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*Mcr*I: a novel class-II restriction endonuclease from *Micrococcus cryophilus* recognizing 5'-CGRY/CG-3'

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A new class-II restriction endonuclease, *Mcr*I, with a novel sequence specificity as isolated from the Gram-positive eubacterium *Micrococcus cryophilus*. *Mcr*I recognizes the palindromic hexanucleotide sequence

5'-C G R ↓Y C G-3'
3'-G C Y R G ↑C-5'.

The novel enzyme in the presence of Mg^{2+}-ions cleaves specifically both strands as indicated by the arrows. The staggered cuts generate 3'-protruding ends with single-stranded 5'-RY-3' dinucleotide extensions. The *Mcr*I recognition sequence was deduced from mapping data on DNAs of bacteriophages ΦX174RF and M13mp18RF characterized by one and four cleavage sites, respectively. The cut positions within both strands of the recognition sequence were determined in sequencing experiments by analyzing hydrolysis of phosphodiester bonds within a polylinker region of M13mp18RF DNA containing an additional *Mcr*I recognition site including treatment with T4 DNA polymerase. The novel enzyme may be a useful tool for cloning experiments by completion of the enzymes *Ecl*XI (5'-C/GGCCG-3'), *Not*I (5'-GC/GGCCGC-3'), *Pvu*I (5'-CGAT/CG-3') as well as *Eae*I (5'-Y/GGCCR-3') and *Xho*II (5'-Y/GATCR-3') characterized by partly identical sequence specificities.

Restriction endonuclease; Hexanucleotide recognition sequence; 3'-Protruding terminus; CpG-dinucleotide; HTF-island

1. INTRODUCTION

More than 1000 class-II restriction endonucleases have been isolated from eu- and archaebacteria [1-3]. These enzymes represent at least 163 different sequence specificities. Most important are subclass-IIP enzymes [1] recognizing regular tetra, hexa- or octanucleotide palindromes. Out of the 64 possible hexanucleotides, 51 sequences are currently covered by corresponding enzymes.

In addition to enzymes characterized by unequivocally defined hexanucleotides there are also enzymes known recognizing hexanucleotide palindromes with ambiguous nucleotides. Examples are enzymes specified by purine:pyrimidine (R:Y) residues like *Dsa*I (5'-C/CRYGG-3'; [6]) by A/C:G/T (M:K) residues like *Acc*I (5'GT/MKAC-3'; [7]) or by still more complex A/G/T:T/C/A (D:H) nucleotide combinations like *Bsp*1286I (5'-GDGCH/C-3'; [8]). These enzymes complement restriction endonucleases of related unequivocally defined hexanucleotide recognition sequences because additional cleavage sites on DNA may be cut with the mentioned enzymes of more complex sequence specificity retaining cutting frequencies lower than related enzymes of palindromic tetra- or pentanucleotide sequence specificity.

We have discovered a further enzyme, *Mcr*I, recognizing the hexanucleotide palindrome 5'-CGRY/CG-3'. We report here the isolation and characterization of the novel enzyme enlarging the set of class-II restriction endonucleases cutting at GpC-rich hexanucleotides. We designated the new class-II enzyme in accordance with the proposal of Smith and Nathans [4] as *Mcr*I to distinguish it from the series of enzymes isolated from different strains of the genus *Micrococcus* [1,2,5].

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2. MATERIALS AND METHODS

2.1. *Bacterial strain and culture conditions*

Micrococcus cryophilus (DSM 20429/ATCC 15174) originally isolated from pork sausages prepared from frozen meat, belongs to the Gram-negative cocci [9]. Cells were cultured aerobically at 18°C in a mixture of 25 g/l Merck standard medium I at a pH value between 7.2 and 7.6. The organism was cultivated in a 10 litre Giovanola fermenter using a 1% inoculum, 0.08 Vvm aeration and agitated at 450 rpm. After 6 h, the cells were harvested in a late-log phase by centrifugation in a Padberg 41G centrifuge at 20000 rpm and stored at −70°C.

2.2. *Enzymes*

Restriction endonucleases (*Aat*II, *Ava*II, *Bgl*II, *Ksp*I, *Mlu*I, *Nar*I,

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*Nci*I, *Pst*I, *Sna*BI, *Sno*I, *Ssp*I, *Stu*I), calf intestine alkaline phosphatase (CIAP), T4 DNA polymerase (T4DNAP), T4 DNA ligase (T4DNALig), T4 polynucleotide kinase (T4PNK), terminal transferase (TdT) and Klenow enzyme (PolIk) were from Boehringer Mannheim. The enzymes were employed according to the manufacturer's specifications.

2.3. *DNAs, nucleotides and reagents*

Bacteriophage lambda-cI857Sam7, ΦX174RF and M13mp18RF DNA, plasmids pUC18, pBR322 and PBR328, DNA molecular weight markers III and VI, universal M13 sequencing primer, dATP, dGTP, dCTP, dTTP, ddATP, ddGTP, ddCTP and ddTTP were from Boehringer Mannheim. Virus Ad-2 and SV40 DNAs were from BRL.

[α-^{32}P]dATP (approx. 3000 Ci/mmol) and [γ-^{32}P]ATP (approx. 3000 Ci/mmol) were from Amersham; Sephadex-G25, Q-Sepharose CL-6B, S-Sepharose CL-6B and Sephacryl-AcA 34 from LKB-Pharmacia. Agarose was from FMC Corp., Marine Colloids Div./Rockland, ME, low melting temperature agarose (type VII) from Sigma. Dithioerythritol (DTE), dithioerytrit (DTT), and phenylmethylsulfonyl fluoride (PMSF) were from Boehringer Mannheim.

2.4. *Isolation of* McrI

The isolation of *Mcr*I was performed according to standard procedures [10] using Q-Sepharose CL-6B, S-Sepharose CL-6B and Sephacryl-AcA 34 chromatography. The final preparation was stored at −20°C.

2.5. *Enzyme assay*

Assays during the enzyme purification were performed using 1–5 μl of the column fractions, incubated with 1 μg *lambda*-cI857Sam7 DNA in 25 μl buffer H (50 mM Tris-HCl, pH 7.5/37°C; 10 mM $MgCl_2$; 100 mM NaCl; 10 mM DTE; for 1 h at 37°C. Reactions were terminated by addition of 5 μl cold stop solution (7 M urea; 20% (w/v) sucrose; 60 mM EDTA and 0.1% (w/v) Bromophenol blue). The reaction mixtures were resolved by electrophoresis for 3 h at 100 V on 1% (w/v) agarose gels in 40 mM Tris-acetate, pH 8.2/25°C; 1 mM EDTA; 10 μg/ml ethidium bromide.

For exact determination of *Mcr*I activity, varying amounts of the final enzyme preparation were incubated with 1 μg *lambda*-cI857Sam7 DNA in 25 μl incubation mixture for 1 h at 37°C. The reactions were terminated by addition of 5 μl cold stop solution. 25 μl of each reaction mixture was resolved by electrophoresis as above. One unit of *Mcr*I is defined as the amount of enzyme which digests 1 μg of *lambda*-cI857Sam7 DNA within 1 h at 37°C in buffer H.

2.6. *DNA labelling*

Sequencing primer was 5′-endlabeled by treatment with 0.05 units of CIAP per pmol 5′-termini [11] and rephosphorylation with 1 unit T4PNK per pmol 5′-termini in the presence of 50 μCi [γ-^{32}P]ATP [12]. The efficiency of labeling was determined by measuring the rate of incorporation of radioactivity into acid-insoluble material in a liquid scintillation counter after collecting acid-precipitable material on glass filters.

2.7. *Determination of cleavage positions*

Determination of *Mcr*I cleavage sites within both strands using the enzymatic method was according to [13] and [14]. Nucleotide sequence analysis was performed using a 5′-phosphorylated sequencing primer and incorporation of [γ-^{32}P]dATP following the enzymatic sequencing method described in [15]. Treatment with T4DNAP was performed as described in [14]. The lengths of both 5′-labelled strands starting from the 5′-labelled universal sequencing primer generated by additional *Mcr*I digestion were determined by electrophoresis under denaturing conditions in 5% (w/v) polyacrylamide gels containing 8 M urea, and comparison with corresponding bands of a sequence ladder.

2.8 *Computer analysis*

Computer data were generated on a Microvax II (Digital Equipment Corp.) under VAX/VMS using the UWGCG software package [16]. In addition, computer programs were used which are analogous to those described in [17]. These programs were designed to search for recognition sequences of new restriction endonucleases. This was achieved by comparison of experimentally observed fragment lengths with computer-derived fragment patterns of possible new recognition sequences (P.S. Neumaier, unpublished data). The restriction enzyme databank of the UWGCG software package was updated with the data of [1]. Sequence data for Ad-2 DNA [18] and for *lambda*-cI857Sam7 DNA [19] were obtained from the EMBL data bank [20], Entries AD2 and LAMBDA. The additional nucleotide sequences used for computer analysis were determined by [21,22] for pUC18 DNA and M13mp18RF DNA, by [22–24] for pBR322 DNA, and by [22,25] for pBR328 DNA.

The indication of *Mcr*I recognition sites on the various sequenced DNAs refer to the first adenosine nucleotide of the hexanucleotide recognition sequence of the (+)-strand in accordance with the computer programs described in [16]. However, if cut positions are cited, these data are related to the actual sites of phosphodiester bond hydrolysis.

3. RESULTS

3.1. *Optimal conditions for enzyme activity*

The activity of *Mcr*I is optimal for pH 7.8 at 37°C, and salt concentrations at 200 mM NaCl. The relative activities of *Mcr*I at various temperatures, pH values and salt concentrations as well as relative activities of *Mcr*I in 5 frequently used standard buffers are given in Table I. The enzyme is strictly dependent on Mg^{2+}-ions with an optimum at 10 mM $MgCl_2$, but does not require *S*-adenosyl-methionine or ATP for activity.

Table I

Relative *Mcr*I activity at various temperatures, pH values, salt concentrations and in standardized incubation buffers

Temperature (°C)	25	30	37	42		
Relative activity (%)	50	80	100	40		
pH value	7.0	7.5	7.8	8.0	8.5	
Relative activity (%)	50	75	100	90	60	
C_{NaCl} (mM)	50	100	150	200	250	300
Relative activity (%)	30	60	80	100	60	15
Standard buffer*	A	B	L	M	H	
Relative activity (%)	50	80	70	50	100	

* Composition of standard buffers is according to Maniatis [11] and O'Farrell [31]:

Buffer A = 33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate, 0.05 M dithioerythrit (DTT); pH = 7.5/37°C

Buffer B = 10 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl; pH = 8.0/37°C

Buffer L = 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithioerythritol (DTE); pH = 7.5/37°C

Buffer M = 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithioerythritol (DTE); pH 7.5/37°C

Buffer H = 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol (DTE); pH 7.5/37°C

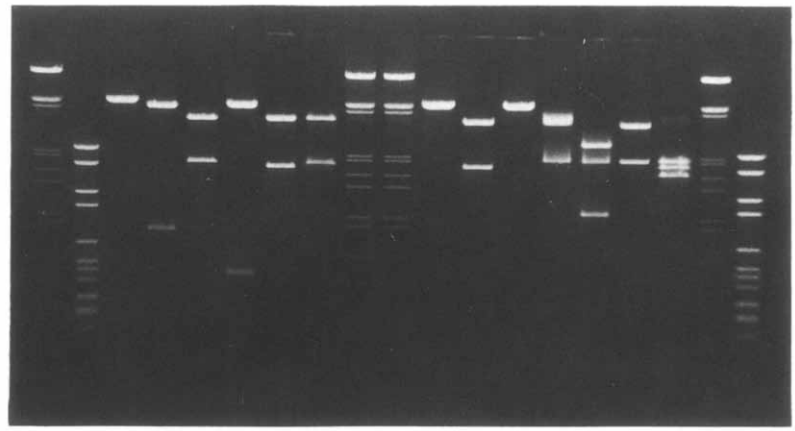

Fig. 1. Double-digestions of ΦX174RF DNA with *Mcr*I and additional restriction enzymes. ΦX174RF DNA was cut with *Mcr*I (lanes 3, 11) and the resulting linearized DNA was additionally cleaved with *Pst*I (lane 4), *Aat*II (lane 5), *Ava*II (lane 6), *Ksp*I (lane 7), *Nci*I (lane 8), *Ssp*I (lane 12), *Stu*I (lane 13), *Mlu*I (lane 15) and *Nar*I (lane 17), respectively. ΦX174RF[*Mlu*I]-fragments are shown in lane 14; ΦX174RF[*Nar*I]-fragments are shown in lane 16. *Lambda*-cI857Sam7[*Eco*RI + *Hin*dIII]-fragments (lanes 1, 9, 10, 18) and a mixture of pBR328[*Bgl*I]-fragments and pBR328[*Hin*fI]-fragments (lanes 2, 19) were used as high-M_r- and low-M_r-molecular weight markers.

Highly purified *Mcr*I is free of contaminated site-specific or unspecific endonucleases as well as exonucleases, since neither *lambda*-cI857Sam7 DNA or pBR322 DNA digested with at least 20 units *Mcr*I for 16 h (320-fold excess of enzyme) gave sharp ends without any smearing after electrophoresis in agarose gels (data not shown). Functional purity is shown by ligation with 0.1 units T4DNALig per μg *Mcr*I-fragments which yields in >95% ligation products; > 95% of the ligation products can be recut with *Mcr*I.

3.2. *Determination of recognition sequence*

The recognition sequence of *Mcr*I was determined by mapping its recognition sites on ΦX174RF DNA at position 4605 and on M13mp18RF DNA at positions 1425, 3882, 6408 and 6524 by double digestions with *Mcr*I and various restriction enzymes cutting around these sites. In detail, with ΦX174RF DNA after linearization of the phage DNA with *Mcr*I, double-digestions were performed with a series of restriction endonucleases including *Aat*II, *Ava*II, *Ksp*I, *Mlu*I, *Nar*I, *Nci*I, *Pst*I, *Ssp*I and *Stu*I (Fig. 1). For all tested enzymes, the resulting fragment sizes are in agreement with *Mcr*I recognition at a hexanucleotide 5′-CGR-YGG-3′ sequence at position 4605 (Table II). The position of the four *Mcr*I recognition sites on M13mp18RF DNA were determined in an analogous way with *Ava*II (pos. 5913), *Bgl*II (pos. 6943), *Sna*BI (pos. 1269) and *Sno*I (pos. 4742) double-digestions. The number of cleavage sites were also determined on DNAs of phage *lambda*-cI857Sam7, virus Ad-2 and SV40, as well as plasmids pBR322 and pBR328 (Fig. 2).

The *Mcr*I recognition sequence 5′-CGRYGC-3′ was confirmed by comparison of these experimentally observed *Mcr*I fragment patterns (see Fig. 2) with the corresponding computer data derived by analysis of the known nucleotide sequence with the program MAP of the UWGCG program package [25] obtained by digestion of *lambda*-cI857Sam7, at recognition sites 2945, 4189, 6677, 7456, 9164, 9305, 10937, 11937, 12925,

Table II

Mapping of the single *Mcr*I recognition sequence on ΦX174RF DNA at position 4605 by double-digestions with additional enzymes

Second enzyme	Position(s) of recognition sites	Experimentally observed fragment sizes of double-digestions (bp)	Computer-derived fragment sizes of double-digestions (bp)
*Pst*I	5386	4600, 800	4605, 781
*Aat*II	2786	3500, 1800	3567, 1819
*Ava*II	5042	5000, 450	4949, 437
*Ksp*I	2862	3600, 1750	3625, 1743
*Nci*I	2801	3600, 1800	3582, 1804
*Ssp*I	1009	3600, 1800	3596, 1790
*Stu*I	4488	5300, 100	5269, 117
*Mlu*I	221, 2146	2500, 1900, 1000	2459, 1925, 1002
*Nar*I	1020, 2977	2000, 1800, 1600	1957, 1801, 1628

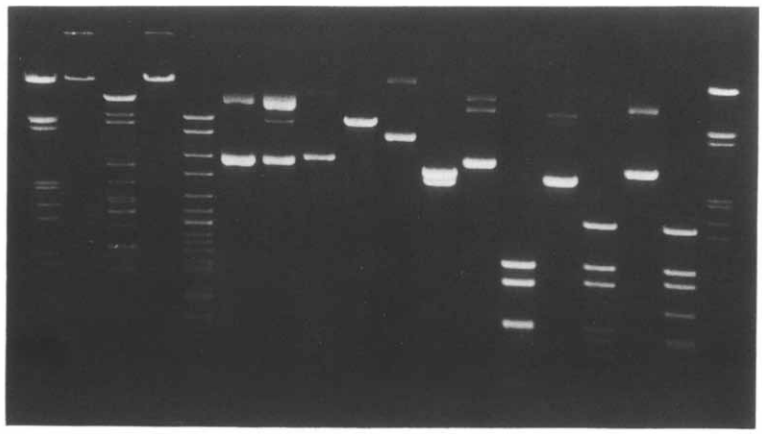

Fig. 2. *Mcr*I cleavage fragment pattern for DNAs of *lambda*-cI857Sam7, Ad-2, SV40, ΦX174RF, M13mp18RF, pUC18, pBR322 and pBR328. M, fragments of *lambda*-cI857Sam7 cut with *Eco*RI + *Hin*dIII were used as low-M_r-molecular weight markers (lanes 1 and 18). 1 μg of viruses Ad-2 and SV40, bacteriophages ΦX174RF and M13mp18RF and plasmids pUC18, pBR322 and pBR328, respectively, is cleaved with 5 units of *Mcr*I in 25 μl incubation mixture for 1 h as described in section 2. The uncleaved DNAs (lanes 2, 4, 6, 8, 10, 12, 14, 16) and the respective *Mcr*I cleavage products (lanes 3, 5, 7, 9, 11, 13, 15, 17) are resolved by electrophoresis for 2 h at 100 V in 1% (w/v) agarose gels using 40 mM Tris-acetate, pH 8.2/25°C; 1 mM EDTA and 10 μg/ml ethidium bromide as electrophoresis buffer. The lengths (in bp) of the resulting *Mcr*I fragments are: 9533, 9189, 5600, 4848, 2488, 2028, 1708, 1632, 1460, 1450, 1244, 1000, 988, 986, 891, 867, 779, 760, 476, 243, 191 and 141 (*lambda*-cI857Sam7); 5500, 4716, 2866, 2285, 1754, 1483, 1309, 1295, 1137, 1045, 954, 849, 782, 747, 605, 553, 550, 546, 517, 484, 433, 432, 426, 413, 402, 401, 396, 302, 253, 246, 239, 218, 189, 186, 170, 167, 165, 160, 155, 120, 89, 80, 60, 53, 40, 24, 9, 8 and 4 (Ad-2); no fragmentation (SV40); 5386 (ΦX174RF); 2526, 2457, 2150 and 116 (M13mp18RF); 923, 747, 443, 424 and 149 (pUC18); 1449, 923, 765, 424, 367, 286 and 149 (pBR322); 1443, 923, 784, 560, 395, 367, 286 and 149 (pBR328).

14375, 15835, 16595, 16786, 17029, 17920, 19948, 20424, 21410, 26258, 35791, 36658 and 45847, Ad-2 DNA at positions 4131, 5885, 6311, 6497, 7980, 8147, 8317, 9454, 9514, 9674, 9763, 9883, 10665, 10689, 10942, 11343, 11498, 12051, 12535, 12968, 12976, 13389, 13429, 13946, 14496, 14714, 16023, 16269, 16349, 17303, 17312, 18357, 18659, 19264, 19696, 20545, 21091, 21487, 21726, 21915, 21919, 22084, 22137, 23432, 23552, 26418, 26820, 27567, 29852 and 35352, pUC18 DNA at positions 280, 723, 1147, 2070 and 2219, pBR322 DNA at positions 290, 657, 943, 2392, 2816, 3739 and 3888, and pBR3288 DNA at positions 290, 657, 943, 1727, 2650, 2799, 4242 and 4802. SV40 DNA is not cut by *Mcr*I.

3.3. *Determination of the cleavage positions*

The exact cleavage position of *Mcr*I was determined by applying the enzymatic sequencing method [13,14]. An M13mp18RF derivative containing an insert with a 5′-CGACCG-3′ *Mcr*I site was used as template for enzymatic sequencing reactions starting with a 5′-phosphorylated universal M13 sequencing primer. In parallel, a universal sequencing primer ^{32}P-endlabeled with T4 PNK and [γ-^{32}P]ATP, was annealed to the same template, and a partial double-stranded DNA was created by treatment with PolIk and all four dNTPs. This double-stranded DNA was used as substrate for *Mcr*I to produce an 5′-endlabeled DNA fragment compared to the sequencing ladder. Samples were analyzed by electrophoresis and subsequent autoradiography as described above (Fig. 3).

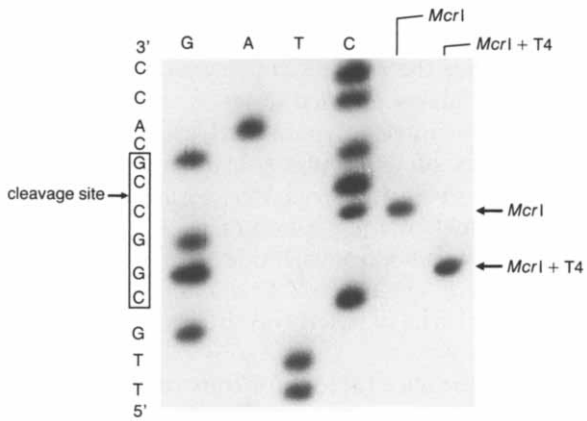

Fig. 3. Autoradiograph of the sequencing ladder around an *Mcr*I recognition site of M13mp18 DNA. Preparation of single-stranded M13mp18RF derivative with a 5′-GCACCG-3′-insert and sequencing reactions were done according to [13]. A partial double-stranded, 5′-endlabeled DNA as substrate for *Mcr*I was prepared and by PolIk-extension of a 5′-endlabeled M13 universal primer annealed to single-stranded M13mp18RF DNA. Lanes G, A, T, C represent the four base-specific sequencing reactions, lane *Mcr*I represents the respective *Mcr*I-treated sample, lane *Mcr*I + T4 DNAP represents the *Mcr*I-treated sample additionally incubated with T4 DNAP and all four dNTPs. The interpretation followed the rule described in [13]. The fragment resulting from *Mcr*I-cleavage has the same mobility as the band of the internal C in the sequencing ladder of the *Mcr*I recognition sequence 5′-CGACCG-3′. This band of the C sequence ladder represents a fragment ending with 5′-[^{32}P----CGAC-3′, and therefore indicates the cleavage mode 5′-CGAC$^{\downarrow}$CG-3′. The generation of dinucleotide 3′-protruding single-stranded ends was confirmed by additional treatment with 10 units T4 DNAP and all four dNTPs for 30 min at 25°C; this treatment results in a fragment which migrates with the 5′-G residue of the *Mcr*I sequence. The observed band is obtained by exonucleolytic shortening of the single-stranded dinucleotide fragment ends for the 3′-protruding two nucleotides.

The cleavage position of *Mcr*I determined by enzymatic sequencing on the first recombinant M13mp18RF DNA derivative is as follows (Fig. 3):

5′-[^{32}P]-----GTGGA*CGAC* ↓ *CG*CGTCC-----3′.

The generation of dinucleotide 3′-protruding single-stranded ends by *Mcr*I treatment was confirmed by shortening the 5′-^{32}P-labeled strand for two nucleotides by an exonucleolytic reaction catalyzed by T4 DNAP in the presence of all four dNTPs resulting in 5′-[^{32}P]-----GTGGA*CG*-3′ chains. From both experiments the specificity of *Mcr*I is concluded as:

5′-C G R Y ↓ C G-3′

3′-G C Y R G C-5′.
↑

4. DISCUSSION

With *Mcr*I from *Micrococcus cryophilus*, a novel class-II restriction endonuclease has been discovered and purified recognizing the hexanucleotide recognition sequence 5′-GCRY/GC-3′. *Mcr*I occurs as the only restriction endonuclease in this particular *Micrococcus* strain and thus the enzyme can be purified by a simple procedure in highly purified form. There are reports on several other restriction endonucleases from 10 different species of the genus *Micrococcus* [1,2,5], but these enzymes all differ from *Mcr*I sequence specificity. However, most of these enzymes recognize like *Mcr*I tetra- penta- and hexanucleotide palindromes, which are rich in G/C nucleotides. This is correlated with the high G:C-content of *Micrococcus* chromosomal DNA [9].

*Mcr*I may be a useful tool for construction of recombinant DNA because it broadens the set of enzymes recognizing palindromic hexanucleotides. In particular, it complements enzymes of related sequence specificity like *Ecl*XI (5′-C/GGCCG-3′; [26]), *Not*I (5′-GC/GGCCGC-3′; [27,28]) and *Eae*I (5′-Y/GGCCR-3′; [29]) all recognizing G/C-rich hexa- or octanucleotide palindromes.

Finally, *Mcr*I may be applied for analysis of G/C-rich DNA sequence stretches, or as low-frequency cutting class-II restriction endonucleases for genomes of high G:C-content [25]. Because *Mcr*I recognition sequence contains two CpG-dinucleotides, it may be also applied for preferential cleavage of human genomic DNA which contains CpG-dinucleotides predominantly in islands of high transcription frequency [30].

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