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New restriction endonucleases from *Flavobacterium okeanokoites* (*Fok*I) and *Micrococcus luteus* (*Mlu*I)

(Class II enzymes; DNA sequencing; $\begin{matrix} GGATGN_9^{\downarrow} \\ CCTACN_{13\uparrow} \end{matrix}$; $A^{\downarrow}CGCGT$)

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SUMMARY

Two new restriction endonucleases have been isolated from *Flavobacterium okeanokoites* IFO12536 and *Micrococcus luteus* IFO12992 and named *Fok*I and *Mlu*I, respectively. Based on analysis of the sequences around the restriction sites, the recognition sequences and cleavage sites of these endonucleases were deduced as below:

*Fok*I: (5′) --N-G-G-A-T-G-N-N-N-N-N-N-N-N-N$^{\downarrow}$pN-N-N-N—N-- (3′)
(3′) --N-C-C-T-A-C-N-N-N-N-N-N-N-N-N-N—N-N-N-Np$_{\uparrow}$N-- (5′)

and

*Mlu*I: (5′) --N-A$^{\downarrow}$pC-G-C-G—T-N-- (3′)
(3′) --N-T G-C-G-Cp$_{\uparrow}$A-N-- (5′)

*Mlu*I introduces double-strand cleavages at unique sequences that are completely two-fold rotationally symmetric like most type II restriction endonucleases. *Fok*I belongs to a class of restriction endonucleases that recognize specific but asymmetric nucleotide sequences and introduce staggered cleavages at appointed positions away from the recognition sequences.

INTRODUCTION

A number of type II restriction endonucleases with different sequence specificities have been isolated from a variety of microorganisms (see Roberts, 1981). In the course of surveying for new enzymes, we found that *Flavobacterium okeanokoites* IFO12536 and *Micrococcus luteus* IFO12992 contain type II restriction endonucleases with specificities different from those of known enzymes.

Abbreviations: bp, base pairs; T4dC, cytosine-substituted T4 coliphage.

MATERIALS AND METHODS

(a) Bacterial strains and their cultivation

Flavobacterium okeanokoites IFO12536 and *Micrococcus luteus* IFO12992 were obtained from Institute for Fermentation, Osaka, Japan. Cells were grown with vigorous shaking at 37°C in L-broth (10 g of Bacto-tryptone, 5 g of yeast extract, 1 g of glucose and 5 g of NaCl per liter, pH 7.2) and harvested by centrifugation when the cell density reached about $A_{260} = 1$. The cell pellets were washed once with buffered saline and stored at −70°C. The yield from 1 liter culture was about 4 g (wet weight).

(b) DNA preparation

T4dC phage and its host bacterial strain, *E. coli* B834, were provided by Dr. H. Takahashi of Institute of Applied Microbiology, University of Tokyo. The source of λ phage was *E. coli* K-12W3350(λ*c*I857*S*7)-lysogen. Phage DNAs were prepared by phenol extraction from phage particles banded in CsCl gradient (Thomas and Davis, 1975; Takahashi et al., 1978). Plasmids, pA02, pA043 and pBR322, were isolated from *E. coli* C600 cells carrying these plasmids by the procedure described earlier (Oka et al., 1979).

(c) Enzymes

Restriction endonucleases *Hap*II, *Hae*III and *Hin*fI were purchased from Takara Shuzo Co., *Ava*II from Bethesda Research Lab., nuclease P1 from Yamasa Shoyu Co. and bacterial alkaline phosphatase from Worthington Biochemical. Polynucleotide kinase was prepared from *E. coli* A19 cells infected with phage T4amN82 according to the method of Richardson (1965).

(d) Preparation procedure for *Fok*I and *Mlu*I

20 g of frozen cells were suspended in 50 ml of 0.05 M Tris · HCl, pH 7.5, and 1 mM 2-mercaptoethanol, disrupted by sonication for total 5 min at 6A output and centrifuged for 60 min at 30 000 rev./min in the Spinco 40 rotor. The clear supernatant was applied directly onto a phosphocellulose column (Whatman P-11, 1.5 cm × 20 cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, 1 mM 2-mercaptoethanol and 5% glycerol (buffer A). After washing the column with 100 ml of buffer A, adsorbed proteins were eluted with a linear gradient of 0 to 1 M KCl in buffer A (400 ml). 10-ml fractions were collected and tested for endonuclease activity by the gel electrophoresis method. Fractions that cleaved DNA into discrete bands were combined and dialyzed against buffer A.

The dialyzed fraction was charged onto a DEAE-cellulose column (Whatman DE-52, 1 cm × 20 cm) previously equilibrated with buffer A. After washing the column with 50 ml of buffer A, adsorbed proteins were eluted with a linear gradient of 0 to 0.4 M KCl in buffer A (200 ml) and 5-ml fractions were collected. Fractions that cleaved DNA into distinct bands were collected, concentrated by dialysis against buffer A containing 0.1 M KCl and 50% glycerol and stored at −20°C.

(e) Assay of endonuclease activity

For the assay of enzyme activities at each step of purification, 2 μl of aliquot from fractions were added to 30 μl of reaction mixtures containing 0.01 M Tris · HCl, pH 7.5, 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol and 1 μg of appropriate DNA. After incubation for 30 min to 2 h at 37°C, reactions were terminated by adding 5 μl of 0.05 M EDTA, 15% Ficoll and 0.02% bromophenol blue at 0°C. Solutions were electrophoresed for 2 h at 10 V/cm on horizontal slab gels consisting of 1 to 1.4% agarose in 90 mM Tris · borate, pH 8.3, 2.5 mM EDTA and 0.5 μg/ml ethidium bromide.

One unit of enzymes was defined as the amount sufficient to digest 1 μg of pBR322 DNA for *Fok*I and 1 μg of lambda DNA for *Mlu*I in 1 h at 37°C.

(f) Nucleotide sequence determination

Restriction fragments were treated with alkaline phosphatase and then rephosphorylated with [γ-^{32}P]-ATP (about 5.5 Ci/μmol: Radiochemical Center, Amersham) and polynucleotide kinase. After separation of the two labeled ends by secondary cleavage with a different restriction endonuclease, the sequence of the 5′-^{32}P-labeled strand was determined by the ladder method of Maxam and Gilbert (1977). Two-dimensional electrophoresis-homochromatog-

raphy (Jay et al., 1974) was also used to identify the 5′-terminal sequences in a mixture of restriction fragments. 5′-Terminal nucleotides were analyzed as described earlier (Sugisaki, 1978).

RESULTS

(a) Purification and properties of *Fok*I and *Mlu*I endonucleases

The approximate KCl concentrations that elute *Fok*I and *Mlu*I enzymes from phosphocellulose and DEAE-cellulose columns are given in Table I. Crude cell extracts contained some nonspecific nucleases, the bulk of which were removed by phosphocellulose column chromatography. The remaining nonspecific nuclease activities were further removed by DEAE-cellulose column chromatography, so that prolonged incubation of DNA with excesses of enzyme preparations at this step did not change the proper gel electrophoresis patterns. The enzyme preparations at this step were sufficiently pure for subsequent experiments. Approximate yields of *Fok*I and *Mlu*I endonucleases at the DEAE-cellulose step are given in Table I. Comparison of the restriction patterns obtained from lambda and plasmid DNAs revealed that the specificities of these enzymes are respectively different from those of known restriction endonucleases.

Full activities were obtained by addition of Mg^{2+} alone. Activities were inhibited at KCl concentrations higher than 0.15 M. Maximum activities were obtained at 0 to 20 mM KCl for *Fok*I and 0.1 M KCl for *Mlu*I, respectively. In the subsequent experiments, therefore, 20 mM KCl was added for *Fok*I digestion and 0.1 M KCl for *Mlu*I digestion.

TABLE I

Approximate KCl concentrations eluting *Fok*I and *Mlu*I from Whatman P-11 and DE-52 columns and yields of the enzymes

Enzymes	P-11 column (M)	DE-52 column (M)	Yields (units/g cells)
*Fok*I	0.30–0.34	0.08–0.10	200
*Mlu*I	0.42–0.46	0.18–0.20	800

(b) Cleavage site specificity of *Mlu*I

Since the entire sequences of a ColE1 derivative pAO2 and pAO2 carrying Tn*903* (pAO43) have been determined (Oka et al., 1979; 1981), cleavage site specificities of enzymes were first analyzed by using these DNAs. *Mlu*I cleaved pAO43 at two sites but not pAO2 (Fig. 1). Restriction analysis of the two fragments produced from pAO43 indicated that the sites are located within the inverted repeats (IR903 region) that occur at both extremities of Tn*903* (Fig. 2). The two fragments, named M1 and M2, were labeled with ^{32}P at their 5′-termini, and the M1 fragment was digested with *Ava*II and the M2 fragment with *Hae*III, respectively. The resulting subfragments were isolated and the 5′-terminal sequences were analyzed by the Maxam and Gilbert method (1977). The sequences obtained were compared with the sequence of IR903 in pAO43, and aligned as shown in Fig. 3. Assuming that *Mlu*I is a six-base cutter, the possible recognition sequence is ACGCGT.

*Mlu*I cleaved T4dC DNA and λ DNA at multisites (Fig. 1). Restriction fragments generated from these DNAs were analyzed by the two-dimensional

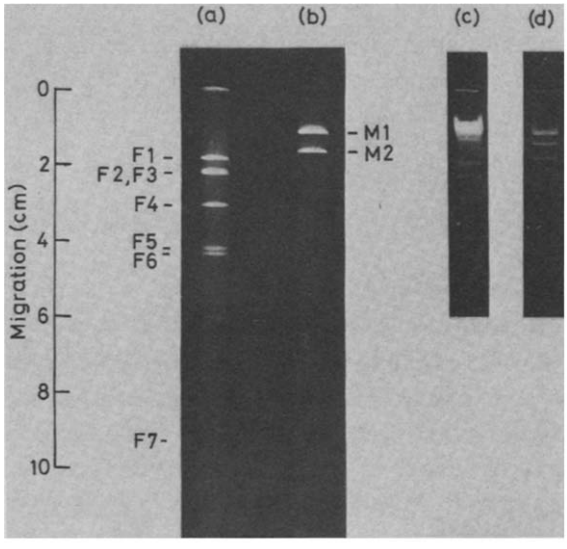

Fig. 1. Gel electrophoretic patterns of fragments produced from pAO43, and phage T4dC and λ DNAs by cleavage with *Fok*I and *Mlu*I. *Fok*I and *Mlu*I digests of pAO43 were electrophoresed on 5% polyacrylamide gels and the gel electrophoretic patterns are shown in lanes (a) and (b), respectively. *Mlu*I digests of phage T4dC and λ DNAs were electrophoresed on 1% agarose gels and the gel electrophoretic patterns are shown in lanes (c) and (d), respectively.

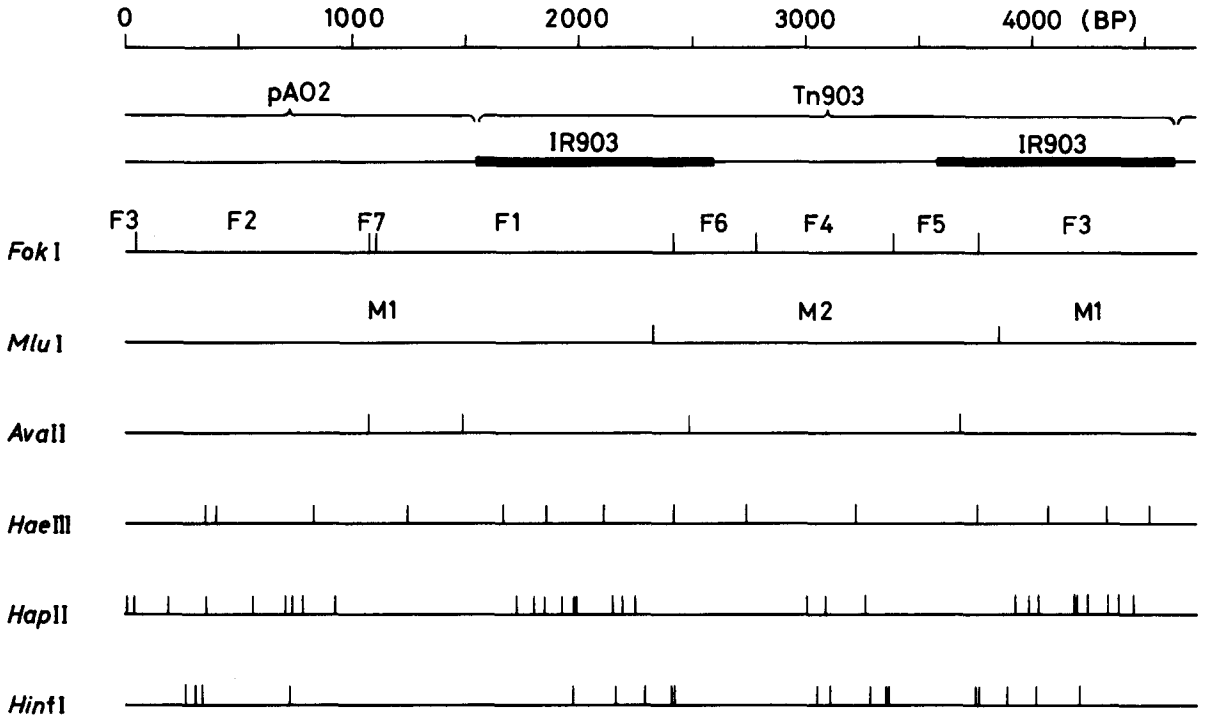

Fig. 2. Map showing the alignment of *Fok*I and *Mlu*I cleavage sites in pAO43. The circular pAO43 DNA molecule is presented in a linear form split at the ligated point of pAO2 (Oka et al., 1977), and the map distance is shown as the number in base pairs. Cleavage sites of *Ava*II, *Hae*III, *Hap*II and *Hin*fI used for secondary cleavage to obtain subfragments labeled at one 5'-terminal are also shown for reference.

mobility shift method, which uses electrophoresis and homochromatography (Jay et al., 1974). An autoradiogram of the resulting fingerprints is shown in Fig. 4. Unique nucleotides appeared up to the fifth position, but all four nucleotides occurred at the sixth position. The sequence was thus read as pCGCGTN---.

Based on the above observations, we concluded that *Mlu*I recognizes the following hexanucleotide sequence and introduces cleavages at the indicated positions.

(5') --A↓pC-G-C-G—T-- (3')
(3') --T— G-C-G-Cp↑A-- (5')

The internal tetranucleotide sequence is the recognition sequence of *Acc*II (Roberts, 1981). It should be mentioned that *Mlu*I does not cleave pBR322 and pAO2, each containing TCGCGA but not ACGCGT (Fuchs et al., 1980).

DNA sequence of pAO43
(5') - - - TGTGCTGGTCATTAAACGCGTATTCAGGCTGACCC - - - (3')
(3') - - - ACACGACCAGTAATTTGCGCATAAGTCCGACTGGG - - - (5')

From 5'-end of M1 fragment
pCGCGTATTCAGGCTGACCC - - - (3')

From 5'-end of M2 fragment
(3') - - - ACACGACCAGTAATTTGCGCp

Fig. 3. Alignment of the sequences obtained for the *Mlu*I cleavage site with pAO43 DNA sequence.

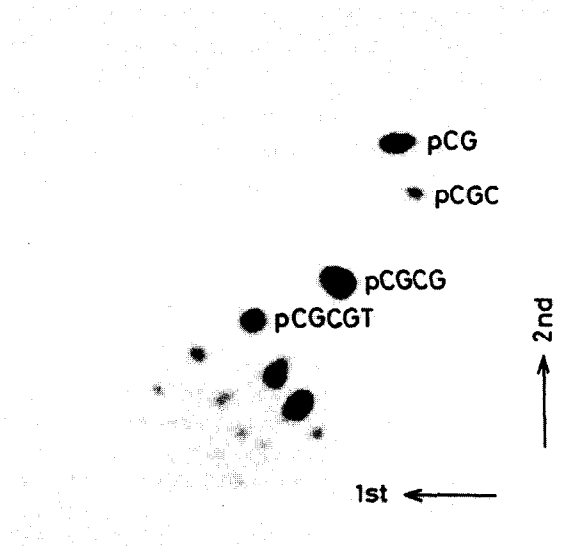

Fig. 4. Analysis of the 5′-terminal sequence created by *Mlu*I digestion of T4dC DNA. 5′-^{32}P-labeled *Mlu*I-restriction fragments of T4dC DNA were partially digested with DNaseI and fractionated by electrophoresis on cellulose acetate film (first dimension), followed by homochromatography on DEAE-cellulose thin-layer plate with homo-mix VI (second dimension). The 5′-^{32}P-oligonucleotides were detected by autoradiography and the sequences, as inferred from their mobility shift, are specified in the fingerprint.

(c) Cleavage site specificity of *Fok*I

*Fok*I cleaved pAO2 at three sites and pAO43 at seven sites. By comparison of the resulting fragments and by cross digestion with other restriction endonucleases, the seven fragments produced from pAO43, named F1 to F7, were allocated on the physical map of pAO43 as shown in Fig. 2. Preliminary analysis, however, indicated that no unique nucleotides are present at the 5′-termini of the fragments. Thus the precise alignment of the *Fok*I fragments on the pAO43 sequence was achieved by analysis of the 5′-terminal sequences. F1 to F6 fragments were labeled with ^{32}P in polynucleotide kinase reaction, and the two labeled ends of each fragment were separated by digestion with other enzymes (*Hap*II for F1 and F4, *Hin*fI for F2 and F3, and *Ava*II for F5 and F6). The 5′-terminal sequences of the subfragments were analyzed by the Maxam and Gilbert method. The 5′-terminal nucleotides produced by nuclease P1 were also determined by PEI-cellulose thin layer chromatography. By comparing the sequences obtained with that of pAO43, the terminal regions of the six fragments were aligned as

Cleavage site

F3/F2	(5′)---TTGTCTGTGAGC	GGATG CCGGGAGCT↓GACAAGCCCGTCAGGGCGCGTCAG---(3′)
	(3′)---AACAGACACTCG	CCTAC GGCCCTCGACTGTT↑CGGGCAGTCCCGCGCAGTC---(5′)
F2/F7	(5′)---TTTAGAAGAGGA	GGATG GTGCGATGGTCCCTCCCTGAACATCACGTATAT---(3′)
	(3′)---AAATCTTCTCCT	CCTAC CACGCTACCAGGG↑AGGGACTTGTAGTGCATATA---(5′)
F7/F1	(5′)---AACCTCCTTGTT	GGATG TCAGGCTAACTATATACGTGATGTTCAGGGAGG---(3′)
	(3′)---TTGGAGGAACAA	CCTAC AGTCCGATTGATA↑TATGCACTACAAGTCCCTCC---(5′)
F1/F6	(5′)---CACTTTCTGGCT	GGATG ATGGGGCGA↓TTCAGGCCTGGTATGAGTCAGCAA---(3′)
& F5/F3	(3′)---GTGAAAGACCGA	CCTAC TACCCCGCTAAGT↑CCGGACCATACTCAGTCGTT---(5′)
F6/F4	(5′)---AAATTCCAACAT	GGATG CTGATTTAT↓ATGGGTATAAATGGGCTCGCGATA---(3′)
	(3′)---TTTAAGGTTGTA	CCTAC GACTAAATATACC↑CATATTTACCCGAGCGCTAT---(5′)
F4/F5	(5′)---GGCAGTTCCATA	GGATG GCAAGATCC↓TGGTATCGGTCTGCGATTCCGACT---(3′)
	(3′)---CCGTCAAGGTAT	CCTAC CGTTCTAGGACCA↑TAGCCAGACGCTAAGGCTGA---(5′)

Fig. 5. Comparison of the sequences around the *Fok*I cleavage sites on pAO43. The sequences are aligned with respect to the cleavage sites (arrows) and the common sequence is boxed.

in Fig. 5. The results of analysis are summarized as follows:

(1) *Fok*I introduces duplex cleavages on double-stranded DNA so as to yield the protruding 5′-ends of four nucleotides.

(2) There is no similarity in the sequences at the cleavage sites.

(3) A common pentanucleotide sequence, GGATG, occurs at nucleotide positions 9 to 13 from the cleavage sites, as shown in boxes in Fig. 5.

It is, therefore, most likely that GGATG is the recognition sequence of *Fok*I. This was further confirmed by digestion of pBR322. According to the sequence data of pBR322 (Sutcliffe, 1979), GGATG occurs at 11 positions. When the fragments produced from pBR322 by *Fok*I digestion were analyzed by gel electrophoresis, 11 fragments were indeed identified. Their sizes were also compatible with those of fragments computed from the sequence data. The mode of *Fok*I cleavage was thus formulated as below.

(5′) --N-G-G-A-T-G-N-N-N-N-N-N-N-N-N↓pN-N-N-N—N-- (3′)
(3′) --N-C-C-T-A-C-N-N-N-N-N-N-N-N-N—N-N-N-Np↑N-- (5′)

DISCUSSION

*Fok*I and *Mlu*I belong to type II restriction endonucleases. *Mlu*I is a six-base cutter and cleaves λ DNA only at several sites. A popular cloning vector pBR322 does not contain the *Mlu*I site. Therefore, this enzyme would become a useful tool for gene manipulation. *Fok*I should be convenient for construction of accurate cleavage maps of DNA. Because the sequences in the protruding 5′-ends differ with each cleavage site and two protruding 5′-ends created at a single cleavage site have complementary sequences, it is possible to identify adjacent fragments from their 5′-terminal sequences.

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