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*Mvn*I: a restriction enzyme in the archaebacterium *Methanococcus vannielii*

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1. SUMMARY

The methanogenic archaebacerium *Methanococcus vannielii* contains a type II restriction endonuclease. The enzyme was purified by a simple three-step procedure resulting in enzyme preparations free of contaminating unspecific nucleases. The restriction enzyme recognizes and cleaves the sequence 5′-CG↓CG-3′ (*Fnu*DII and *Tha*I isoschizomer) and generates DNA fragments with blunt ends. Due to its purity and activity at moderate temperatures, *Mvn*I might be a useful alternative to *Fnu*DII and *Tha*I active at 60 °C.

2. INTRODUCTION

More than 600 restriction endonucleases of eubacterial origin are known [1,2]. Within the archaebacterial kingdom [3] only five restriction enzymes have been described so far: The nucleases of *Thermoplasma acidophilum* [4], *Sulfolobus solfataricus* [5] and *Sulfolobus acidocaldarius* [6], all are isoschizomers of known enzymes. However, the methanogen *Methanococcus aeolicus* contains three different activities with novel recognition sequences [7]. Thus, an investigation on the occurrence of restriction endonucleases in other *Methanococci* was undertaken. The mesophilic strain *Mc. vannielii* [8] might be an organism especially suitable for the development of an archaebacterial transformation and in vitro transcription system. It can be easily grown in mineralic media on a large scale, forms colonies on agar with high plating efficiency [9], its protein cell wall can be lysed by detergents and its DNA-dependent RNA polymerase recognizes promoters specifically in vitro [10–12]. In order to study possible factors which might interfere with these experiments we analyzed extracts of *Mc. vannielii* for the presence of nucleases. In this communication, we report on the identification of a type II restriction endonuclease in this *Methanococcus* strain.

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3. MATERIALS AND METHODS

3.1. DNAs and reagents

λ*c*I857*S*am7 DNA, M13mp18RF DNA, pBR322 DNA, pBR328 DNA, λ · *Eco*RI/*Hin*dIII fragments (DNA M_r marker III), pBR322 · *Hae*III fragments (DNA M_r marker V), alkaline phosphatase from calf intestine (CIAP), T4 polynucleotide kinase (T4 PNK), T4 DNA polymerase (T4 DNAP), T4 DNA ligase (T4 DNA *Lig*) and the restriction enzymes *Pst*I and *Sal*I were products of Boehringer Mannheim GmbH. *Tha*I was obtained from BRL, *Fnu*DII from NEBL. All enzymes were used according to the specifications of the suppliers.

High specific activity [γ-^{32}P]ATP (~ 3000 Ci/mmol) was obtained from Amersham, DEAE-Cellulose and Heparin-Cellulose was from PL-Pharmacia, Agarose from FMC Corporation, Marine Colloids Div./Rockland, ME, and Low-Melting Temperature Agarose, Type VII, from Sigma, MO.

3.2. Strain cultivation

Methanococcus vannielii was cultivated by using the technique described by Balch and Wolfe [13] in a medium containing 0.5 g/l KH_2PO_4; 0.4 g/l $MgSO_4$; 0.4 g/l NaCl; 0.4 g/l NH_4Cl; 0.05 g/l $CaCl_2 \times 2H_2O$; 0.01 g/l $FeSO_4 \times 7H_2$; 6 ml/l trace minerals [14]; 1 ml/l 0.1% resazurine; 6.5 g/l $NaHCO_3$; 0.5 g/l L-cysteine; 0.5 g/l $Na_2S \times (7–9)H_2O$; pH 6.5. 1 μM Na_2WO_4 was added after the sterilisation of the medium.

For fermentation the organism was grown at 37 °C in a 300 l enamel-coated fermenter (Bioengineering) using a 3.5% inocolum. The fermenter was gassed with 6 l H_2 and 1.5 l CO_2 per minute at 600 rpm agitation.

3.3. Preparation of a high speed supernatant (S 100)

2 g cells (wet weight) were suspended in 4 ml TMK buffer (50 mM Tris-HCl, pH 8.0/4 °C; 10 mM $MgCl_2$; 50 mM KCl; 40% (v/v) glycerol) and disrupted by a French Pressure cell at 100 000 kPA. The crude extract was centrifuged for 1 h at 38 500 rpm in a 50 Ti rotor (Beckman).

3.4. Purification of the restriction endonuclease Mvn*I* from Mc. vannielii

The S 100 supernatant was applied to a DEAE-cellulose column equilibrated with TMK buffer. After washing with two column volumes of TMK buffer, protein fractions were eluted in three steps with two column volumes each of TMK buffer containing 0.2 M, 0.4 M and 0.8 M KCl. The combined active fractions were desalted by dialysis and applied to a Heparin-Cellulose column. Protein was eluted with a linear salt gradient (0.05–0.8 M KCl in TMK buffer). The endonuclease activity eluting at 0.18 M KCl was dialysed against TMK buffer containing 50% (v/v) glycerol, and stored at −20 °C.

3.5. Enzyme assay

Fractions (1–2 μl) were incubated with 1 μg λ*c*I857*S*am7 DNA in a 20 μl reaction mixture (80 mM Tris-HCl, pH 8.0/37 °C; 10 mM $MgCl_2$; 50 mM KCl; 0.1 mM EDTA; 100 μg/ml bovine serum albumin) for 1 h at 37 °C. The reactions were terminated by adding 5 μl of cold stop solution (7 M urea; 20% (w/v) sucrose, 60 mM EDTA and 0.01% (w/v) bromophenol blue). The complete reaction mixtures were resolved by electrophoresis for 3 h at 100 V on 0.8% (w/v) agarose gels using 40 mM Tris-acetate (pH 8.2/25 °C), 2 mM EDTA, and 1 μg/ml ethidium bromide as electrophoresis buffer.

3.6. Sequence analysis

Nucleotide sequence analysis was performed following the chemical method of Maxam and Gilbert [15] as described by Brown and Smith [16]. The A/G-reaction was modified according to Gray et al. [17]. Interpretation of sequence data follows the rules given by Tomizawa et al. [18], McConnell et al. [19] and Brown and Smith [16].

The indication of *Mvn*I cleavage sites refers to the first cytidine nucleotide of the tetranucleotide recognition sequence of the (+)-strand in accordance with the UWGCG-computer programs of Devereux et al. [20]. However, if cut positions are cited these data are related to the actual sites of phosphodiester bond hydrolysis between the central guanosine and cytidine nucleotides (5′-CG↓CG-3′).

The sequence of pBR328 DNA [21] was obtained from VecBase data bank [22] integrated into the UWGCG-program package.

4. RESULTS AND DISCUSSION

4.1. Identification and characterization of the Mc. vannielii *restriction endonuclease*

The S 100 supernatant was fractionated by DEAE Cellulose chromatography. Nuclease activity hydrolyzing DNA unspecifically was found in the 0.2 M KCl eluate. However, the flow through contained an activity which cleaves DNA into defined fragments (Fig. 1). The activity of this enzyme fraction was found to be optimal at a temperature of 37 °C in the presence of 50 mM KCl and 10 mM $MgCl_2$. The activity does not require *S*-adenosylmethionine or ATP. This indicates that this enzyme is a type II restriction endonuclease. It was designated *Mvn*I in a slight modification of the standard nomenclature of restriction enzymes [23] to distinguish it from *Mva*I from *Micrococcus varians* [24]. The activity of the final preparation was 10 units/μl (Fig. 1, lanes 2–5).

The absence of unspecific nucleases in the *Mvn*I preparation was shown by incubation of at least 30 units *Mvn*I with 4 μg DNA for 16 h (480-fold excess of enzyme). Under these conditions, no detectable hydrolysis of the *Mvn*I restriction fragments could be detected (Fig. 1, lanes 7, 8). Ligation of 1 μg $\lambda \cdot$ *Mvn*I-fragments with 1 unit T4 DNA *Lig* for 16 h at 4 °C resulted in more than 80% ligation products. Subsequent digestion with *Mvn*I re-generated more than 90% of the original *Mvn*I-fragments.

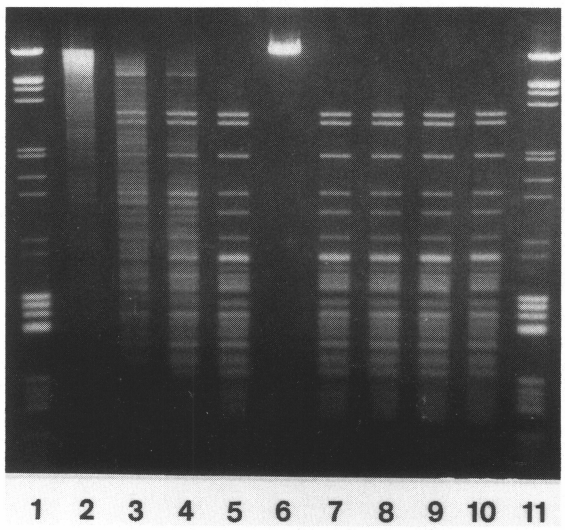

Fig. 1. Digestion patterns of bacteriophage λ*c*I857*S*am7 DNA obtained with *Mvn*I (lanes 2–5, 7, 8), *Tha*I (lane 9) and *Fnu*D II (lane 10), respectively. The reaction mixtures containing 1 μg λ*c*I857*S*am7 DNA were incubated for 1 h at 37 °C with 0.1, 0.3, 0.6 and 1.0 units *Mvn*I, respectively (various dilutions of the final enzyme preparation with 10 U/μl, lanes 2–5), with 10, 30 units *Mvn*I, respectively (=160-, 480-fold excess of enzyme, lanes 7, 8), and for 1 h at 60 °C with 1.0 unit *Tha*I or 1.0 unit *Fnu*DII, respectively (lanes 9, 10). The resulting fragments were analyzed on a 0.8% (w/v) agarose gel by electrophoresis for 3 h at 100 V. Undigested DNAs used as control: λ*c*I857*S*am7 DNA (lane 6), and a mixture of λ·*Eco*RI/*Hin*dIII and pBR322·*Hae*III fragments.

3.2. Identification of the recognition sequence

Bacteriophage λ*c*I857*S*am7 DNA [25] was digested with *Mvn*I, and the resulting *Mvn*I fragments were separated by agarose gel electrophoresis (Fig. 1, lanes 5, 7, 8). By applying computer programs designed to search for recognition sequences on the basis of physical mapping data, the observed fragment pattern was found to be specific for the recognition sequence 5′-CGCG-3′. This result could be confirmed by digestion of λ DNA with the isoschizomers *Tha*I [1,4] and *Fnu*DII [1,27] resulting in identical fragment pattern after digestion of λ*c*I857*S*am7 DNA (Fig. 1, lanes 9, 10).

Furthermore, *Mvn*I, *Tha*I and *Fnu*DII show identical cleavage specificities on Adeno-2 DNA (303 cleavage sites). All three enzymes are also characterized by the lack of cleavage sites on virus SV40 DNA (data not shown). In addition, *Mvn*I is identical to *Tha*I and *Fnu*DII with respect to hydrolysis of φX174RF and M13mp18RF phage DNAs, as well as plasmids pBR322 and pBR328 [1,21,22,28].

3.3. Identification of the cleavage positions

The cutting sites within both strand of the *Mvn*I recognition sequence 5′-CGCG-3′ at position 946 were determined by the chemical method

including trimming with T4 DNAP as described by brown and Smith [16]. pBR328 [21,22] was linearized with *Sal*I at position 651, and terminally labeled at the 5′-ends with T4 PNK and [γ-^{32}P]ATP. Monolabeled sub-fragments were generated by a second digestion with *Pst*I at position 2524. The resulting fragments, 3034 bp and 1873 bp in size, were separated by electrophoresis on a 1% (w/v) low melting agarose gel. The smaller 1873 bp fragment containing a cleavage site for *Mvn*I at position 946, was extracted, and four aliquots were treated according to the Maxam and Gilbert chemical sequencing reactions [15]. Further aliquots of the above fragments were cleaved with *Mvn*I alone (A), or with *Mvn*I followed by treatment with T4 DNAP (B) as described in [16]. The resulting fragments were analyzed on an 8% (w/v) sequencing gel containing 8 M urea (Fig. 2). The 5′-labeled 296 bp pBR328 · *Myn*I/*Sal*I fragment (A) appears alongside the band of the 5′-sequence ladder which represents the internal C-residue of the *Mvn*I recognition sequence 5′-CG*C*G-3′ (Fig. 2). However, the chemically derived DNA chain has lost its 3′-terminal C-residue as a consequence of chemical cleavage. Therefore, this DNA chain actually end with its 5′-neighbouring G-residue at position 947. This shows that *Mvn*I cleaves between the internal G-residue at position 947 and the C-residue at position 948, thus creating blunt ends.

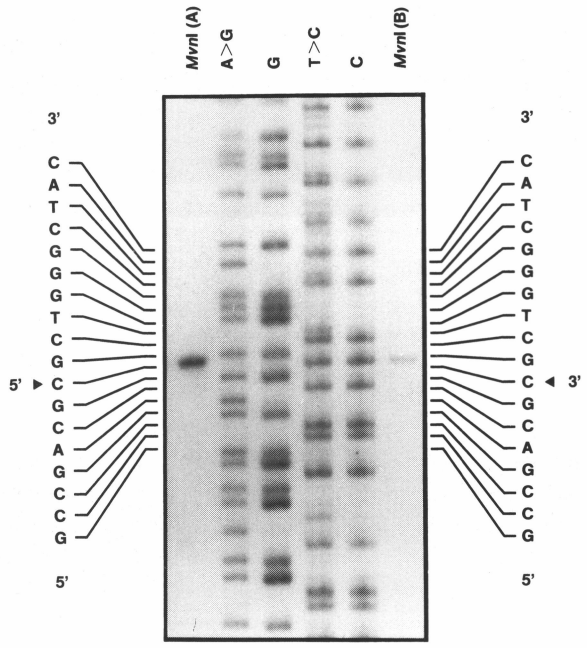

Fig. 2. Determination of the cleavage position of *Mvn*I on both strands of pBR328 DNA at position 946. The lengths of the 5′-labeled pBR328·*Mvn*I/*Sal*I-fragments with (B) and without (A) T4 DNAP treatment (lanes 1, 6) were determined by comparing their migration distances with the positions of the various bands of the 5′-sequencing ladder of the 5′-labeled (+)-strand of the pBR328·*Pst*I/*Sal*I fragment between positions 2524 and 651 (lanes 2–5). Position 1 of pBR328 DNA is defined as the first T of the sequence 5′-*T*TCTCATGTT-3′ within the (+)-strand as described by Pfeiffer et al. [22]. Labeling of the protruding 5′-end of the (+)-strand of the pBR328·*Sal*I fragment, dephosphorylated with 0.04 units CIAP per pmol 5′-termini (32), was performed with 2 units of T4 PNK and 10 pmol [γ-^{32}P]ATP (~ 3000 Ci/mmol) per μg DNA according to [33]. All labeled fragments were purified under native conditions by electrophoresis in 1% (w/v) low melting temperature agarose gels. The sequencing procedure followed the protocol of the chemical method described by maxam and Gilbert [15,33]. Electrophoresis under denaturing conditions was performed in 8% (w/v) polyacrylamide gels containing 8 M urea.

The pBR328 · *Mvn*I/*Sal*I fragment (B) treated with T4 DNAP migrated alongside the same band of the 5′-sequence ladder, representing the internal C residue of the *Mvn*I recognition sequence 5′-CG*C*G-3′ as was observed with the pBR328 · *Mvn*I/*Sal*I fragment (A) (Fig. 2). This confirms that *Mvn*I produces blunt ends by hydrolyzing the phosphodiester bond between the G′-residue at position 947 and the C′-residue at position 948. In case of protruding ends, the pBR328 · *Mvn*I/*Sal*I fragment would either be extended (5′-protruding ends) or degraded (3′-protruding ends) by at least one nucleotide after action of T4 DNAP.

The sequencing data indicate that *Mvn*I cuts its recognition sequence in both strand between the G- and C-nucleotides at positions 947 and 948 producing blunt ends as follows:

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      ↓
5′-CG CG-3′
3′-GC GC-5′.
      ↑
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Although *Mvn*I shares the recognition sequence with two already described restriction enzymes, it might be a useful tool for experiments in molecu-

lar biology. It can be purified by a rapid and simple procedure in high purity and shows – in contrast to *Tha*I from a thermophilic archaebacterium – its optimal activity at standard incubation temperature.

The occurrence of high levels of restriction endonucleases in *Methanococci* suggests a possible role of these nucleases in protecting the cells from bacteriophage infections. Bacteriophages indeed have been found in *Halobacteria* [29], S^0-dependent arachaebacteria [29], representatives of the order *Methanobacteriales* [30,31], and might be also present in the genus *Methanococcus.*

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