Biological Sciences Section

Pakistan J. Sci. Ind. Res., Vol. 30, No. 11, November 1987

PRESENCE OF NEW TYPE II SPECIFICITY RESTRICTION ENZYMES IN LOCAL BACTERIA

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(Received September 1, 1987; revised November 23, 1987)

Eight bacterial strains isolated from local environments have been screened for the presence of new Type II restriction enzymes by analyzing lambda(λ) DNA fragments resulting from protein DNA interactions. Partially purified protein extracts of two of these strains, namely *Pseudomonas aeruginosa* A and *Citrobacter freundii* A_4 contain endonuclease activities which have been highly purified by a combination of gel filtration, ion exchange and affinity chromatography. The identity of the new enzymes, designated as PaeAI and $CfrA_4I$, have been confirmed by analyses of incised λ, ϕ x 174RFI, pBR322, adeno-2 and M13 mp 19RFI DNA substrates as truly Type II restriction enzymes. The isolated enzymes, by analogy with known enzymes, seem to recognize CCGCGG and CTGCAG base sequences respectively.

Key words: Local bacterial isolates, Restriction enzymes, Isoschezomers.

INTRODUCTION

Genetic restriction and modification have been identified in a variety of prokaryotes as two component system involving a restriction endonuclease and a cognate methylase enzyme [1-4]. The system has been divided into three types on the basis of biochemical and catalytic properties of the component enzymes [5]. Type I and III enzymes are complex multifunctional proteins that are able to cleave as well as methylate unmodified DNA [6-8]. Although both types of enzymes require ATP for DNA cleavage, extensive hydrolysis of ATP has been observed only with Type I enzymes. Requirement of S-adenosyl-methionine in endonuclease reaction is absolute for Type I and stimulatory for Type III enzymes [9,10]. The DNA sequences recognized by these proteins are asymmetric. Type III sequences consist of five to six bases [11-13], whereas Type I sequences are hyphenated with two constant domains of three to four bases separated by a nonspecified spacer of six or eight bases. In both cases, DNA methylation occurs at the recognition sequence, whereas DNA cleavage occurs 25-27 bases away in the case of Type III enzymes [13] and several hundred bases away in the case of Type I enzymes [14].

Type II enzymes are structurally simple proteins, requiring Mg^{++} alone for catalytic properties [6-8, 15]. These enzymes cleave within or very close to the recognition sequence [15]. The cognate modification enzyme is a separate protein which catalyze the transfer of the methyl group from S-adenosylmethionine to one of the bases (A or C) in the recognition sequence [2,16].

The uses of Type II restriction endonucleases as molecular scissors in gene splicing has revolutionized studies in basic molecular biology and simultaneously contributed to the development of biotechnology. Scientists have used these novel enzymes as reagent proteins in gene mapping [17], cloning [18] and DNA sequencing [19, 20]. Those enzymes which recognize a specific sequence but cleave a few nucleotides away from that sequence provide a means to correctly reconstruct a genome by religating a vast mixture of DNA sequences and thus exercise a vital role in genetic engineering.

According to the latest directory of restriction endonucleases, 810 enzymes have been discovered which recognize only 124 different base specificities [21]. On the basis of theoretical considerations it can be postulated that there are many more potential sites for which specific cleaving enzymes must exist in the vast microflora as yet unexplored. In our laboratory we have been engaged in a search for new Type II specificity restriction endonucleases from microorganisms collected from different ecological environments in Pakistan. We report here the discovery of two new restriction enzymes in locally isolated strains, namely, *Pseudomonas aeruginosa* A, and *Citrobacter freundii* A4. The new enzymes have been highly purified and have been designated as *PaeAI* and *$CfrA_4$I* respectively.

MATERIALS AND METHODS

Chemicals and reagents. DEAE cellulose, cellulose phosphate and agarose Type IV, were purchased from Sigma Chemical Company. Biogel A 0.5M and purified

grade of cellulose (Munktell No. 410) were obtained from Bio Rad Company. All the inorganic chemicals and reagents were obtained from E.Merck. Substrates DNA and restriction enzymes required in these studies were prepared in our own laboratory according to standard procedures.

Bacterial strains and media. Bacterial strains obtained from different local environments and used in the present studies are described in Table 1. Strains 1-6 were isolated in our laboratory from marine samples collected from the Karachi seashore and strains 7-8 were recovered from sludge samples obtained from petrol storage tanks in Lahore. Standard bacterial strains, used in the isolation of Type II enzymes, were obtained from American Type Culture Collection (ATCC) through Prof. R.J. Roberts Laboratory, Cold Spring Harbor Labs, New York, USA. GM 1519, C 1857 S_7, lysogen in GM 119 were obtained from G.M. Marinus, University of Massachusetts Medical School, Worcester, USA JM 107 was obtained from J. Messing, University of Minnesota, USA. Bacterial growth media were used as described in the text and the various ingredients were obtained from Difco Laboratories.

Preparation of substrate DNA. Bacteriophage lambda (λ) DNA was isolated from GM 1519, lysogen in GM 119. Phages were purified by a series of cesium chloride gradients DNA was isolated by phenol chloroform extraction of purified phages at 10^{13} particles/ml [22] and dialysed against TE buffer (0.01M Tris-HCl pH 7.8, 0.001M EDTA). A typical preparation gave an average yield of 3 mg DNA from 1 litre cells.

M13mp19RFI DNA was prepared from *E.coli* JM107 after infection with Phage M13mp19. From a culture grown to the stationary phase, bacterial cells were separated by centrifugation and M13mp19RFI DNA was extracted by the alkaline extraction method [23].

pBR322 plasmid DNA was prepared by amplifying log phase GM119 culture by the addition of 170 μg/ml chloramphenicol. Extraction of plasmid DNA was achieved by alkaline extraction method [23]. Both pBR322 and M13mp19RFI DNA were purified by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient.

ϕ x 174RFI DNA was prepared by infecting actively growing *E.coli* HF4704 cells with phage ϕ x 174 am3 at an moi of 5 and RFI DNA was isolated and purified on a neutral sucrose gradient as described in a recent publication [24].

Adeno-2 DNA was kindly supplied by Professor R.J. Roberts of Cold Spring Harbor Laboratories, N.Y., USA.

Assay procedure. 1-2 μg of lambda or adeno-2 DNA was digested with 5-10 μl protein in 50 μl assay mixture (0.006M Tris-HCl pH 7.5, 0.006M $MgCl_2$, 0.006M 2-Mercaptoethanol). After incubation for 1 hr at 37°, 8-10 μl of a solution containing 50 % glycerol, 0.5 % SDS, 0.1M EDTA and 0.1 % bromophenol blue was added to the reaction. The enzyme DNA products were resolved by electrophoresis on 1.4 % agarose gel in a buffer (0.04M Tris, 0.005M NaAOC, 0.001M EDTA) containing 0.5 μg/ml ethidium bromide. DNA band patterns were visualized through a short-range UV illuminator [25].

The number of cleavage sites in different DNA substrates was determined by the digestion of 1-2 μg of lambda, adeno-2, pBR322, M13mp19RFI and ϕ x 174RFI DNA with purified enzyme concentrates. DNA fragments were resolved on 1.0 % agarose gel in TBE buffer (0.089M Tris-borate, 0.089M boric acid, 0.002M EDTA) by electrophoresis; the number of sites and band patterns obtained were compared to the computer patterns of already known enzymes obtained from Cold Spring Harbor Laboratory, New York, USA.

One unit of enzyme is defined as the amount of enzyme required for the complete digestion of one μg of λ DNA in one hr. at 37° in 50 μl reaction mixture.

Isolation, purification and characterization of enzymes. Unless otherwise indicated, all operations were performed at 4°.

Growth of cells. Cells were grown under forced aeration at an appropriate temperature in a Microferm laboratory fermenter (New Brunswick Scientific) in 4 litres of a defined medium (Table 1). Cells were harvested by centrifugation, washed with buffer A (0.01M Tris-HCl pH 7.5, 0.01M 2-mercaptoethanol) and stored at -20°.

Preparation of extract. Frozen cells (10 g) were suspended by homogenization in 10 ml of buffer A. The cell suspension was disrupted by sonic irradiation for 1.5-2.0 min. at half power with a Branson sonifier. Cellular debris and high molecular weight DNA were removed by centrifugation at 80,000 g and the salt concentration in the supernatant was raised to IM, by adding solid NaCl (fraction I; 12 ml).

Gel filtration. A column of biogel A 0.5M (4.91cm^2 x 50cm) was prepared and washed with buffer B (1.0M NaCl, 0.01M Tris-HCl pH 7.5, 0.01M 2-mercaptoethanol). Crude extract (Fraction I) was layered on to the column and eluted with buffer B at a flow rate of 0.5ml/min. Seventy 5 ml fractions were collected and every third fraction was assayed for the presence of Type II endonucleolytic activity on lambda DNA by agarose gel electrophoresis as described above (fraction II; 50-60 ml).

Phosphocellulose chromatography. A column of phosphocellulose (0.95cm^2 x 20cm) was equilibrated by

washing with 1 litre of buffer C (0.01M K-PO_4 pH 7.2, 0.01M 2-mercaptoethanol, 0.0001M EDTA, 10% glycerol). Fraction II, after dialysis against ten volumes of buffer C, was applied to the column with an adjusted flow rate of 5 ml/hr and washed with 3 column volume of buffer C. The protein was eluted in 5 ml fractions with a 200 ml linear gradient of 0.01M-1.0M KCl in buffer C. On completion of the gradient, column fractions together with column flow through and wash, were assayed for the presence of Type II endonucleolytic activity on lambda DNA by agarose gel electrophoresis. Most active fractions showing discrete DNA banding pattern were pooled for further purification (fraction III; 40 ml).

DEAE-Cellulose chromatography. A column of DEAE cellulose (0.95 cm^2 x 20 cm) was prepared and equilibrated with buffer D(0.01M K-PO_4 pH 7.6, 0.01M 2-mercaptoethanol, 0.0001M EDTA, 10% glycerol). Fraction III was loaded on to the column with an adjusted flow rate of 8ml/hr and washed with 3 column volumes of the same buffer. Proteins adsorbed to the column were eluted in 5ml fractions with a 200ml linear gradient of 0.01-0.5M KCl in buffer D. (fraction IV; 25ml).

DNA-Cellulose chromatography. DNA cellulose was prepared from cellulose (Munktel No. 410) as described elsewhere (26) and used to yield highly purified enzyme. A column (1.1cm^2 x 9 cm) after equilibration with buffer E (0.1M NaCl, 0.01M Tris-HCl pH 7.5, 0.01M 2-mercaptoethanol, 0.0001M EDTA, 5% glycerol) was used. Fraction IV was loaded at a flow rate of 4ml/hr. and the adsorbed protein was eluted in 2.0ml fractions with 100ml linear gradient of 0.1M-1.0M NaCl in buffer E.

RESULTS AND DISCUSSION

Eight bacterial strains isolated from samples collected from different local environments have been characterized in our laboratory according to standard procedures and entered in our culture collection. Table 1 shows the source of isolated strains, optimum growth temperature, nutritional requirement and the presence or absence of restriction enzymes. Rhizobium strains grow optimally between 25 and 28° whereas the remaining strains have optimum growth temperature of 37°. These strains are preserved in the culture collection laboratory of the centre and are available to the scientific community on request.

Presence of Type II restriction enzyme activities in various fractions eluting from the biogel A 0.5M column was examined by changes in the electrophoretic mobility of lambda DNA after digestion with a suitable amount of the protein. (Table 1). Six strains, namely, *Azospirillum brasilense, Rhizobium trifolii, R. meliloti, R. leguminosarum R. cowpea* and *Pseudomonas stutzeri* PS37, did not show any observable change in the electrophoretic mobility of lambda DNA (unpublished data), whereas two strains namely *Pseudomonas aeruginosa A* and *Citrobacter freundii* A_4 exhibited a discrete DNA band pattern typical of Type II restriction endonucleases.

Chromatographic properties of PaeAI and CfrA_4I. Table 2 summarizes elution properties of the two enzymes during various purification steps. When the purification scheme described under the experimental section was applied to a protein extract of *Pseudomonas aeruginosa* A and *Citrabacter freundii* A_4, endonuclease activities were eluted in fractions 16-18 and 44-54 respectively. Since the elution properties of various proteins through a biogel column depend on their molecular sizes, the present results show that enzyme PaeAI has a molecular size 2-3 fold higher than CfrA_4I. The difference in molecular sizes was further confirmed when precise experiments were carried out to determine the stokes radius and sedimentation properties of these proteins (unpublished data). During ion exchange chromatography, the two enzymes exhibited similar adsorption properties on positively charged DEAE cellulose column eluting between 0.15 to 0.22M KCl concentration. Further, purification of the enzymes was carried out either on DNA cellulose or phosphocellulose. PaeAI, which was purified by DNA cellulose chromatography as a final purification step, required an NaCl concentration of 0.24-0.29M for elution (Table 2). However, CfrA_4I was purified on phosphocellulose to which it binds tenaciously, requiring a salt concentration of 0.37 to 0.42M for elution.

Properties of purified enzymes

Stability. Both enzymes after extensive purification were very stable when stored in 50% glycerol at −20°. no serious losses in enzyme activity were observed over a period of six months.

Purity. The purified enzymes were not contaminated with non-specific exonucleases since the banding pattern did not appreciably change during incubation of a variety of DNA substrates from one-half of an hour to 18 hr. at twenty fold enzyme concentration. Further, there was no loss to back ligation of incised DNA, with phage T-4 polynucleotide ligase.

Requirement. Both enzymes exhibit pH optima between 7 – 7.6 in either Tris-HCl or potassium phosphate buffer. No activity is observed below pH 4 or above pH 10. Both enzymes essentially require magnesium ions for

Table 1. Growth characteristics and source of isolation of bacterial strains used in the present studies

CAMB* Cat. No.	Bacterial strains	Isolate No.	Source	Optimum growth (temp.) °C	Growth medium	Type II enzyme
7	*Azospirillum brasilense*	–	Marine water	37°	LB ¶	–
2537	*Citrobacter freundii*	A4	"	"	"	+
3001	*Rhizobium* cowpea	–	"	28°	YMA §	–
3004	*R. leguminosarum*	–	"	"	"	–
3006	*R. meliloti*	–	"	"	"	–
3012	*R. trifolii*	–	"	"	"	–
2549	*Pseudomonas aeruginosa*	AI	Pet. Pump Sludge	37°	LB	+
2534	*P. stutzeri*	PS37	"	"	"	–

* The strains are available to the scientific community under these code numbers.

¶ Luria-Bertani medium.

§ Yeast Mannitol Agar.

Table 2. Purification of enzymes.

Strain	Enzyme	Columns used	Eluent	Enzyme eluted at
Pseudomonas aeruginosa A	PaeAI	Biogel A 0.5M	Buffer B	–
		DEAE cellulose	0.01-0.5M KCI in buffer C	0.15-0.2M
		DNA cellulose	0.01-1.0M NaCI in buffer E	0.24M-0.29M
Citrobacter freundii A_4	$CfrA_4I$	Biogel A 0.5M	Buffer B	
		DEAE cellulose	0.01-1.0M KCI in buffer D	0.15-0.22M
		Phosphocellulose	0.01-1.0M KCI in buffer C	0.37-0.42M

normal levels of activity which completely disappears in its absence.

Incision characteristics of enzymes. Fig. 1 presents incision patterns of PaeAI on a variety of DNA substrates. It appears that the enzyme cleaves at four unique sites on λ DNA, 33 sites on adeno-2 DNA, a single site on ϕx174RFI DNA and does not cleave pBR322 and M13mp19RFI DNA. When the incision pattern of the isolated enzyme is compared with those available in the literature, there is a clear resemblance with the incision properties of SacII. This was further confirmed by double digest analysis, PaeAI produced no extra bands with different DNA substrates in the presence of SacII (Fig. 2).

The band pattern of lambda, adeno-2, pBR322, M13mp19RFI and ϕx174RFI DNAs digested with $CfrA_4I$ are shown in Fig. 3. It appears that lambda and adeno-2 DNA contain 28 and 30 cleavage sites respectively, whereas pBR322, M13mp19RFI and ϕx174RFI DNA are cleaved at a single unique site. These incision properties of $CfrA_4I$ resemble the DNA banding pattern of the PstI enzyme. When $CfrA_4I$ and PstI were used individually or together they resulted in the same band pattern on different DNA

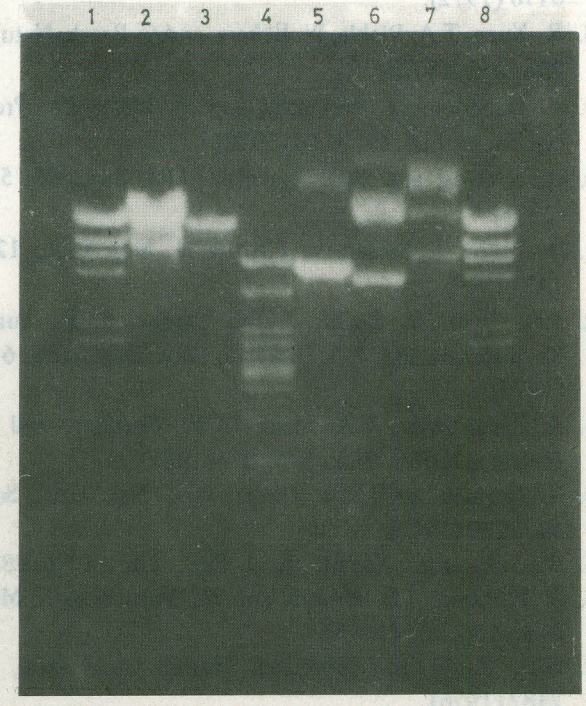

Fig. 1. Characterization of PaeAI using different DNA substrates. Lanes 2 and 3 contain λ DNA whereas Lanes 4,5,6 and 7 contain Ad_2, ϕx174RFI, pBR322 and M13mp19RFI DNAs digested with PaeAI respectively. Lanes 1 and 8 contain Digests of λ DNA with Hind III, used as marker.

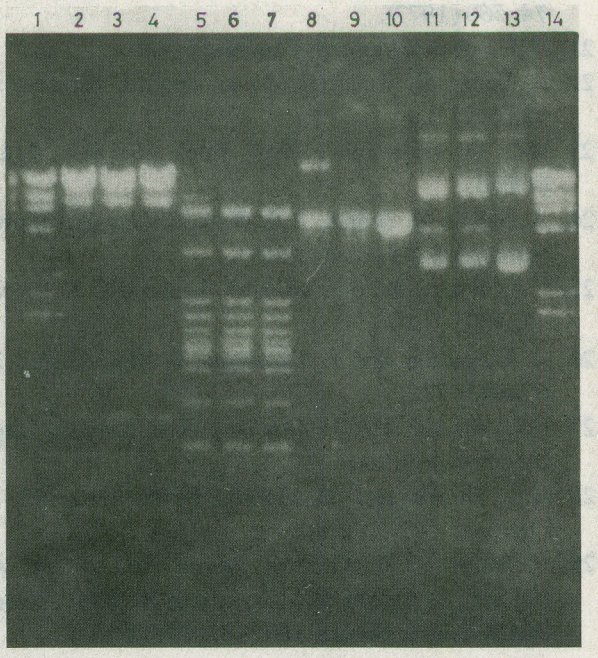

Fig. 2. Characterization of PaeAI by double digestions. Lanes 1 and 14 contain digests of DNA with HindIII. Lane 2,5,8 and 11 contain λ, Ad_2, ϕ174RFI and pBR322 DNA digested with PaeAI respectively. Lanes 4,7,10 and 13 contain λ, Ad_2, ϕx174 and pBR322 DNA digested with SacII respectively. Lanes 3,6,9 and 12 contain double digests of λ, Ad_2, ϕx174RFII and pBR322 DNA with PaeAI and SacII.

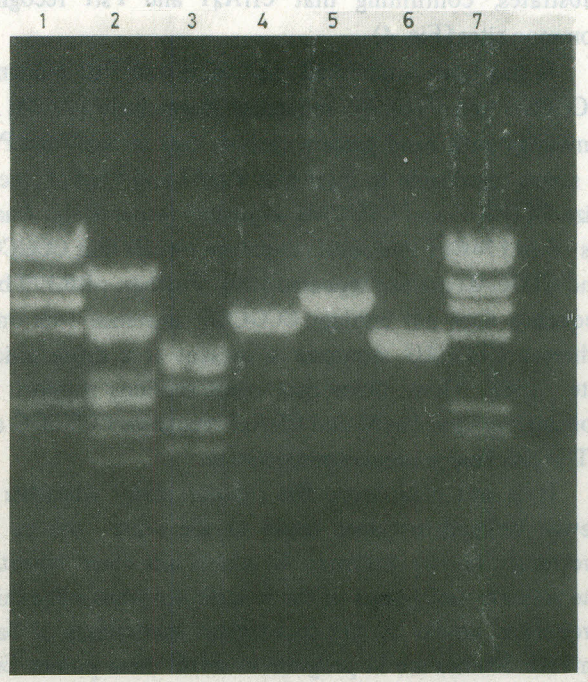

Fig 3. Characterization of $CfrA_4I$ using various DNAs. Lanes 2,3,4,5 and 6 contain λ, Ad_2, ϕ174RFI, M13mp19RFI and pBR322 DNA digested with $CfrA_4I$ respectively. Lanes 1 and 7 are digests of DNA with HindIII.

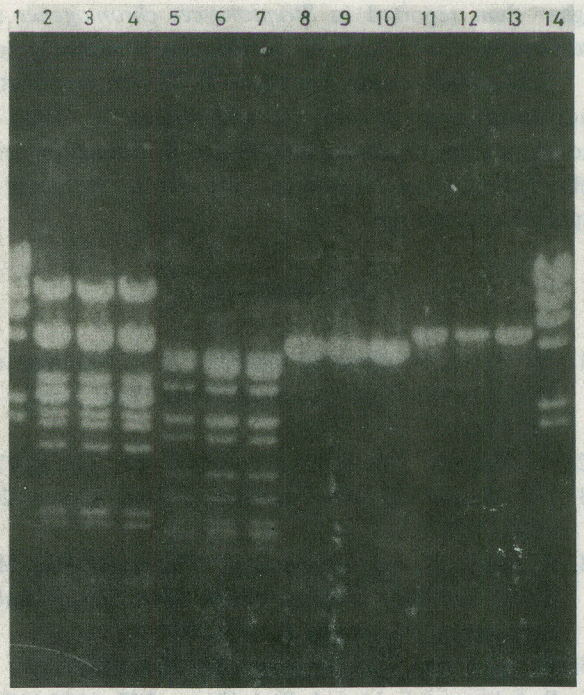

Fig. 4. Characterization of $CfrA_4I$ by double digestions. Lanes 1 and 14 are digests of DNA with HindIII. Lanes 2,5,8 and 11 contain digests of λ, Ad_2, pBR322 and ϕx174RFI DNA with CcII respectively. Lanes 4,7,10 and 13 are double digests of λ, Ad_2 pBR322 and ϕ174RFI DNA with PstI respectively. Lanes 3,6,9 and 12 are double digests of λ, Ad_2, pBR322 and ϕx174RFII DNA with $CfrA_4I$ and PstI.

substrates, confirming that $CfrA_4I$ and PstI recognize common sites (Fig. 4).

It has been established that SacII and PstI recognize CCGCGG and CTGCAG sequences respectively [27,28]. In similarity with band pattern analysis data of SacII and PstI, it seems reasonable to infer that PaeAI and $CfrA_4I$ (recognize CCGCGG and CTGCAG sequences respectively. Analysis of ϕx174RFI nucleotide sequence reveals that CCGCGG and CTGCAG are not repeated [29]. Further, pBR322 base sequence does not contain CCGCGG sequence even once, whereas CTGCAG sequence is present at a single unique site [30]. These theoretical considerations support the conclusions that PaeAI and $CfrA_4I$ recognize CCGCGG and CTGCAG base sequences respectively.

It is not clear from the present study wheather the newly isolated enzymes incise at the same site as the previously known enzymes. It is also not clear wheather the cognate methylases of the isolated enzymes will exhibit properties similar to the counterpart methylases of SacII an PstI. Studies are in progress to settle these points. Even if the newly isolated enzymes show exactly the same recognition sequence and cleavage site, these enzymes have important uses in the recombinant DNA work because the levels of PaeAI and $CfrA_4I$ in the new host is 10-20 fold higher than reported in *Streptomyces chromogenes* and *Providencia stuartii* and the purification is relatively easy.

Acknowledgements. We thank F. Jabeen for isolation and identification of local bacterial strains, M. Amjad for the preparation of substrate DNA and Z. Ahmed for many helpful discussions. Thanks are also due to A. Sattar for typing the manuscript.

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