 100 g cells from 10 ml medium in 3 weeks.

The various materials and methods used for determining the cleavage specificity at the *A. oscillarioides* enzymes were as in [3]. Endo R.*Tha* was purchased from Bethesda Res. Labs, Rockville, MD.

2.1. *Enzyme purification*

The initial stages of the purification procedure were as in [3]. To every 100 ml extract (made 0.1 M with respect to NaCl) 11 ml of a 10% solution of Polymin P (at pH 7.9) were added dropwise with stirring. After centrifugation the supernatant (containing the bulk of the enzyme activity) was adjusted to 70% saturation with ammonium sulfate at 0°C. After standing for 30 min the precipitate was collected by centrifugation at 25 000 × *g* for 60 min, dissolved in 50 ml buffer B (20 mM potassium phosphate (pH 7.4) 0.1 mM EDTA, 0.2 mM $MgCl_2$, 2 mM mercaptoethanol) and dialyzed against 2 × 2 l of the same buffer containing also 10% glycerol (buffer Bg). The precipitate formed after addition of polymin P was not discarded but extracted with buffered 0.6 M NaCl as in [3] and inspected for endonuclease activity as well.

Phosphocellulose chromatography of the dialyzed preparation, as for *Acy*I [3], resulted in the separation of two DNA-cleavage activities eluting in the second half of the 0–0.5 M NaCl gradient in the order *Aos*II, *Aos*I.

2.1.1. Purification of *Aos*I

The active fractions eluting last from the phosphocellulose column were dialyzed against 2 × 2 l buffer Ag (10 mM Tris–Cl (pH 7.4), 0.1 mM EDTA, 0.2 mM $MgCl_2$, 2 mM mercaptoethanol, 10% glycerol). The enzyme was purified further on DEAE–Sephadex A50 and DEAE-cellulose (DE 52, Whatman) as for *Acy*I [3].

2.1.2. Purification of *Aos*II

The fractions showing cleavage activity that eluted in front of *Aos*I were concentrated by adsorption onto a 4 × 1 cm column of hydroxyapatite (Biorad) equilibrated with 10 mM KPO_4 (pH 7.0) – 2 mM mercaptoethanol and eluted with 0.4 mM potassium phosphate (pH 7.0). The enzyme (5 ml), still heavily contaminated with an exonuclease was diluted in small portions with 6 vol. Ag buffer containing 30 μg/ml of autoclaved gelatin and applied to a 8 × 1.5 cm column of heparin–Sepharose [5]. Active fractions appeared halfway through an 80 ml gradient of 0.15–0.80 M NaCl in Ag buffer. These fractions were diluted 5-fold with Bg buffer containing 30 μg/ml of autoclaved gelatin, applied to a 4 × 1 ml phosphocellulose column and eluted in 1 ml with 0.3 M NaCl in Bg buffer. Both enzymes (*Aos*I and II) were stored in an ice–salt bath at –8°C.

2.2. *Localization of cleavage sites in DNAs of known nucleotide sequence*

Three such DNA molecules served this purpose: plasmid pBR322 DNA [6,7], the replicative form of bacteriophage φX174 DNA [8,9] and the left terminal fragment of adenovirus 5 DNA, *Hpa*I–E [10,11]. Agarose gel patterns obtained following single digests of these DNA molecules with endo R.*Taq*I, *Hin*fI, *Hae*III, *Alu*I *Hha*I or *Acy*I [3] were compared to patterns obtained after double digests of the DNAs with one of these endo Rs and either *Aos* enzyme. Inspection of the patterns revealed which of the fragments contained cleavage sites for an *Aos* endonuclease.

2.2.1. Determination of cleavage specificity

Three approaches (two of which were treated extensively in [3]) were used:

1. The nucleotide order at 5′-termini generated by *Aos*I or II cleavage was determined by labeling with [γ-^{32}P]ATP and polynucleotide kinase [12], digesting with pancreatic DNase and separation of the oligonucleotides by two-dimensional homochromatography;
2. Nucleotide sequences of specific, 5′-[^{32}P]DNA fragments generated by *Aos*I or II were determined [13] and aligned with the known nucleotide sequence;
3. The 'fifth lane' method [14,15] was applied to a kinase-labeled *Taq*I/*Ava*I fragment of pBR322 containing an *Aos*I site. An aliquot containing 5% of this fragment was digested with *Aos*I and run besides a dideoxy sequence ladder [16] of the *Taq*I/*Ava*I fragment, thus yielding the *Aos*I recognition and cutting pattern.

3. Results

3.1. *Cleavage pattern of* Aos*I*

This enzyme cleaves adenovirus 5 DNA at ~15 sites, λ DNA at ~10 sites, bacteriophage φX174 RF DNA once and SV40 DNA not at all. Sites of cleavage in pBR322 were located in three areas near nucleotides 270, 1400 and 3600. When looking for common sequences near those points we noted that only at positions 262, 1357, 1455 and 3588 in pBR322 DNA [7] the symmetrical hexanucleotide sequence 5′ TGCGCA 3′ occurs (fig.1a). In order to prove that these sequences are indeed recognized by *Aos*I, pBR322 was cleaved with this enzyme and the sites of cleavage were labeled with ^{32}P using polynucleotide kinase. Fragments containing only one labeled 5′-terminus were obtained by a second cleavage with endo R.*Tha*I [15,16]. The nucleotide sequence from one such fragment is depicted in fig.2. Comparison

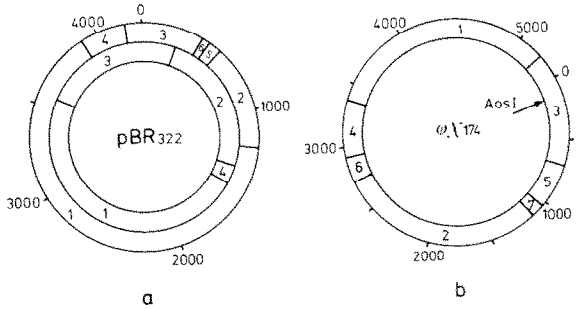
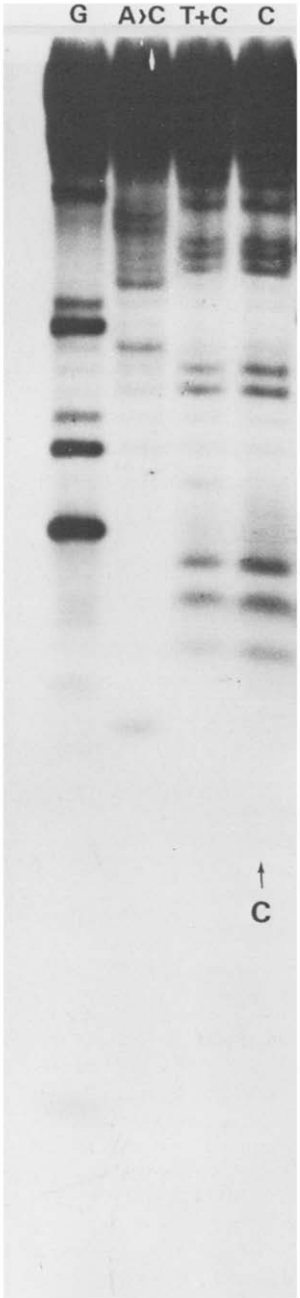


Fig.1. Maps of endo R.*Aos*I and *Aos*II cleavage sites in pBR322 DNA and φX174 RF DNA. (a) The inner circle gives the number and size of pBR322 DNA fragments generated by *Aos*I. The positions of the nucleotides at the 3′-termini of the cleavage sites are 262, 1357, 1455 and 3588. The outer circle gives the number and size of pBR322 fragments obtained following digestion by *Aos*II. The coordinates are 414, 435, 548, 1205, 3903 and 4285. (b) Fragmentation of φX174 RF DNA by endo R.*Aos*II is shown in double circle. The cleavage coordinates are: 718, 1020, 1134, 2783, 2977, 3364 and 5226. The single cut by *Aos*I at position 157 is indicated by the arrow.

with the sequence of pBR322 [7] shows that the sequence is that predicted by the above assumption, and furthermore indicates that the cleavage occurs in the middle of the recognition sequence 5′ TGC↓GCA 3′. The observation that *Aos*I cuts φX174 RF DNA once [19] is also in line with these findings (fig.1b). The cleavage point was confirmed by controlled digestion with pancreatic DNase of 5′-^{32}P-labeled *Aos*I fragments of pBR322 DNA. The two-dimensional homochromatogram is depicted in fig.3. The 5′-



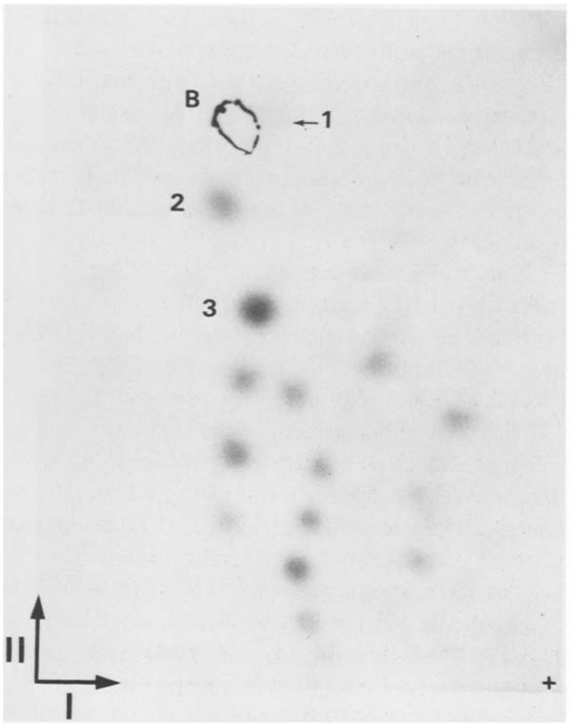


Fig.3. Two-dimensional homochromatogram showing 5′-^{32}P-labeled oligonucleotides derived from sequences cut by endo R.*Aos*I. (1) pG; (2) pGpC; (3) pGpCpA; (B) blue marker (xylene cyanol FF). The identity of the remaining spots can be established by applying the 'wandering spot rules' [17] and is in accord with the structures near the three *Aos*I sites in pBR322 DNA (262, 1357, 1455) which were studied here (I) direction of electrophoresis; (II) chromatography.

Fig.2. Nucleotide sequence determination of the pBR322 fragment proceeding from the *Aos*I site at coordinate 1455 to the *Tha* site at position 1416. pBR322 DNA was digested with *Aos*I, labeled with ^{32}P at the 5′-termini and further cleaved with *Tha*I. Isolated fragments were chemically cleaved and the products were separated on a 20% polyacrylamide denaturing gel [13]. The lanes are: G only, A>C, T+C and C only, respectively. The sequence partially reads, from the bottom up, GCACCCGTGGCCAGGACCCAA.

terminal nucleotide at these cleavage sites was identified as pG by further degradation of a portion of the DNase digest with snake venom exonuclease followed by electrophoresis on Whatman no. 1 paper. 5′-Oligonucleotides were deduced to be pGpC, pGpCpA and higher homologs by applying the mobility shift rules described [17] to the homochromatogram. The 'fifth lane' technique (see section 2) was also used to demonstrate clearly the cleavage site of *Aos*I. By using the dideoxy technique [16] to generate the sequence ladder, the 3′-terminal nucleotide at the *Aos*I cleavage site is found by reading from the band in the *Aos*I cleavage lane directly across the sequence ladder to C in the centre of the sequence 5′ TGC↓GCA 3′ (fig.4).

3.2. *Cleavage pattern of* Aos*II*

It was noted that *Aos*II cleaves φX174 RF DNA at several sites but does not cut any of the seven φX174 RF DNA fragments [19] produced by *Acy*I [3]. Subsequent experiments showed that the cleavage patterns of both φX174 RF DNA and pBR322 DNA are identical for *Aos*II and *Acy*I (fig.1). From this we concluded that *Aos*II must cleave Ad5 *Hpa*I–E DNA at coordinates 815, 1303, 1320 and 1458 or very near those sites. This was verified by sequencing fragments of Ad5 *Hpa*I–E DNA from the *Aos*II cleavage sites [3,11]. An identical approach was made with two fragments of pBR322 DNA (terminating at nucleotide 1207 and 3905, respectively) produced by the action of *Aos*II (results not shown). This confirms the conclusion that endo R.*Aos*II and *Acy*I are isoschizomers, and also shows that the actual site of cleavage within the sequence GPu↓CGPyC is as indicated by the arrow. The result of a two-dimensional chromatogram (fig.5) of a DNase digest of *Aos*II fragments labeled by kinase was identical to that obtained with *Acy*I [3], thus fully supporting the above conclusion.

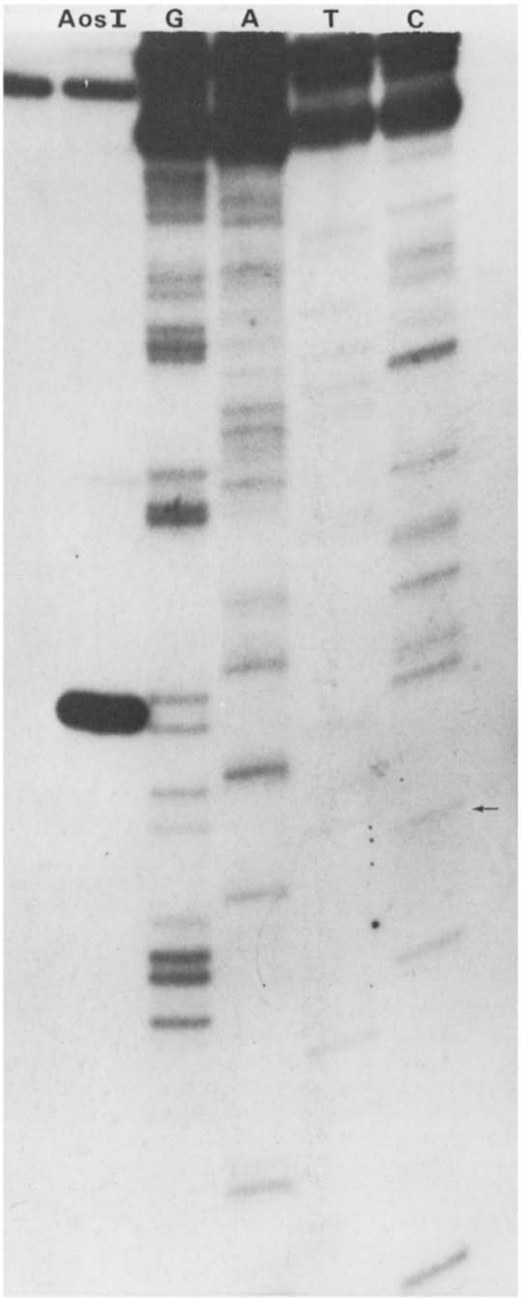


Fig.4. Determination of the *Aos*I recognition and cleavage site between nucleotides 1415 and 1416 in pBR322 DNA using the 'fifth lane' method. pBR322 DNA was digested with *Taq*I, labeled with ^{32}P at the 5′-termini and cleaved with *Ava*I. The 157-nucleotide fragment extending from nucleotide 1268 to nucleotide 1424 was isolated, a 5% aliquot was digested with *Aos*I, and the remaining DNA was enzymatically processed for sequencing [16]. The *Aos*I and dideoxy-generated fragments were separated on a 0.6 mm × 20 cm × 40 cm 12% polyacrylamide denaturing gel (19:1 acrylamide: *N,N′*-methylene diacrylamide, 2.5 mM ammonium peroxy disulfate, 7 M urea) in 90 mM Tris–borate (pH 8.3) 1 mM EDTA at 1000–1200 V. The lanes are: *Aos*I digestion, G, A, T and C, respectively. The sequence, from the arrow at nucleotide 1347 up reads: CTGTGAATGCGCA. Comparison with the 'ladder' indicates that the cut is made in TGC↓GCA as shown.

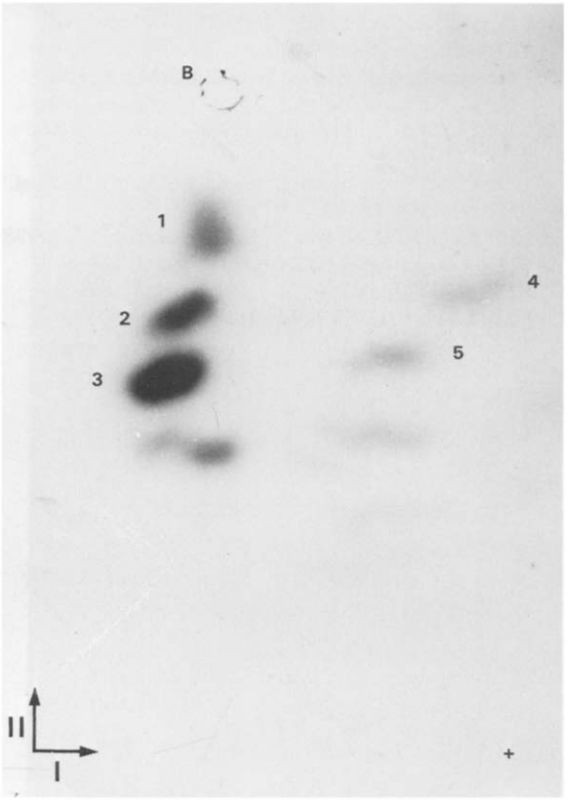


Fig.5. Isolation of ^{32}P-labeled oligonucleotides originating from the *Aos*II cleavage sequence. (1) pCpG; (2) pCpGpC; (3) pCpGpCpC; (4) pCpGpT; (5) pCpGpTpC; (B) blue marker (xylene cyanol FF). The figure shows that the specificity is lost after the fourth nucleotide. (I) direction of electrophoresis; (II) homochromatography.

4. Discussion

*Aos*I cleaves infrequently in DNAs as diverse as bacteriophage λ DNA and the human adenovirus 5 DNA. Thus it should be a useful tool to those seeking to cleave DNA at only one or a few sites. The sequence recognized by *Aos*I (TGCGCA) is the same as that suggested for an enzyme (*Mst*I) recently isolated from a different blue–green alga (genus *Microcoleus*, family Oscillatoriaceae) [4]. The probable recognition sequence for *Mst*I was deduced using a computer-assisted method [18], but to our knowledge neither the recognition sequence nor the actual point of cleavage by *Mst*I in the TGCGCA sequence has been biochemically established [4].

The second system, *Aos*II, cleaves DNAs more frequently than does *Aos*I. It is apparently identical with the endonuclease *Acy*I found in *Anabaena cylindrica* [3]. Its recognition sequence GPuCGPyC shows the same degeneracy as *Acy*I, as becomes clear when inspecting the structures of the seven cleavage sites [19] in φX174 RF DNA: GG↓CGCC at sites 1020 and 2977, GG↓CGTC (=GA CGCC) at sites 718, 1134, 3364 and 5226 and GA↓CGTC at site 2783.

It is concluded that *Anabaena oscillarioides*, strain CCAP 1403/11 is a useful microorganism to investigators who wish to have both of these unique endonucleases at their disposal.

Acknowledgements

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