Characterization of DNA binding activities of over-expressed *Kpn*I restriction endonuclease and modification methylase

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The genes encoding the *Kpn*I restriction endonuclease and methyltransferase from *Klebsiella pneumoniae* have been cloned and expressed in *Escherchia coli* using a two plasmid strategy. The gene for *Kpn*I methylase with its promoter was cloned and expressed in pACYC184. Even though the methylase clone is in a low copy number plasmid pACMK, high level expression of methylase is achieved. A hyper-expressing clone of *Kpn*I endonuclease, pETRK was engineered by cloning the R gene into the T7 expression system. This strategy resulted in over-expression of *Kpn*I endonuclease to about 15–30% of cellular protein. Both the enzymes were purified using a single chromatographic step to apparent homogeneity. The yield of purified endonuclease and methylase from one liter of culture was approximately 30 and 6 mg respectively. Electrophoretic mobility shift assays show that both the enzymes are capable of binding to specific recognition sequence in the absence of any cofactors. The complexes of *Kpn*I methyl transferase and endonuclease with their cognate site exhibit distinctive behaviour with respect to ionic requirement.

1. Introduction

The primary function of restriction–modification (R–M) systems in bacteria is to serve as defense mechanisms against intruding DNA molecules (Linn and Arber 1968). R–M systems are composed of two separate enzymatic activities. One is a restriction endonuclease (Enase) that cleaves DNA at a specific recognition sequence. The second is a DNA methyltransferase (Mtase), which is able to methylate the same sequence and render it refractive to cleavage by the corresponding Enase. The Enases are defined as double-strand nucleases that recognize specific DNA sequences and cleave at a defined point within or close to that sequence. These enzymes are classified into three main groups according to their cofactor requirements and complexity of the reaction (Roberts and Halford 1993). The type II Enases require only Mg^{2+} as a cofactor and by far the simplest ones. Most of the enzymes of this group recognize palindromic sequence which generally vary between four to eight base pairs in length (Wilson and Murray 1991). There has been a great degree of interest to isolate and characterize new type II Enases due to their wide usage in recombinant DNA

technology (Roberts and Macelis 1997). A number of R–M systems have been cloned, sequenced and hyper-expressed primarily because of their application potential and commercial benefits. The cloning methods are generally based on bacteriophage infection (Mann *et al* 1978; Walder *et al* 1981), Mtase selection (Kiss *et al* 1985), induction of the DNA-damage inducible SOS response by the *mcr* and *mrr* systems, in the presence of methylated DNA (Piekarowicz *et al* 1991) and transferring the plasmid encoding R–M genes into *Escherichia coli* cloning vectors (Bougueleret *et al* 1984; Gingeras and Brooks 1983; Blumenthal *et al* 1985). Although recognition sequences for a large number of Enases are known, less information is available on their structure–function relationship and biochemical properties. By virtue of their remarkable sequence specificity, they also serve as very good model systems for studying protein–DNA interactions.

Sequence comparison of R genes has revealed surprising results. Very little sequence conservation is observed amongst Enases except few residues involved in catalysis. In contrast, distinct motifs are found representing characteristic patterns of different sub-classes viz., N^6 adenine, N^4 cytosine or C^5 cytosine Mtases (Posfai *et al* 1989; Kumar *et al* 1994). Another noteworthy feature is the absence of any sequence similarity between Mtases and cognate Enases. Thus, Enases and the corresponding Mtases constitute a unique class of DNA binding proteins which recognize same sequence, yet catalyze totally different type of enzymatic reactions. Delineation of molecular interaction parameters of the two non-homologous proteins which recognize same sequence would constitute an important study in the area of nucleic acid-protein interactions. In this direction, we have over-expressed *Kpn*I Enase and Mtase genes and studied the DNA binding properties of the purified enzymes.

2. Materials and methods

2.1 *Bacterial strains and plasmids*

Klebsiella pneumoniae strain (ATCC 4970) was used for cloning *Kpn*I R–M system. *E.coli* DH10B [*mcr*AD (*mrr hsd* RMS *mcr*BC) *end*A1 f 80 *dlac*Z D M15 D *lac* X74 *rec*A1 *deo*RD *(ara, leu)* 7697*ara* D139 *gal*U *gal*K *nup*G *rps*L] and plasmids pACYC184 (cam^R + tet^R) (Chang and Cohen 1978) and pTrc99C (amp^R) (Amann *et al* 1988) were from our laboratory collection. pET11d was obtained from Novagen. *K. pneumoniae* and other *E. coli* strains were grown in Luria Bertani (LB) medium.

2.2 *Enzymes and chemicals*

Restriction enzymes, T4 DNA ligase, Klenow polymerase, *Taq* DNA polymerase, T4 polynucleotide kinase, oligonucleotides, deoxynucleotidetriphosphates and IPTG were obtained from Bangalore Genei Pvt. Ltd. Shrimp alkaline

phosphatase, [methyl-^{3}H]-S-adenosyl-L-methionine ([H^{3}] AdoMet) and [g -^{32}P]ATP were purchased from Amersham.

2.3 *DNA preparation*

Total chromosomal DNA was isolated from *K. pneumoniae* cells by SDS-proteinase K lysis followed by phenol–chloroform extraction (Ausubel *et al* 1987). Small and large scale plasmid isolations were done by alkaline-lysis method (Sambrook *et al* 1993).

2.4 *PCR amplification of Mtase and Enase genes*

All PCR reactions were performed using GeneAmp PCR System (Perkin Elmer). *kpniM* (*Kpn*I methylase) gene was amplified as a 1.3 kb fragment using a forward primer which has a *Stu*I site and a reverse primer which carries a *Hin*dIII site (table 1, primers 1 and 2). The reaction mixture contained 10 mM TAPS (3-tris[hydroxy methyl] aminopropane sulphonic acid) pH 8·8, 50 mM potassium chloride, 1·5 mM magnesium chloride, 0·01% gelatin, 200 m M deoxynucleotidetriphosphates, 1·5 units of *Taq* DNA polymerase, 100 ng of *K. pneumoniae* genomic DNA and 0·25 m g each of the forward and reverse primers. *kpniR* (*Kpn*I endonuclease) gene was amplified as a 0·7 kb fragment using two primers which carry *Nco*I and *Bam*HI sites (table 1, primers 3 and 4).

2.5 *Expression of KpnI Enase and Mtase*

E. coli cells harbouring the Mtase clone pACMK were grown for 12–16 h at 37° C and harvested by centrifugation for 10 min at 10,000 *g*. *E. coli* containing cloned *kpniR* gene in the presence of pACMK was grown in LB medium containing 100 m g/ml ampicillin and 20 m g/ml chloramphenicol at 37° C until the absorbance at 600 nm reached 0·6. Then the cells were induced with 1 mM IPTG (0·3 mM IPTG in the case of pETRK clone) and harvested after 3 h incubation at 37° C by centrifugation for 10 min at 10,000 *g*. Protein samples were analysed by SDS-PAGE as described by Laemmli (1970).

2.6 *Detection of Mtase activity*

The chromosomal DNA was isolated and purified from *E. coli* carrying the plasmid for Mtase gene. One m g of the chromosomal DNA was incubated with excess of *Kpn*I Enase for 2 h at 37° C to check the cleavage. The enzyme was also assayed by transfer of methyl group from AdoMet to the substrate DNA. The assay reaction contained 50 mM Tris-HCl (pH 7·4), 5 mM EDTA, 5 mM b -mercaptoethanol, 100 m g/ml BSA, 1·5 m g of l DNA, 0·1 m Ci of [methyl-^{3}H] AdoMet (sp.activity, 263 Beq/ mmol) and different amounts of enzyme in a 20 m l reaction mixture. The reactants were incubated at 37° C for 30 min and 5 m l of the mixture was taken onto a GF/C filter, dried, washed with 10% TCA and acid precipitable counts measured using liquid scintillation counter.

2.7 *Detection of Enase activity in vitro*

An induced culture (100 ml) was prepared and the cells were harvested by centrifugation for 10 min at 10,000 *g*. The pellet was resuspended in 10 ml of 10 mM Tris-HCl (pH 7·4), 0·1 mM EDTA, 7 mM b -mercaptoethanol and 2 mM phenyl methyl sulphonyl fluoride (PMSF) and lysed by sonication for 5 min. A crude extract was prepared by centrifugation of the sonicated sample for 30 min at 20,000. Different concentrations of the extract were incubated with 1 m g of l DNA in 50 m l reaction mixture containing 10 mM Tris-HCl (pH 7·4), 10 mM magnesium chloride and 5 mM b -mercaptoethanol at 37° C for 1 h. The reactions were terminated by adding 5 m l stop buffer [20% Ficoll, 50 mM EDTA, bromophenol blue (0·1%) xylene cyanol (0·1%)] and then resolved by electrophoresis for 1 to 2 h at 100 V on 0·8% agarose gels in 40 mM Tris-acetate (pH 7·8), 1 mM EDTA, 0·5 m g/ml ethidium bromide. The unit for the enzyme activity was estimated by incubating various amounts of enzyme with 1 m g of l DNA under the standard assay conditions. One unit of the *Kpn*I Enase is defined as the amount of enzyme required to digest 1 m g of l DNA.

2.8 *Purification of KpnI Enase and Mtase*

For the purification of Enase, 4 g of cells were resuspended in 15 ml of buffer A [10 mM potassium phosphate (pH 7·0), 1 mM EDTA, 7 mM b -mercaptoethanol] with 2 mM PMSF. Cells were lysed by sonication for 30 min and centrifuged at 100,000 *g* for 2 h. The supernatant was treated with 1% polyethyleneimine (PEI) in the presence of 250 mM KCl. The sample was centrifuged at 20,000 *g* for 15 min and the supernatant was subjected from 0 to 50% ammonium sulphate fractionation. The pellet was dissolved in 5 ml of buffer A and dialyzed against buffer A. The dialyzed sample was loaded onto a 5 ml Hi-Trap heparin column and the enzyme was eluted with buffer A containing 0–0·8 M KCl. The fractions containing the enzyme (between 200–350 mM KCl) were pooled and dialyzed against buffer B [10 mM Tris-HCl (pH 7·4), 0·1 mM EDTA, 50 mM KCl, 5 mM b -mercaptoethanol and 50% glycerol]. The protein estimation was done by dye binding method using bovine serum albumin as standard (Bradford 1976).

The *Kpn*I Mtase was purified from 10 g of overnight grown cells. The cells were sonicated with 20 ml of buffer A containing 2 mM PMSF and centrifuged at 100,000 *g* for 2 h. The crude extract was treated with 1% PEI in the presence of 250 mM KCl. The sample was centrifuged and the supernatant was subjected to 0–30% ammonium sulphate fractionation. The pellet was dissolved in a 5 ml of buffer A, dialyzed and was loaded onto a 5 ml heparin column and eluted with 0–1 M KCl. The fractions containing the enzyme (between 0·65–0·9 M KCl) were pooled and dialyzed against buffer B [10 mM Tris-HCl (pH 7·4), 0.1 mM EDTA, 50 mM KCl, 5 mM b -mercaptoethanol and 50% glycerol].

2.9 *Electrophoretic mobility shift assay*

The substrate used for DNA binding assays was a 38 mer double stranded oligonucleotide which contains *Kpn*I site at the middle (table 1, primers 5 and 6). One of the strands was labelled using T4 polynucleotide kinase and [g -^{32}P] ATP (sp. activity, 5000 Ci/mmol). The two strands were annealed by heating the sample to 80° C and then cooling to room temperature slowly. The protein-DNA complexes were formed by incubation of the enzyme with 10 nM labelled double stranded oligonucleotide containing Tris-HCl (pH 7·4), 5 mM EDTA, 7 mM 2-mercaptoethanol and different concentrations of either *Kpn*I Mtase or Enase (2 nM–500 nM). The reaction mixture was incubated at room temperature for 15 min followed by the addition of 2 m l of stop buffer [20% Ficoll, 50 mM EDTA, bromophenol blue (0·1%) xylene cyanol (0·1%)]. The samples were then loaded on to 6% (or 8%) native acrylamide gel (30 : 0·8) and electrophoresed using 1 × TBE (pH 8·3) or with 50 mM HEPES (pH 7·4) and 2 mM EDTA at a constant voltage of 10 V/cm for 1 h at 4° C. The complexes were detected by autoradiography of the dried gel.

3. Results

3.1 *Organization of regulatory elements in KpnI R–M systems*

The genes for *Kpn*I Enase and Mtase are present within the close proximity to each other in *K. pneumoniae* genome (Chatterjee *et al* 1991). *Kpn*I Enase recogni-zes and cleaves the DNA sequence 5¢ –GGTAC‾ C–3¢ (Hammond *et al* 1990). The *Kpn*I Mtase recognizes the same sequence and methylates adenine residue at N^6 position (Kiss *et al* 1991). Like other R–M systems, the *Kpn*I Enase and Mtase open reading frames (ORFs) do not show any considerable sequence similarity inspite of recognizing the same sequence element. The gene organization and promoter elements for *Kpn*I R–M system is depicted in the figure 1. The R and M genes are arranged divergently and separated by 167 bp. The intergenic region contains all the regulatory elements required for the expression of both the genes. The promoters are separated by 57 bp and present in opposite strands having typical characteristic features of s -70 promoters of *E. coli* (Hawley and McClure 1983; Nagaraja 1993). Moreover codon usage pattern of both Mtase and Enase ORFs is similar to that of *E. coli*. These characteristics suggest that expressing functional proteins of *Kpn*I R–M system in *E. coli* is feasible.

3.2 *Strategy employed for cloning*

Further analysis of promoter elements of *Kpn*I Enase and Mtase (figure 1) suggest that their relative strengths could vary to a significant extent. The Mtase promoter appeared to be stronger having six out of six nucleotide consensus at –

10 sequence (5¢ –TATAAT–3¢) and four out of six to the – 35 consensus sequence (5¢ –TTGACA–3¢). In contrast, only three and four nucleotides out of six consensus sequence found in – 10 and – 35 elements respectively in the case of *Kpn*I Enase (figure 1). Thus, the strategy for cloning the genes involved, prior cloning of the Mtase gene with its own promoter sequences in a low copy number plasmid. This would allow sufficient over-expression of Mtase to ensure complete protection of the genomic DNA when gene for cognate Enase is cloned into the strain containing the cloned Mtase gene. The *kpniR* gene cloning however, would be as a promoter-less fragment downstream of a strong *trc* or T7 promoter to ensure very high level expression.

3.3 *KpnI Mtase hyper-expression and purification*

The *kpniM* gene amplified with its own promoter elements was cloned into pACYC184 (figure 2A). The introduction of the gene between *Eco*RV and *Hin*dIII sites results in the inactivation of *tet* gene and also constitutive expression of the Mtase. The expression of functional Mtase is assayed by the resistance to *Kpn*I cleavage of the DNA isolated from the cells harbouring Mtase plasmid. The enzyme activity was directly assayed by transfer of methyl group to substrate DNA using [^{3}H]-labelled AdoMet (§ 2). The expression of Mtase was monitored by analysing the protein samples in SDS-PAGE at different time intervals during the growth (not shown). Over-night cultures (12–16 h) of the pACMK containing cells showed considerable over-production of the Mtase (figure 2B). The over-expressed *Kpn*I Mtase has relative molecular mass of 44 kDa corresponding the size derived from its ORF. Using the pACMK clone, the *Kpn*I Mtase was purified by facile purification procedure involving single chromatography step (§ 2, figure 2C). From one liter of the culture, 6 mg of purified protein was obtained.

3.4 *KpnI Enase hyper-expression and purification*

In contrast to *Kpn*I methylase cloning, Enase gene was cloned as a promoter-less fragment into two separate expression vectors. The cloning strategy of the PCR product into pTrc99C and pET11d is shown in figure 3A. While both the systems are inducible by IPTG, the former is driven by *trc*- a *trp-lac* hybrid promoter (Amann *et al* 1988) while latter is driven by T7 RNA polymerase-T7 promoter system (Studier and Moffatt 1986). Although the inducible *trc* promoter is expected to produce large amounts of protein, the pTrcRK construct did not show high level of expression (see discussion). The total *Kpn*I Enase activity in this clone was about 10^7 units per 1 litre culture which is only 20 times more than that obtained from the wild type strain. This prompted us to pursue the alternate approach of using T7 expression system to obtain high levels of expression. The cloned genes from this expression system are expressed from a strong T7 promoter. Further, the presence of *lac*I gene ensures efficient repression of the basal transcription levels. The time course of IPTG induction using pETRK clone is shown in figure 3B . Most of the protein was expressed within 2–3 h after the induction. The protein has approximate molecular mass of 32 kDa. Analysis of the protein profile from the pellet and supernatant fractions of the lysed cells showed that the most of the protein was in the latter fraction (not shown). The enzyme has been purified to near homogeneity using heparin sepharose chromatography (figure 3C). The total activity estimated from this clone was at least 2×10^8 units per 1 litre culture which approximately corresponds to the specific activity > 2×10^6 units/mg with l DNA as a substrate. The specific activity is comparable to that of other type II Enases having hexameric recognition sequences (Maxwell and Halford 1982; Theriault *et al* 1985; Brooks *et al* 1989).

3.5 *Analysis of enzyme-DNA complexes*

The purified *Kpn*I Enase and Mtase were used to probe their interaction with specific recognition sequence in
electrophoretic mobility shift assays. For this purpose a 38 mer duplex oligonucleotide was designed having 5¢ –GGTACC–3¢ sequence at the centre. A single *Kpn*I Enase enzyme-DNA complex is detected when the enzyme is incubated with substrate oligonucleotide (figure 4A); the protein-DNA complex was not observed when the protein was incubated with 38 mer duplex DNA which does not contain the *Kpn*I site (not shown) indicating that the complex was specific to *Kpn*I Enase. The enzyme-DNA complex was formed over a broad pH range of 6·5, 7·4 and 8·3 (not shown). The effect of monovalent cation, such as Na^+ was studied on the complex formation. The interaction was salt sensitive as complex formation was observed only between 0 to 75 mM NaCl concentrations. Beyond this concentration of NaCl, detectable enzyme-DNA complex was not observed (figure 4B). The binding was maximum in the absence of salt or very low NaCl concentrations. This is in agreement with cleavage characteristics of the enzyme; maximum cleavage activity was obtained with buffers having low ionic strength. Addition of 5 mM Mg^{2+} to DNA-protein complex produced dramatic

change in the interaction pattern. Instead of the slower moving complex (figure 4C, panel B, lane 1), a faster moving cleavage product (lane 2 and 3) appears, as Mg^{2+} is a cofactor for the enzymatic reaction. The enzyme cleaves the DNA in the presence of Mg^{2+} even at 4° C (figure 4B, panel B, lane 2). These results taken together reflect high degree of specific interaction of the enzyme with the substrate prior to the addition of cofactor.

Incubation of *Kpn*I Mtase with the same 38 mer substrate DNA leads to the formation of DNA-protein complex (figure 5). In contrast to Enase-DNA complex, the Mtase-DNA complex is resistant to different concentrations of monovalent cation; complex was detected at 300 mM NaCl concentration (figure 5, lane 8). DNA-protein complex was observed in absence of S-adenosine homocysteine (AdoHcy) unlike many other Mtases
(figure 5, lane 1). The presence of AdoHcy did not influence the complex formation to a great extent (figure 5, lane 3).

4. Discussion

In this paper, we describe the cloning and over-expression of R–M system from *K. pneumoniae*. Further we show that DNA binding properties of the purified proteins exhibit certain distinctive characteristics.

One major objective of this study was to express large amounts of *Kpn*I Enase and its cognate Mtase for detailed biochemical and structural analysis. Thus, the strategy involved cloning of *kpniR* gene from the genomic DNA into suitable expression vectors and transform into cells which were already harbouring the Mtase plasmid. Prior expression of Mtase would ensure stable maintenance and expression of R gene. Although Mtase was cloned into a low copy number vector, high level expression was achieved. This is possibly due to the presence of promoter elements bearing resemblance to strong promoters and perfect Shine-Dalgarno sequence apart from other features appropriate for over-expression in *E. coli* (Makrides 1996). Further, there seems to be continued accumulation of the enzyme when the cells were grown over long periods of time. The hyper-expression and the convenient purification of functional Mtase would facilitate the purification of the enzyme in large scale for structural studies. Inspite of high level expression, the *Kpn*I Enase is completely soluble. This is some what unexpected as high level expression in *E. coli* using T7 expression system often leads to inclusion bodies which is a frequently encountered problem. One possible explanation for high solubility of *Kpn*I Enase is similar codon usage in both *K. pneumoniae* and *E. coli,* thus minimizing the mistranslation products. The hyper-expression of *Kpn*I Enase would facilitate the purification of the protein in large quantities. This would enable studies on structure function relationships, solution structure analysis and detailed protein-DNA interaction studies. Several major efforts are underway to determine the

solution structures of Enases. The structural information is available for *Eco*RI (Kim *et al* 1990), *Eco*RV (Winkler *et al* 1993; Winkler 1992), *Bam*HI (Newman *et al* 1994), *Pvu*II (Cheng *et al* 1994), *Cfr*10I (Bozic *et al* 1996), *Fok*I (Wah *et al* 1997) and *Bgl*I (Newman *et al* 1998). While these enzymes have distinct structures, a notable common feature seems to be the organization of conserved catalytic sequence motifs (PD and D/EXK-) at the reaction centre (Stahl *et al* 1998).

As a prelude to detailed structural analysis, we have assessed the complex formation of *Kpn*I Mtase and Enase with DNA substrate having the recognition sequence. *Kpn*I Enase forms stable complex with 38 mer specific duplex oligonucleotide in the absence any cofactor. In the presence of magnesium there was a cleavage product seen even at 4° C. The absence of complex formation with a non-specific 38 mer duplex oligonucleotide indicates that the enzyme readily discriminates between specific and non-specific sites unlike in the case of *Eco*RV, *Taq*I and *Cfr*9I where the specificity is brought about by magnesium ion (Taylor *et al* 1991; Zebala *et al* 1992; Siksnys and Pieckkaityte 1993). *Kpn*I Enase forms specific complex at broad pH values 6·5, 7·4 and 8·3 (not shown). In contrast, *Mun*I Enase does not exhibit specific binding at pH 8·5, but shows considerable sequence specificity at pH 6·5 (Lagunavicius *et al* 1997). *Eco*RV also exhibits pH dependent variation in sequence specific interaction at its recognition sequence (Engler *et al* 1997). In the case of Mtases, addition of cofactor or competitive inhibitor to the binding reaction is known to facilitate the formation of specific enzyme-DNA complex (Dubey and Roberts 1992; Chen *et al* 1991; Wyszynski *et al* 1993). *Kpn*I Mtase, however, forms specific complex in the absence AdoHcy (figure 5). A notable feature is the formation of *Kpn*I Mtase-DNA complex even at high concentrations of monovalent cations. In contrast, ionic interactions do not seem to contribute in a significant manner for the formation of specific DNA-Enase complex. Thus, the complexes of these enzymes with DNA respond in very different manner to the ionic environment. If these contrasting features in DNA recognition by the two enzymes could be considered as pointers of their differential interaction, a detailed analysis should unravel the molecular secrets of specificity determination in target recognition.

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References

Amann E, Ochs B and Abel K J 1988 Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*; *Gene* 69 301–315

Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A and Struhl K 1987 *Current protocols in molecular biology* (New York: John Wiley)

Blumenthal R M, Gregory S A and Cooperider J S 1985 Cloning of a restriction-modification system from *Proteus vulgaris* and its use in analyzing a methyl-sensitive phenotype in *Escherichia coli; J. Bacteriol*. 164 501–509

Bougueleret L, Schwarzstein M, Tsugita A and Zabeau M 1984 Characterization of the genes coding for the *Eco*RV restriction and modification system *of Escherichia coli*; *Nucleic Acids Res*. 12 3659–3676

Bozic D, Grazulis S, Siksnys V and Huber R 1996 Crystal structure of *Citrobacter freundii* restriction endonuclease Cfr10I at 2·15 Å resolution; *J. Mol. Biol.* 255 176–186

Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding; *Anal. Biochem.* 72 248–254

Brooks J E, Benner J S, Heiter D F, Silber K R, Sznyter L A, Jager-Quinton T, Moran L S, Slatko B E, Wilson G G and Nwankwo D O 1989 Cloning the *Bam*HI restriction modification system; *Nucleic Acids Res*. 17 979–997

Chang A C Y and Cohen S N 1978 Construction and characterization of amplifiable Multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid; *J. Bacteriol.* 134 1141–1156

Chen L, MacMillan A M, Chang W, Ezaz-Nikpay K, Lane W S and Verdine G L 1991 Direct identification of the active-site nucleophile in a DNA (Cytosine-5)-methyltransferase; *Biochemistry* 30 11018–11025

Cheng X , Balendiran K, Schildkraut I and Anderson J E 1994 Structure of *Pvu*II endonuclease with cognate DNA; *EMBO J.* 13 3927–3935

Chatterjee D K, Hammond A W, Blakesley R W, Adams S M and Gerard G F 1991 Genetic organization of *Kpn*I restriction and modification system; *Nucleic Acids Res.* 19 6505–6509

Dubey A K and Roberts R J 1992 Sequence specific DNA binding by the *Msp*I methyltransferase; *Nucleic Acids Res.* 20 3167–3173

Gingeras T R and Brooks J E 1983 Cloned restriction/ modification system from *Pseudomonas aeroginosa*; *Proc. Natl. Acad. Sci. USA* 80 402–406

Engler L E, Welch K K and Jen-Jocobson L 1997 Specific binding by *Eco*RV endonuclease to its cognate recognition site GATATC; *J. Mol. Biol.* 269 82–101

Hammond A W, Gerard G F and Chatterjee D K 1990 Cloning the *Kpn*I restriction-modification system in *Escherichia coli*; *Gene* 97 97–102

Hawley D K and McClure W R 1983 Compilation and analysis of *Escherichia coli* promoter sequences; *Nucleic Acids Res*. 11 2237–2255

Kim Y, Grable J C, Choi P J, Greene P and Rosenberg J M 1990 Refinement of *Eco*RI endonuclease crystal structure: a revised protein chain chasing; *Science* 249 1307–1309

Kiss A, Kinta C and Venetianer P 1991 M.*Kpn*I is an adenine-methyltransferase; *Nucleic Acids Res*. 19 3460

Kiss A, Posfai G, Keller C C, Venetianer P and Roberts R J 1985 Nucleotide sequence of *Bsu*RI restriction-modification system; *Nucleic Acids Res*. 13 6403–6421

Kumar S, Cheng X, Klimasauskas S, Mi S, Posfai J, Roberts R J and Wilson G G 1994 The DNA (cytosine-5) methyltransferases; *Nucleic Acids Res*. 22 1–10

Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4; *Nature (London)* 227 680–685

Lagunavicius A, Grajulis S, Balciunaite E, Vainius D and Siksnys V 1997 DNA binding specificity of *Mun*I restriction endonuclease is controlled by pH and calcium ions: involvement of active site carboxylate residues; *Biochemistry* 37 11093–11099

Linn S and Arber W 1968 Host specificity of DNA produced by *Escherichia coli*, X. *In vitro* restriction of phage FD replicative form*; Proc. Natl. Acad. Sci. USA* 59 1300–1309

Makrides S C 1996 Strategies for achieving high-level expression of genes in *Escherichia coli*; *Microbiol. Rev.* 60 512–538

Mann M B, Rao R N and Smith H O 1978 Cloning of restriction and modification genes in *Escherichia coli*. The *Hha*II system from *Haemophilus haemolyticus; Gene* 3 97– 112

Maxwell A and Halford S E 1982 The *Sal*GI restriction endonuclease. Purification and properties; *Biochem. J*. 23 77–84

Nagaraja V 1993 Control of transcription initiation; *J. Biosci.* 18 13–25

Newman M, Strzeleckka T, Dorner L F, Schildkraut I and Aggarwal A K 1994 Structure of *Bam*HI endonuclease bound to DNA: partial folding and unfolding on DNA binding; *Science* 269 656–663

Newman M, Lunnen K, Wilson G, Greci J, Schildkraut I and Philips S E 1998 Crystal sturucture of restriction endonuclease *Bgl*I bound to its inerrupted DNA recognition sequence; *EMBO J.* 17 5466–5476

Piekarowicz A, Yuan R and Stein D C 1991 A new method for the rapid identification of genes encoding restriction and modification enzymes; *Nucleic Acids Res*. 19 1831–1835

Posfai J, Bhagwat A S, Posfai G and Roberts R J 1989 Predictive motifs derived from cytosine methyltransferases; *Nucleic Acids Res.* 17 2421–2435

Roberts R J and Halford S E 1993 Type II restriction enzymes; in *Nucleases* (eds) S M Linn, R S Lloyd and R J Roberts (New York: Cold Spring Harbor Laboratory Press) pp 35–88

Roberts R J and Macelis D 1997 REBASE-restriction enzymes and methylases; *Nucleic Acids Res*. 25 248–262

Sambrook J, Fritsch E F and Maniatis T 1993 *Molecular cloning*. *A laboratory manual* 2nd edition (NewYork: Cold Spring Harbor Laboratory Press)

Siksnys V and Pieckkaityte M 1993 Catalytic and binding properties of restriction endonuclease *Cfr*9I; *Eur. J. Biochem.* 217 411–419

Stahl F, Wende W, Jeltsch A and Pingoud A 1998 The mechanism of DNA cleavage by the type II restriction enzyme *EcoRV*: Asp36 is not directly involved in DNA cleavage but serves to couple indirect readout to catalysis; *Biol. Chem.* 379 467– 473

Studier F W and Moffatt B A 1986 Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes; *J. Mol. Biol*. 189 113–130

Theriault G, Roy P H, Howard K A, Benner J S, Brooks J E, Waters A F and Gingeras T R 1985 Nucleotide sequence of the *Pae*R7 restriction modification system and partial characterization of its protein products; *Nucleic Acids Res*. 13 8441–8461

Taylor J D, Badcoe I G, Clarke A R and Halford S E 1991 *Eco*RV restriction endonuclease binds all DNA sequences with equal affinity; *Biochemistry* 30 8743–8753

Wah D A, Hirsch J A, Dorner L F, Schildkraut I and Aggarwal A K 1997 Structure of the multimodular endonuclease *Fok*I bound to DNA; *Nature (London)* 388 97–100

Walder R Y, Hartley J L, Donelson J E and Walder J A 1981 Cloning and expression of the *Pst*I restriction-modification system in *Escherichia coli; Proc. Natl. Acad. Sci. USA* 78 1503–1507

Wilson G G and Murray N E 1991 Restriction and modification systems; *Annu. Rev. Genet.* 25 585–627

Winkler F K 1992 Structure and function of restriction endonucleases; *Curr. Opin. Struc. Biol.* 2 93–99

Winkler F K, Banner D W, Oefner C, Tsernoglou D, Brown
R S, Heathman S P, Bryan R K, Martin P D, Petratos K and Wilson K S 1993 The

crystal structure of *Eco*RV endonuclease and of its complexes with cognate and non-cognate DNA fragments; *EMBO J.* 12 1781–1795

Wyszynski M W, Gabbara S, Kubareva E A, Romanova E A, Oretskaya T S, Gromova E S, Shabarova Z A and Bhagwat
A S 1993 The systeine conserved among DNA cytisine methylases is required for methyl transfer, but not for specific DNA binding; *Nucleic Acids Res*. 21 295–301

Zebala J A, Choi J and Barany F 1992 Characterization of steady state, single turnover and binding kinetics of the *Taq*I; *J. Biol. Chem.* 267 8097–8105

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