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A new restriction endonuclease from *Citrobacter freundii*

A.A.Janulaitis, P.S.Stakenas, E.N.Lebedenko* and Yu.A.Berlin*

All-Union Research Institute of Applied Enzymology, Vilnus 232028, and *Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, USSR

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ABSTRACT

CfrI, a new restriction endonuclease of unique substrate specificity, has been isolated from a Citrobacter freundii strain. The enzyme recognizes a degenerated sequence PyGGCCPu in double-strand DNA and cleaves it between Py and G residues to yield 5′-protruding tetranucleotide ends GGCC.

INTRODUCTION

Although a great number of site-specific endonucleases (class II and III restriction endonucleases) have been described (1), the search for new restrictases remains urgent due to the outstanding role these enzymes play in structural and functional studies of nucleic acids. In course of wide screening of microbial cultures we discovered a new site-specific endonuclease from a Citrobacter freundii strain (2). We designated the enzyme as CfrI in accordance with the nomenclature of restrictases (3), though no evidence of the enzyme being a component of a restriction-modification system is so far available. This paper describes the isolation of the CfrI endonuclease and the structure elucidation of its recognition site.

MATERIALS AND METHODS

The following commercial materials have been used: peptone (Difco), nuclease-free bovine serum albumine (BSA) A grade, 2-mercaptoethanol (ME), dithiothreitol (DTT), dCTP, dGTP (Calbiochem), [^{32}P]nucleoside-5′-triphosphates (Amersham), phosphocellulose P11 and cellulose CF11 (Whatman), Sephadex G-50 s.f.(Pharmacia), cellulose acetate strips (Schleicher & Schüll), hydroxylapatite, agarose, acrylamide and methylene-bis-acrylamide

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(BioRad), tris (Fluka). Other reagents were p.a. Bacterial DNA was isolated from E.coli B (4) and immobilized on cellulose CF 11 (5); the preparation contained 4 mg/g DNA as determined by the method (6). Phage λcI857S7 DNA was isolated from the purified phage (7), the isolation of supercoiled DNAs of ϕX174am3 (RF) and pBR322 was carried out by buoyant density centrifugation in caesium chloride-ethidium bromide (8). Enzymes were isolated according to usual procedures (T4 DNA ligase and polynucleotide kinase, BAP, restrictases EcoRI, BamHI, SalI, PstI, BspI) or obtained from Worthington (VPDE, SPDE, pancreatic DNAse, micrococcal nuclease) and Boehringer (E.coli DNA polymerase). Heparin-agarose synthesised by the method (9) was kindly supplied by O.Sudjuvene (Vilnus).

Culture growth.

A strain of C.freundii RFL2 isolated from natural sources and identified by S.Bardzilauskene (Vilnus) was grown at 37°C in 10 l of intensively aerated cultural broth containing - g/l: peptone - 5, meat extract - 8, NaCl - 5 (pH 6.8-7.0) up to the A_{540} value of 2.8. Cells collected by centrifugation (ca.24 g) were kept at -20°C.

Isolation, purification and analysis of the CfrI restrictase.

All operations were carried out at 4°C. Frozen cells (20 g) were thawed, suspended in 40 ml 10 mM K-phosphate pH 7.4, 7 mM ME, 1 mM EDTA, 10% (v/v) glycerol (A buffer), homogenized by sonication (300 w, 22 KHz; 6x1 min) and centrifugated for 1 hr at 48000 g. The supernatant was diluted by an equal volume of A buffer containing 0.4 M NaCl and chromatographed on a P-cellulose column (1.5x22 cm; 0.2 to 0.6 M NaCl, 6 ml fractions), see Fig.1. Fractions 23 to 27 eluted at 0.32 to 0.37 M NaCl were pooled, diluted with an equal volume of A buffer and chromatographed on a heparin-agarose column (1x12 cm; 0.2 to 0.7 M NaCl, 2.5 ml fractions; see Fig.2). Fractions 21 to 26 eluted at 0.45 to 0.53 M NaCl were chromatographed on a hydroxylapatite column (1x10 cm) in A buffer containing 0.3 M NaCl in the linear gradient of K-phosphate pH 7.4 (0.01 to 0.3 M, 2.5 ml fractions), see Fig.3. Fractions 10 to 17 eluted at 0.07 to 0.12 M K-phosphate were pooled and dialized against A buffer containing 0.1 M NaCl and 50% (v/v) glycerol; the same buffer with 100

mcg/ml BSA was used to dilute the enzyme preparations.

To determine the CfrI endonuclease activity, λ DNA (2 mcg) or immobilized E.coli DNA (140 mcg) were incubated in a buffer containing 10 mM NaCl, 5 mM $MgSO_4$, and 75 mM tris-HCl pH 8.6 (in case of the immobilized substrate 100 mcg/ml BSA were added) at 37°C. Incubation mixtures were analysed by electrophoresis in 1% agarose gel in 0.1 M sodium borate pH 8.2, 2 mM EDTA, 1 mcg/ml ethidium bromide (10), or by spectrophotometry at 260 nm (5), respectively. An activity unit is the amount of CfrI which fully hydrolyses 1 mcg λ DNA in 40 mcl incubation mixture for 1 hr at 37°C (electrophoretic activity unit, eau), or, after 15 min incubation with the excess of the immobilized DNA, causes A_{260} increase of 0.1 (spectrophotometric activity unit, sau; correlation between analysed amount of the enzyme and absorbance increase is linear up to A_{260} 0.15), see (5). Effect of pH value, ionic strength, temperature, metal ions and other factors on the CfrI activity was estimated by visual control of agarose electrophoregrammes of λ DNA restricts. Protein concentration was determined by the method (11).

pBR322 DNA hydrolyses with combinations of restrictases were run at 37°C in 50 mM NaCl, 6 mM $MgCl_2$, 6 mM ME, 6 mM tris-HCl pH 7.5, excesses of enzymes (up to 50-100 eau/mcg) and long incubations (3 to 5 hr) were used. The hydrolysates were analysed by 5% PAGE in 30 mM Na-phosphate, 1 mM EDTA, 36 mM tris-HCl pH 7.7.

Structure elucidation of the CfrI recognition site.

Dephosphorylation and [^{32}P]phosphorylation of restriction fragments. CfrI restricts of λ DNA (5 mcg) were incubated 1 hr at 60°C in a 80 mcl solution containing 50 mM tris-HCl pH 8.9, 10 mM $MgCl_2$ and 100 u BAP followed by phenol deproteinization, ether extraction and ethanol precipitation. The DNA was then 5'-^{32}P-phosphorylated in a 70 mcl solution containing 50 mM tris-HCl pH 8.8, 10 mM $MgCl_2$, 10 mM DTT, 20 mcCi [γ-^{32}P]ATP (2000 Ci/mmole) and 7 u T4 polynucleotide kinase (80 min at 37°C). After gel-filtration on a Sephadex G-50 column in 1 mM tris-HCl pH 7.5, 1 mM NaCl, 0.1 mM EDTA, the 5'-labelled fragments were lyophilised and dissolved in 75 mcl 20 mM tris-HCl pH 7.6, 5 mM $MgCl_2$.

FIG. 1

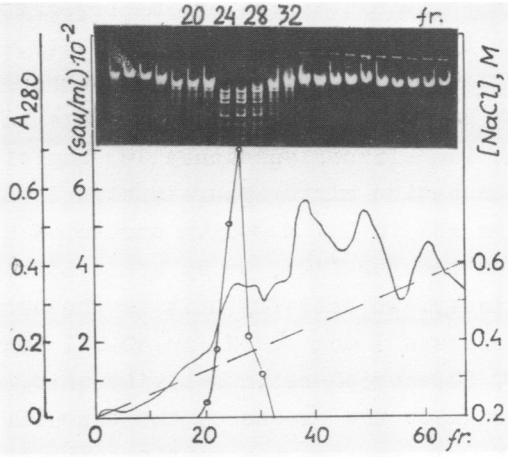


FIG. 2

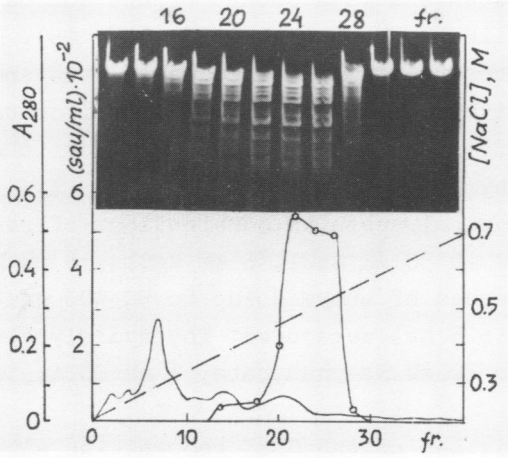
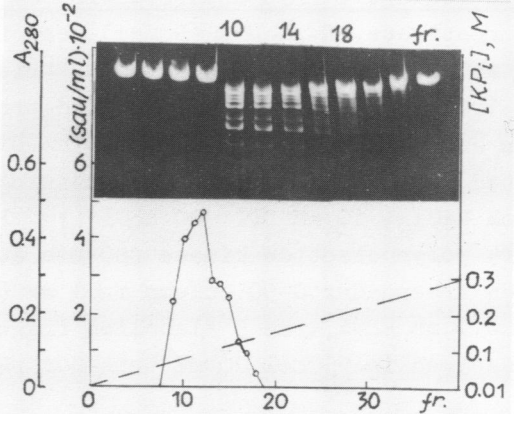


FIG. 3



Complete exonuclease hydrolysis. An aliquote of the solution (2 mcl containing 0.13 mcg λ DNA fragments) was incubated for 1 hr at 37°C with 1 mcl (1 mcg) DNAse, then 0.1 mcl 0.1 M $MgCl_2$, 1 mcl 0.1 M tris-base and 4 mcl (4 mcg) VPDE were added and incubation at 37°C continued for 2 hr. The products were analysed by paper electrophoresis on Whatman 1 in pyridine acetate pH 3.5, the spots being cut out and radioactivity measured in toluene scintillator.

Partial DNAse hydrolysis. Six mixtures each containing 2 mcl 5'-^{32}P-restricts and 1 mcg DNAse were incubated at 20°C for 1.5, 10, 20, 40, 60, and 60 min, resp. The last solution was mixed with 0.6 mcl 0.1 M tris-base and 3 mcl (3 mcg) VPDE and incubated again for 1 hr. All the mixtures were pooled and two-dimensionally separated by electrophoresis on a cellulose acetate strip 3x55 cm (pyridine acetate pH 3.5, 100 V/cm) and homochromatography (homomixture VI (12)); see Fig.4.

Nearest-neighbour analysis. Mixture of 0.2 mcg λ DNA CfrI-restricts, 3 mcCi (15 pmoles) of a labelled precursor ([α-^{32}P] dCTP or -dGTP), 15 pmoles of an unlabelled precursor (dGTP or dCTP, resp.), and 1.5 u DNA polymerase in 30 mM tris-HCl pH 7.6, 5 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT (total volume 45 mcl) was incubated for 1 hr at 20°C, the precursors were separated by gel-filtration, the labelled polynucleotides lyophilised and dissolved in 30 mcl of water. An aliquote (5 mcl) was exhaustively hydrolysed with DNAse and then VPDE (cf. above) or by micrococcal nuclease (0.3 u; 50 mM glycine pH 8.9, 10 mM $CaCl_2$; 1.5 hr at 37°C) and then SPDE (neutralization with 3 mcl 0.1 M AcOH; buffer containing 50 mM $AcONH_4$, 1 mM EDTA and 0.25% Tween-80, pH 5.6; 0.01 u SPDE; 1.5 hr at 37°C). The products were analysed by paper electrophoresis (see above).

FIGS 1-3. Chromatographic purification of the CfrI endonuclease: (o—o) enzymatic activity (sau/ml; 5 mcl aliquotes of fractions), (——) A_{280}, (— —) NaCl concentration. INCERTS: 2 mcl aliquotes, 1 hr incubation with λ DNA at 37°C, agarose gel electrophoresis (see MATERIALS AND METHODS).
1 - phosphocellulose,
2 - heparin-agarose,
3 - hydroxylapatite.

RESULTS AND DISCUSSION

The data on the CfrI isolation (Figs 1-3) are summarized in Table 1. Amount of the enzyme in cell-free extracts could not be determined because of non-specific nucleases. The final yield is about 30 000 eau (1500 e_{au} per gram of wet cells). The enzyme preparation is practically free of non-specific endonucleases (16 hr incubation of λ DNA with 50-fold excess of the enzyme does not affect the normal electrophoretic pattern) and exonucleases or phosphatases (after 2 hr incubation of DNA with 10-fold excess of the enzyme, T4 DNA ligase treatment leads to over 90% ligation, the DNA formed being quantitatively cut again with the CfrI restrictase). Stored in the above-mentioned buffer at -20°C, the enzyme retains partially its activity after at least six months.

The CfrI endonuclease is active in the pH range 7 to 11 with maximum at 8.6 and 37°C (pH values determined at 37°C). ATP and S-adenosyl-L-methionine do not affect the enzyme activity, while magnesium cations are essential with the optimum concentration 5 mM. Cations Co^{2+}, Mn^{2+}, and Zn^{2+} in 0.25 to 1 mM concentration can be substituted for Mg^{2+} (the activity partially retained); 10 mM Ca^{2+} and Ba^{2+} largely inhibit the enzyme, while in case of 5 mM Cu^{2+} or 10 mM Ni^{2+} the inhibition is complete. Sodium chloride inhibits the enzyme partially at 75 mM and completely at 0.3 M concentration. The optimum conditions for CfrI activity were found to be 75 mM tris-HCl pH 8.6, 5 mM $MgSO_4$, 10 mM NaCl at 37°C.

The CfrI sites were located on the pBR322 DNA as follows (see Table 2 and Fig.5). One of the six CfrI fragments (900 bp) of the DNA is cleaved with the EcoRI restrictase to give sub-

Table 1

Isolation of the CfrI endonuclease (from 20 g of wet cells)

Purification step	Total activity, sau	Total protein, mg	Specific activity, sau/mg	Yield, %
Cell-free extract	-	2 500	-	-
Phosphocellulose	23 000	9.6	2 400	100
Heparin-agarose	16 700	0.7	23 900	73
Hydroxylapatite	9 400	0.3	31 400	41

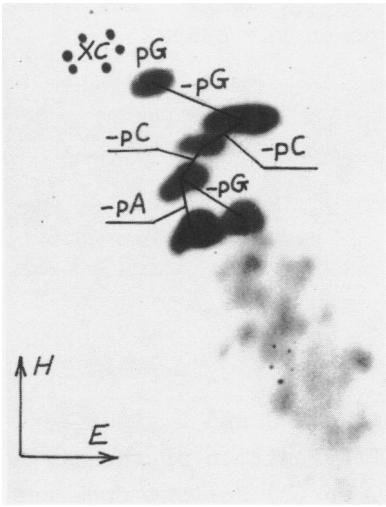


FIG. 4. Two-dimensional separation of the partial DNAse hydrolysis products of 5'-^{32}P-labelled CfrI-restricts of λ DNA (E - electrophoresis, H - homochromatography, XC - xylene cyanole FF as a marker).

fragments 295 and 590 bp, but not with BamHI, the latter cutting another CfrI fragment (105 bp) to yield a subfragment 80 bp. Since the EcoRI and BamHI sites are 375 bp apart (13), these data show positions and orientations of two CfrI fragments (I and II, CfrI sites A, B, and C, resp.) on the pBR322 DNA. Another CfrI fragment (405 bp) is cleaved by the SalI restrictase into subfragments 120 and 280 bp. The distance between SalI and EcoRI sites being 640 bp, this must be fragment IV flanked

Table 2
CfrI restriction fragments of pBR322 DNA (bp)*

CfrI (fragment No.)	CfrI + EcoRI	CfrI + EcoRI +		
		BamHI	SalI	PstI
2000 (VI)	2000	2000	2000	2000
900 (I)	590	590	590	600
480 (V)	480	480	480	480
405 (IV)	405	405	295	405
130 (III)	295	295	280	295
105 (II)	130	130	130	155
	105	80**	120	130
			105	105

*Determined by 5% PAGE, except for (I) (by 1.4% agarose electrophoresis) and (VI) (by subtraction), in direct comparison with BspI restricts of pBR322 DNA.
**The second subfragment (25 bp) not identified.

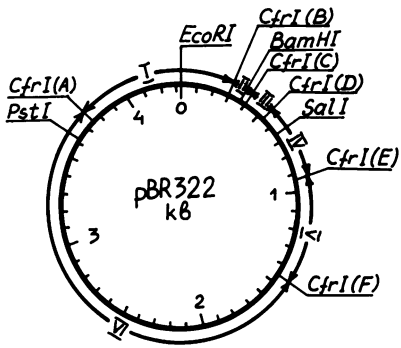


FIG. 5. Positions of CfrI sites on the pBR322 DNA.

with sites D and E, whereas distance between C and D sites (130 bp) corresponds to the CfrI fragment III. Position of the sixth CfrI site (F) flanking the fragment 480 bp (V) follows from the fact that PstI restrictase treatment of the CfrI restricts gives a new subfragment (155 bp) but does not affect the 480 bp fragment.

Comparison of the primary structure of pBR322 DNA (13) in proximity to the located CfrI sites along with identity of electrophoretic patterns of BspI and BspI+CfrI digests of pBR322 DNA suggested a common nucleotide sequence 5′PyGGCCPu as the recognition site of the CfrI endonuclease. This is in agreement with formation of two CfrI fragments of the ϕX174 DS DNA, the shorter of which is ca.550 bp long (its calculated length (14) is 552 bp).

To locate the phosphodiester bond cleaved by the CfrI endonuclease, CfrI restricts of λ DNA were dephosphorylated with BAP, then 5′-^{32}P-phosphorylated by means of T4 polynucleotide kinase and [γ-^{32}P]ATP and, finally, hydrolysed with pancreatic DNAse and VPDE. The only radioactive product of the exhaustive hydrolysis (5′-terminal nucleotide) was identified by paper electrophoresis as d^{32}pG, whereas partial hydrolysis products were two-dimensionally separated to give a fingerprint (Fig.4) demonstrating the common sequence 5′ GGCC(A,G). On the other hand, 5′-protruding ends of the restricts were evened by means of E.coli DNA polymerase and two deoxynucleoside-5′-triphosphates, dCTP and dGTP, one of which contained [α-^{32}P]phosphate. After separation of triphosphates the mixture of polynucleotides was sub-

jected to nearest-neighbour analysis. In case of [α-^{32}P]dCTP as a labelled precursor, dC^{32}p and dG^{32}p in equivalent amounts were identified, whereas the labelled phosphate from [α-^{32}P] dGTP was found in dG^{32}p, dC^{32}p and dT^{32}p in the ratio 2.6:1:1.

The above data unequivocally prove that the CfrI restriction endonuclease recognizes 6-membered deoxynucleotide duplexes

5′ Py↓G-G-C-C-Pu

3′ Pu-C-C-G-G↑-Py

and cleaves phosphodiester bonds between pyrimidine nucleoside and deoxyguanosine residues to yield protruding tetranucleotide 5′-ends G-G-C-C. Although this is apparently similar to a CdiII enzyme referred to in the Tables (1) as having recognition site Py↓GGCCG, the CfrI endonuclease is so far unique in site-specificity. It is noteworthy that CfrI restriction fragments can be cloned in XmaIII sites (C↓GGCCG) and vice versa, this being an additional means of selection.

On the whole, the CfrI endonuclease, due to the size and structure of the recognition site and protruding ends, might be of interest for DNA mapping and sequencing, as well as for recombinant DNA construction. It should be noted that only once a Citrobacter strain had been searched for site-specific endonucleases with no activity found (15). Data presented in this paper rehabilitate the Citrobacter genus as a potential source of restriction endonucleases.

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