Volume 12 Number 6 1984 Nucleic Acids Research

Three new restriction endonucleases *MaeI*, *MaeII* and *MaeIII* from *Methanococcus aeolicus*

Kurt Schmid[1], Michael Thomm[1], Axel Laminet[1], Frank G.Laue[2], Christoph Kessler[2], Karl O.Stetter[1+] and Rüdiger Schmitt[1+]

[1]Institut für Biochemie, Genetik und Mikrobiologie, Universität Regensburg, D-8400 Regensburg, and [2]Boehringer Mannheim GmbH, Biochemical Research Center, D-8132 Tutzing, FRG

Received 31 January 1984; Accepted 20 February 1984

ABSTRACT

Three type II restriction endonucleases, MaeI, MaeII and MäeIII, with novel site specificities have been isolated and purified from the archaebacterium Methanococcus aeolicus PL-15/H. The recognition sequences of these enzymes are

C↓T A G (MaeI)

A↓C G T (MaeII)

↓G T N A C (MaeIII)

with the sites of cleavage as indicated by the arrows. The sequences were confirmed by restriction and computer analyses on sequenced DNA's of plasmid pBR322, bacteriophages λ and ØX174 and virus SV40.

INTRODUCTION

Site-specific endonucleases have become indispensable tools in the analysis and manipulation of nucleic acid molecules. Although a large number of restriction endonucleases have been described (1), additional enzymes with new specificities are required for these studies. We have examined a number of methanogenic bacteria for the presence of restriction endonucleases. In Methanococcus aeolicus PL-15/H, a new archaebacterial strain to be described elsewhere (K.O. Stetter, in preparation), we have found three restriction enzymes with different sequence specificities. In this communication we report the isolation and characterization of these enzymes which have new recognition sequences. In accordance with the proposal of Smith and Nathans (2) we designated these restriction endonucleases as MaeI, MaeII, and MaeIII.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The archaebacterium Mc.aeolicus PL-15/H belongs to the order

methanococcales as determined by its morphology, its substrate specificity, the GC content of its DNA, and by its protein cell envelope (K.O. Stetter, to be published). Cells were cultured anaerobically at 45°C for three days in medium 3 (3) supplemented with 1.5% formate and harvested in the stationary phase by centrifugation.

Enzymes and DNAs

DNA polymerase I (Klenow enzyme) and restriction enzymes were products of Boehringer Mannheim (FRG). pBR322 DNA was purified from an E.coli dam⁻ background (4) by CsCl-EtBr equilibrium centrifugation as described previously (5). Phage DNAs were supplied by Boehringer Mannheim (FRG).

Enzyme assay

Routinely, 1-5 µl samples of an enzyme preparation (column fraction) were incubated with 1 µg pBR322 DNA in 25 µl of the incubation mixture containing 50 mM Tris-HCl (pH 8.0/45°C), 6 mM $MgCl_2$, 7 mM β-mercaptoethanol, 0.01% Triton X100 and 250 mM NaCl (MaeI) or 175 mM NaCl (MaeII) or 350 mM NaCl (MaeIII). Mixtures were incubated at 45°C for 60 min (MaeI) and for 16 hours (MaeII and MaeIII), respectively. Reactions were terminated by adding 5 µl of stop mixture [7 M urea, 20% (w/v) sucrose, 60 mM EDTA, 0.01% bromphenol blue]. Digests were resolved by gel electrophoresis on 1% agarose in 0.04 M Tris-acetate (pH 8.2), 2 mM EDTA, and 1 µg/ml ethidium bromide.

Isolation and purification of MaeI, MaeII, and MaeIII

Frozen cells (35 g wet weight) were suspended in 70 ml of buffer (40 mM Tris-HCl, pH 8.0/4°C; 0.1 mM EDTA; 7 mM β-mercaptoethanol; 0.2 mM phenylmethylsulfonylfluoride) and disrupted at 4°C by two passages through a French pressure cell at 23,000 lb/in^2. The resulting homogenate was adjusted to 0.3 M NH_4Cl. DNA was precipitated by the addition of 7 ml of a 10% (v/v) Polymin P (BASF, Ludwigshafen, FRG) solution and, after 30 min at 4°C, removed by centrifugation (60 min at 23,000 x g). Ammonium sulfate fractionation of the supernatant at 4°C precipitated most of MaeI and partially MaeII at 60% saturation and most of MaeIII plus the rest of MaeII at 95% saturation. The two fractions were sedimented each by centrifugation after standing at 4°C for 16 hours. MaeII present in both fractions was separated in parallel by sub-

sequent molecular sieve fractionations.

The two precipitates were separately dissolved in TEMG buffer [40 mM Tris-HCl, pH 8.0/4°C; 0.1 mM EDTA; 7 mM β-mercaptoethanol; 10% (v/v) glycerol] plus 0.5 M NaCl and fractionated on Ultrogel AcA 34 columns (3 x 100 cm; LKB) using the same buffer for elution. MaeI and MaeII fractions from eluate 1 and MaeIII and MaeII fractions from eluate 2 were separately pooled and the MaeII fractions of both eluates were combined. The resulting three pools that contained MaeI, MaeII, and MaeIII activity, respectively, were dialyzed against TEMG buffer and then separately purified by fractionation on DEAE Sephacel columns (2 x 10 cm; Pharmacia), equilibrated with TEMG buffer using 0-1 M NaCl linear gradients. Enzyme activities were eluted at 0.1-0.3 M NaCl (MaeI), 0.15-0.35 M NaCl (MaeII), and 0.1-0.15 M NaCl (MaeIII), respectively. Active fractions were pooled, dialyzed against TEMG buffer and fractionated on phosphocellulose P11 columns (1 x 10 cm; Whatman) using 0-1 M NaCl linear gradients. Enzyme activities were eluted at 0.4-0.6 M NaCl (MaeI), 0.3-0.5 NaCl (MaeII), and 0.55-0.65 M NaCl (MaeIII), respectively. After dialysis of the pooled fractions against TEMG buffer MaeI and MaeIII were submitted to a final fractionation on Heparin Sepharose CL-6B columns (1 x 5 cm; Pharmacia) employing 0-1 M NaCl (MaeI) or 0-1.5 M NaCl (MaeIII) linear gradients for elution. Enzyme activities appeared at 0.4-0.6 M NaCl (MaeI) and 0.65-0.85 M NaCl (MaeIII), respectively. The final pools of MaeI, MaeII, and MaeIII were dialyzed for 4 hours against storage buffer [20 mM Tris-HCl, pH 8.0/4°C; 0.1 mM EDTA; 10 mM β-mercaptoethanol, 100 mM NaCl; 50% (v/v) glycerol, 0.01% (w/v) Triton X100], which was exchanged every hour and stored at -20°C.

Starting from 35 g of cells the procedure yielded the following total activities: 10,000 units of MaeI, 330 units of MaeII, and 95 units of MaeIII. One unit is defined as the amount of enzyme that cleaves 1 µg pBR322 DNA per hour under optimal assay conditions.

DNA sequencing

Nucleotide sequences at the cleavage sites were determined according to Maxam and Gilbert (6).

Table 1 Optimal conditions for MaeI, MaeII, and MaeIII restriction activities

	MaeI	MaeII	MaeIII
$MgCl_2$ (mM)	12-18	2-6	10-18
NaCl (mM)	250	175	350
β-mercaptoethanol (mM)	6-12	2-8	0-20
pH (50 mM Tris-HCl/45°C)	8.0	8.0	8.0
Temperature (°C)	45-48	45-48	45-48

RESULTS

Optimal conditions for enzyme activity

The temperature optimum for MaeI, MaeII and MaeIII is at 45-48°C, the pH optimum is 8.0. The three enzymes are strictly dependent on Mg^{++} ions, but do not require S-adenosyl-methionine or ATP for activity. Optimal concentrations of $MgCl_2$, NaCl and β-mercaptoethanol determined for each enzyme are listed in Table 1.

Determination of recognition sequences

The recognition sequences of MaeI, MaeII and MaeIII were determined by mapping their restriction sites on pBR322, a 4,363 bp plasmid of known DNA sequence (7-9), using double digestions with known restriction enzymes. By analyzing nucleotide sequences around these sites, either visually or by computer programs designed to search for new restriction sites on the basis of physical mapping data (P.S. Neumaier, pers. communication), common palindromic sequences have been identified. These are CTAG for MaeI, ACGT for MaeII and GTNAC for MaeIII, where N stands for any of the four nucleotides. The frequency of cleavage and the fragment sizes generated by MaeI, MaeII and MaeIII on adenovirus-2 DNA (Ad-2), plasmid pBR322 DNA, bacteriophage lambda DNA (λ), simian virus 40 DNA (SV40) and phiX174 Rf DNA (ØX174) were determined (Fig.1 and Table 2). In the latter four cases, the sites were confirmed by computer search of the published sequences (7-14).

A physical map of pBR322 with the location of MaeI, MaeII and MaeIII restriction sites is shown in Fig.2.

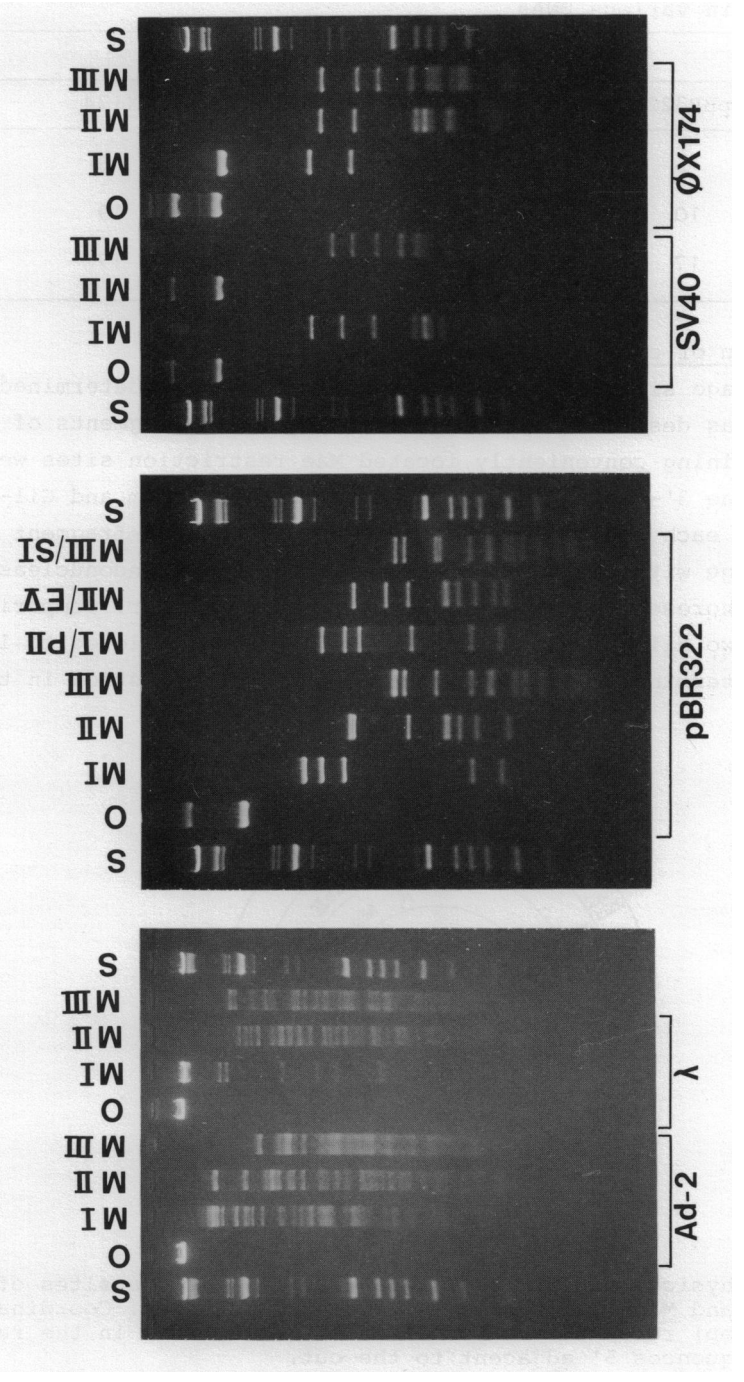

Figure 1 Restriction patterns of various DNAs (as marked) after digestion with MaeI (MI), MaeII (MII), MaeIII (MIII) or double digestion using EcoRV (EV), PvuII (PII) or SalI (SI) as indicated (O = no digestion). EcoRI/HindIII-digested λ DNA plus HinfI-digested pBR322 DNA was used as molecular weight standard (S).

Table 2 Number of cleavage sites of Mae restriction enzymes in various DNAs

Enzyme	DNA				
	pBR322	λ	Ad-2	SV40	ØX174
MaeI	5	14	>35	12	3
MaeII	10	143	>35	0	19
MaeIII	17	156	>35	14	17

Determination of cleavage sites

The cleavage sites of MaeI, MaeII and MaeIII were determined essentially as described by McConnel et al. (15). Fragments of pBR322 containing conveniently located Mae restriction sites were labeled at one 3'-end and sequenced according to Maxam and Gilbert (6). In each case a second sample of the labeled fragment after cleavage with the appropriate Mae restriction endonuclease was electrophoresed in parallel to the sequence ladder. Comparison of the two aligned samples on the sequence gel allowed to locate the 5'-terminal nucleotide generated by Mae digestion in the

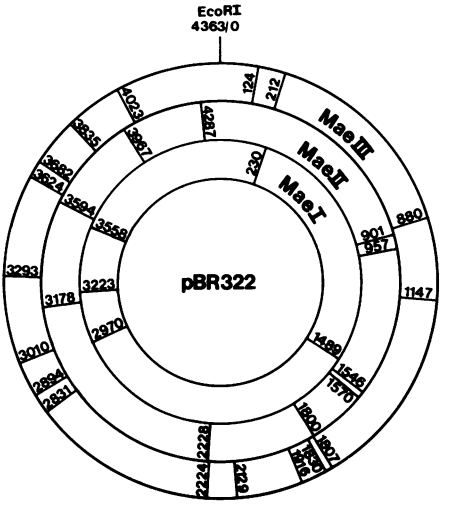

Figure 2 Physical map of pBR322 with the restriction sites of MaeI, MaeII and MaeIII arranged in concentric circles. Coordinate numbers (in bp) refer to the positions of nucleotides in the recognition sequences 5' adjacent to the cut.

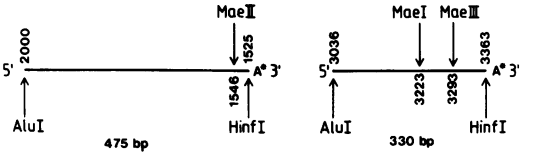

Figure 3 pBR322 fragments used for determining the cleavage sites of MaeI, MaeII and MaeIII. A* marks the ^{32}P-labeled adenosine nucleotide. Relevant restriction sites are indicated by arrows. Numbers refer to the nucleotide positions on pBR322 (ref. 7-9) defined as in Fig.2 (fragment sizes include sticky ends).

sequence.

The 517 bp and 506 bp HinfI fragments B and C of pBR322 (7-9) were isolated from an 1% agarose gel, 3'-labeled by using Klenow DNA polymerase I and [α-^{32}P]-dATP (Amersham Buchler, Braunschweig, FRG) and then cleaved with AluI. After separation by electrophoresis on 7.5% polyacrylamide gels, the 475 bp and 330 bp fragments (including sticky ends) were isolated and purified. As shown in Fig.3, these were cleaved with MaeII (475 bp AluI/HinfI-fragment) or with MaeI and MaeIII, respectively (330 bp AluI/HinfI-fragment). Aliquots of both AluI/HinfI-fragments were chemically modified and cleaved for sequencing (6) and electrophoresed in parallel to corresponding samples obtained by Mae digestion. The results are shown in Figure 4.

The HinfI/MaeI fragment (Fig.4A, "MI") migrates with the C in the MaeI recognition sequence. In the sequence ladder the fragment representing a C (Fig.4A, "C") has actually lost this 5'-terminal C as a consequence of the chemical cleavage (6) and therefore ends with the 3'-neighbor, in this particular case a T-residue. MaeI thus cleaves between T and C in the recognition sequence like C$^{\downarrow}$TAG. Similar considerations apply to the other two cleavage sites, analyzed in Figures 4B and 4C. The HinfI/MaeII fragment (Fig.4B, "MII") migrates together with the nucleotide A (Fig.4B, "A>G") in the MaeII recognition sequence ACGT, thus locating the cleavage between A and C (A$^{\downarrow}$CGT). The HinfI/MaeIII fragment (Fig.4C, "MIII") comigrates with an A (Fig.4C, "A>G"), the 5'-nearest neighbor of the MaeIII recognition sequence GTTAC. Thus MaeIII cleaves at the 5'-side of the recognition sequence, like $^{\downarrow}$GTNAC.

Figure 4 Comparison of electrophoretic mobilities of sequence ladders containing a Mae recognition site and aligned restriction fragments cleaved by the corresponding enzyme. AluI/HinfI fragments of pBR322 were 3'-end labeled and cleaved as diagrammed in Fig.3. Autoradiographs show sequence ladders (marked according to ref.6) that comprise the MaeI (A), MaeII (B) and MaeIII (C) recognition sites (brackets), and in each case the aligned fragment cleaved by the corresponding enzyme (marked MI, MII or MIII).

DISCUSSION

Three restriction endonucleases with novel specificities have been separated and purified from Mc.aeolicus PL-15/H. According to the in vitro activity MaeI is the major species, whereas MaeII and MaeIII appear as minor forms. The three enzymes were classified as type II restriction endonucleases, owing to their strict dependence on Mg^{2+} ions, their independence of ATP and S-adenosylmethionine and to the dyad symmetry of their recognition sites, that are also the substrates for cleavage. MaeI and MaeII recognize the palindromic tetranucleotides C↓TAG and A↓CGT, respectively, with the sites of cleavage as indicated by the arrows. The recognition sequence of MaeIII contains a nondefined nucleotide N in the center of the otherwise pälindromic pentanucleotide ↓GTNAC, and is cleaved next to the 5'G as indicated. The three enzymes produce 5'-protruding ends, a feature facilitating 3' and 5'-end labeling and useful for cloning.

The cultivation of Mc.aeolicus under strictly anaerobic conditions and the separation of three endonuclease activities from one strain are somewhat tedious. On the other hand, each of the enzymes has a novel sequence specificity and is thus potentially useful for elucidating DNA structure and function. MaeI appears especially interesting, since it cleaves DNAs of eubacterial sources at substatistical frequencies (Table 2). It is tempting to speculate that palindromic sequences with a central TA are rare in these DNAs and that restriction enzymes with corresponding specificities had therefore little selective advantage in evolution. On the other hand, the recognition sequence C'TAG contains the amber codon UAG implying that statistically MaeI cleaves before every fourth amber stop codon. In contrast to MaeI, the other two endonucleases cleave DNA of prokaryote and eukaryote origin with about the frequencies expected for enzymes that recognize unique tetranucleotides.

Mc.aeolicus, the source of the new restriction enzymes, has been classified as an archaebacterium (K.O. Stetter, to be published). Archaebacteria constitute a "third kingdom" of organisms and differ in many respects from eubacteria (16). Restriction enzymes have been also detected in Thermoplasma acidophilum (15) and in the sulfur-dependent archaebacteria Sulfolobus, Desulfuro-

coccus and Thermococcus (P.McWilliams, unpublished). However, all these endonucleases turned out to be isoschizomers of eubacterial enzymes (1; P.McWilliams, pers. communication). The finding of MaeI, MaeII and MaeIII in Mc.aeolicus, however, should encourage systematic screening of other archaebacterial strains, which may reveal additional restriction endonucleases with novel specificities.

ACKNOWLEDGEMENTS

We thank Peter S.Neumaier (Boehringer Mannheim GmbH) for conducting the computer analysis, Gerta Gebhardt, Gerda Pensel, Helmut Rausch and Birgit Wöhrl for excellent technical assistance, Helene Beier for artwork and Josef Altenbuchner for his advice.

+To whom correspondence should be addressed

REFERENCES

[1] Roberts, R.J. (1983) Nucl. Acids Res. 11, r135-r167
[2] Smith, H.O. and Nathans, D. (1973) J. Mol. Biol. 81, 419-423
[3] Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R. and Wolfe, R.S. (1979) Microbiol. Rev. 43, 260-296
[4] Hattman, S., Brooks, J.E. and Masurekar, M. (1978). J. Mol. Biol. 126, 367-380
[5] Wiebauer, K., Schraml, S., Shales, S.W. and Schmitt, R. (1981) J. Bacteriol. 147, 851-859
[6] Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564
[7] Sutcliffe, J.G. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 77-90
[8] Livneh, Z. (1983) Proc. Natl. Acad. Sci. USA 80, 238-241
[9] Peden, K.W.C. (1983) Gene 22, 277-280
[10] Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. (1982) J. Mol. Biol. 162, 729-773
[11] Fiers, W., Contreras, R., Haegemann, G., Rogiers, R., van de Voorde, A., van Heuwerswyn, J., van Herreweghe, J., Volckaert, G. and Isebaert, M. (1978) Nature 273, 113-120
[12] Van Heuwerswyn, J. and Fiers, W. (1979) Eur. J. Biochem. 100, 51-60
[13] Sanger, F., Air, G.M., Barrell, B.G., Brown, N.L., Coulson, A.R., Fiddes, J.C., Hutchinson, C.A. III., Slocombe, P.M. and Smith, M. (1977) Nature 265, 687-695
[14] Sanger, F., Coulson, A.R., Friedman, T., Air, G.M., Barrell, B.G., Brown, N.L., Fiddes, J.C., Hutchinson, C.A. III., Slocombe, P.M. and Smith, M. (1978) J. Mol. Biol. 125, 225-246
[15] McConnel, D.J., Searcy, D.G. and Sutcliffe, J.G. (1978) Nucl. Acids Res. 5, 1729-1739
[16] Woese, C.R., Magrum, L.J. and Fox, G.E. (1978) J. Mol. Evol. 11, 245-252