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Cloning, production and characterisation of wild type and mutant forms of the R·*Eco*K endonucleases

Marie Weiserova, Pavel Janscak, Oldrich Benada, Josef Hubácek, Vitaly E.Zinkevich[1], Stuart W.Glover[1] and Keith Firman[2,*]

Laboratory of Molecular Genetics of Bacteria, Institute of Microbiology, Czechoslovak Academy of Sciences, Videnska 1083, 142 20 Prague 4, Czechoslovakia, [1]Department of Biochemistry and Genetics, The Medical School, Catherine Cookson Building, The University, Newcastle upon Tyne, NE2 4HH and [2]Biophysics Laboratories, School of Biological Sciences, University of Portsmouth, St. Michael's Building, White Swan Road, Portsmouth PO1 2DT, UK

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ABSTRACT

The *hsdR*, *hsdM* and *hsdS* genes coding for R·*Eco*K restriction endonuclease, both with and without a temperature sensitive mutation (*ts-1*) in the *hsdS* gene, were cloned in pBR322 plasmid and introduced into *E.coli* C3-6. The presence of the $hsdS_{ts-1}$ mutation has no effect on the R-M phenotype of this construct in bacteria grown at 42°C. However, DNA sequencing indicates that the mutation is still present on the pBR322-hsd_{ts-1} operon. The putative temperature-sensitive endonuclease was purified from bacteria carrying this plasmid and the ability to cleave and methylate plasmid DNA was investigated. The mutant endonuclease was found to show temperature-sensitivity for restriction. Modification was dramatically reduced at both the permissive and non-permissive temperatures. The wild type enzyme was found to cleave circular DNA in a manner which strongly suggests that only one endonuclease molecule is required per cleavage event. Circular and linear DNA appear to be cleaved using different mechanisms, and cleavage of linear DNA may require a second endonuclease molecule. The subunit composition of the purified endonucleases was investigated and compared to the level of subunit production in minicells. There is no evidence that HsdR is prevented from assembling with HsdM and $HsdS_{ts-1}$ to produce the mutant endonuclease. The data also suggests that the level of HsdR subunit may be limiting within the cell. We suggest that an excess of HsdM and HsdS may produce the methylase *in vivo* and that assembly of the endonuclease may be dependent upon the prior production of this methylase.

INTRODUCTION

The *Escherichia coli* enzyme R·*Eco*K is comprised of three subunits encoded by the genes *hsdR*, *hsdM* and *hsdS*. *Eco*K is a member of the K family of type I restriction and modification (R-M) enzymes, which includes enzymes of the related systems *Eco*B and *Eco*D from *E.coli* and StySP and StySB from *Salmonella* (for review see Ref. [1]). Complementation tests have shown that the HsdM and HsdR subunits from related systems are interchangeable and the HsdS polypeptide dictates DNA sequence specificity [1, 2]. The products of all three genes are required for restriction [3], while for modification only the products of genes *hsdS* and *hsdM* are needed [4, 5]. The endonuclease requires ATP, *S*-adenosyl methionine (SAM) and Mg^{2+} as cofactors. ATP and SAM serve as both cofactors and as allosteric effectors and determine whether restrictase, or methylase, activity occurs, dependent upon the state of methylation of the DNA substrate [6]. The methylase requires only SAM as a cofactor. The gene order is *hsdR,M,S*, the *hsdR* gene has its own promoter and the *hsdM* and *hsdS* genes are transcribed from a single promoter situated between genes *hsdR* and *hsdM* [7].

The DNA target sequences recognised by type I R-M enzymes consist of two specific components, one of 3-bp and another of 4- or 5-bp, separated by a non-specific spacer,

e.g. $AAC(N)_6GTGC$ for *Eco*K [8]
$TGA(N)_8TGCT$ for *Eco*B [9]

The DNA is cut at a random sites after translocation past the enzyme in an ATP dependent reaction. DNA translocation and cleavage can occur in both directions from the host specificity site, at which R·*Eco*K remains bound [10]. In one model of the mechanism of cleavage, R·*Eco*K is believed to make a single-stranded cut in the DNA molecule; therefore, a complete double-

* To whom correspondence should be addressed

stranded cleavage requires two endonuclease molecules [11]. The requirement for two enzyme molecules is also emphasised by another model which suggests that cleavage occurs only when two neighbouring, translocating enzymes meet. In the case of a DNA molecule with only one recognition site the cut would require co-operation between the specifically bound enzyme and excess enzyme in the cytoplasm[12].

Sequence comparisons between the *hsdS* genes of the *Eco*K, B and D systems revealed two extensive variable regions of about 450-bp in length and two other regions, 100-bp in the centre and 250-bp at the distal end, which show high degrees of conservation. It was suggested that the conserved regions are involved in protein-protein interactions and the variable regions in DNA recognition [7]. Recent studies have shown that the proximal variable region specifies the trinucleotide component of the DNA target sequence. DNA recognition specificity lies not in the entire variable region but only in domains within the region [13].

One approach to the study of functional domains in the HsdS polypeptide is to use defined mutations in the *hsdS* gene and to test their function in combination with *hsdM* and *hsdR* genes. Two temperature-sensitive mutations have been described [14, 15]. The results presented in this paper focus on a temperature-sensitive, trans-dominant *hsdS* mutation [15]. It was suggested that this mutation, where C^{1245} is replaced by T and as a consequence of this transition Ser^{340} is replaced by Phe in the distal variable region of the mutant HsdS polypeptide, influences the assembly of the HsdM,S methylase complex with the HsdR subunit [16]. We have cloned the *hsdR,M,S* genes, active as both wild type and temperature-sensitive R·*Eco*K endonucleases, into the plasmid pBR322 . This has allowed us to purify the wild type and mutant endonuclease enzyme and to analyse the effect of the temperature-sensitive mutation *in vitro*. This is the first isolation of the *Eco*K restriction endonuclease from genes cloned on a multicopy plasmid that results in over-production of the enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophage

The *E.coli* strains and plasmids used in this study are described in Table 1. Previously described recombinant plasmids used in this work are all derivatives of the vector pBR322 [17]. The virulent mutant of phage λ [18] was used for the *in vivo* testing of restriction and modification following previously described methods [3, 19]. pGEM-7Zf(+) phagemid was from Promega, Wisconsin, USA.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Hsd phenotype	Other characteristics	Source or reference
Strain			
E.coli C	R^-M^-	prototroph	[39]
*E.coli*C3-6	R^-M^-	*rec*A56 derivative of *E.coli* C	This work
E.coli C600	R^+M^+	*thr, leu, thi*	[40]
E.coli WA802	R^-M^+	*met, gal, hsdR2, mer*A	[27]
E.coli JC5088		*thr, ilv, rec*A56	[41]
Plasmid			
pBg3		$hsdR^+$, $hsdM^+$	[26]
pMS_K14		$hsdS^+$	[28]
pVMΔ14		*Eco*RI-*Hpa*I deletion of pMS^K14	This work
pVM11		$hsdS_{ts-1}$	[16]
pVM39		$hsdR^+$, $hsdM^+$, $hsdS_{ts-1}$	This work
pVMC3		$hsdR^+$, $hsdM^+$, $hsdS^+$	This work

Media and microbial procedures

Phage buffer, complete LB medium, M9 medium, L-amino acids, vitamin supplements were as previously described [3]. The solid medium is LB or M9 with agar added at 1.5%. Soft agar overlay is LB with agar added at 0.6%. Ampicillin was used at a concentration of 50 mg/ml. Transformations were carried out using the $CaCl_2$/heat shock method [20]. The *rec*A derivative of *E.coli* C (*E.coli* C3-6) was prepared using the chromosomal linkage of *thy* and *rec*A genes. Trimethoprim was used to select a thy^- mutant of *E.coli* C. This mutant was conjugated with HfrH JC5088 and thy^+ recombinants were selected. From these recombinants the transconjugant thy^+ $recA^-$ was selected by its sensitivity to ultraviolet light.

Nucleic acid methods

Plasmid DNA for ethidium bromide-CsCl equilibrium density gradient centrifugation and for rapid small scale isolation was prepared by the alkaline lysis method. Restriction enzyme digests, ligations, gel electrophoresis and elution of DNA fragments from agarose gels by direct extraction were performed using standard techniques [20]. ssDNA from the phagemid pGEM-7Zf(+) was prepared following induction with KO7 helper phage [21] and sequenced using the universal primer [22]. Random heteroduplex DNA was prepared by denaturing a mixture of equal amounts (0.5 mg each) of unmodified and modified pBR322 plasmid DNA [23].

Protein production and analysis

Protein synthesis in minicells harbouring the appropriate plasmid(s) was detected with ^{35}S methionine [24] and analysed by 10% SDS-PAGE [25]. The protein bands were visualised by autoradiography. The mutant and wild type endonucleases were both purified using the previously described method [23]. The mutant endonuclease was prepared from *E.coli* C3-6[pVM39] grown at 42°C to produce the enzyme $EcoK_{ts-1}^+$ and at 30°C to produce $EcoK_{ts-1}$.

Endonuclease assay: Digestion of the unmodified pBR322 DNA with wild type and temperature-sensitive *Eco*K was performed at 30°C and 42°C in a buffer containing 0.1M HEPES pH 8.0, 0.25mM EDTA, 12mM mercaptoethanol and 6.5mM $MgCl_2$. The endonuclease activity was assayed in a volume of 100ml that contained 8mg of DNA, 0.8mg of *Eco*K enzyme, 0.1mM SAM and 50mg/ml BSA. After 3 minutes pre-incubation at the appropriate temperature, the reaction was started by the addition of ATP at a final concentration of 2mM. Immediately before, and at appropriate intervals after the addition of ATP, 5ml aliquots were mixed with 1.5ml of stop solution (0.1M EDTA, 5% SDS) and placed at 65°C for 5 min. The samples were subjected to electrophoresis in a 0.8% agarose gel in TBE buffer for 16 hr at 1.0 V/cm at 20°C. The gels were photographed following staining with ethidium bromide (0.5mg/ml).

Methylase assay: The methylase activity at 30°C and 42°C was measured in the same buffer used for the endonuclease reactions. The 370ml of reaction mixture contained 2.4mg of enzyme, 3.7mg heteroduplex DNA, 1mM [methyl ^3H] *S*-adenosyl methionine (85Ci/mmol). After pre-incubation for 2 min at the appropriate temperature ATP was added to a final concentration of 5mM. At appropriate intervals 50ml aliquots were mixed with

5ml 0.5M EDTA pH 8.0 and the samples were extracted with phenol-chloroform. The aqueous phase was loaded on Whatman DE82 paper (4 cm^2) and dried. The filters were washed two times in 50ml of 0.5 M sodium phosphate, pH 6.8 for 10 min to remove the unincorporated radioactivity. After washing with ethanol and drying, the amount of incorporated label was determined by scintillation counting.

RESULTS

Construction and function of the endonuclease operon(s)

The source of the *Hin*dIII fragment carrying the intact *hsdR* and *hsdM* genes, and part of the *hsdS* gene, was pBg3 [26]. This fragment was isolated from an agarose gel, and inserted at the *Hin*dIII site in pVM11 [16], a plasmid carrying the mutant $hsdS_{ts-1}$ gene. This results in restoration of an intact *hsdS* gene. This DNA was used to transform *E.coli* WA802 *hsdR*2 [27]. Plasmid DNA was isolated from transformants and screened by size. Several plasmids larger than pBg3 were analysed physically and one of them, named pVM39, complemented restriction in *E.coli* strains WA802 *hsdR*2 and WA803 *hsdS*3, and contained the *Hin*dIII fragment in the required orientation (Figure 1).

In the construction of the recombinant plasmid with the wild type *hsd* operon, a *Hin*dIII digest of the plasmid pVMD14 (the structural analogue of pVM11), prepared from plasmid pMS_K14 [28] by *Hpa*I-*Eco*RI deletion, was ligated to the *Hin*dIII fragment described above (Figure 1), again restoring an intact *hsdS* gene. The resultant plasmid was named pVMC3.

Both plasmids were introduced into *E.coli* C, a strain that has been shown to be deleted for the chromosomal *hsd* genes [29],

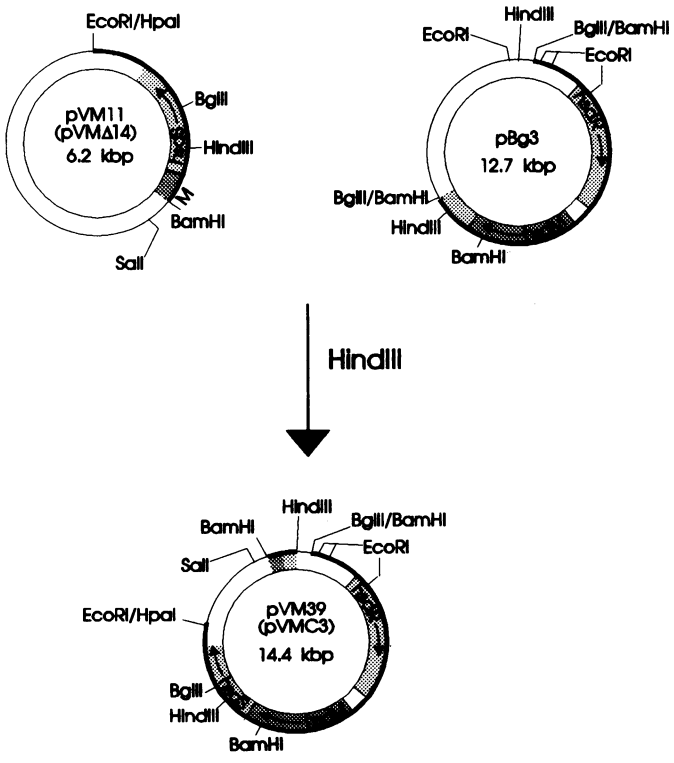

Figure 1. Construction of the endonuclease operon. The plasmid pVM39 carries the endonuclease operon with the mutant *hsdS* gene and its structural analogue with wild *hsdS* gene is pVMC3; ——— pBR322 DNA, ▬▬ chromosomal fragments.

and tested for R-M function. A number of transformants carrying the cloned *hsd* genes were found to display genetic instability resulting from deletion of the cloned fragment (data not shown). To eliminate *rec*A-dependent recombination as one cause of this genetic instability a *rec*A derivative of *E.coli* C (*E.coli* C3-6) was used as a host for both recombinant plasmids. The plasmid pVM39 was introduced into the *rec*A *E coli* strain C3-6. It was found that the $hsdS_{ts-1}$ gene, present on this plasmid, failed to produce the temperature-sensitive R-M phenotype; the efficiency of plating of bacteriophage lambda was reduced by approximately four orders of magnitude to give the same efficiency of plating observed with the wild type R-M system. However, all transformants were stable. This transformation was repeated several times and always produced a wild type phenotype.

Transformation of *E.coli* C3-6 with plasmid pVMC3 (wild type *hsdS* gene) produced the normal R^+M^+ phenotype, and again the transformants were all stable. The frequency of transformation with both pVM39 and pVMC3 plasmids was about three orders of magnitude lower than that obtained for pBR322 alone. When plasmids coding for an endonuclease enter the *E.coli* C cells with an unmodified chromosome the K-modification is

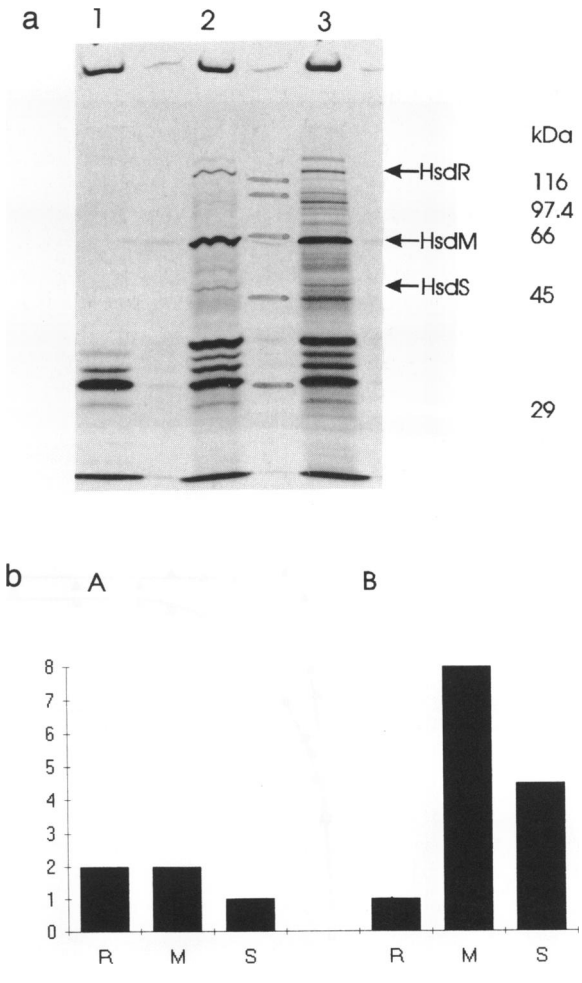

Figure 2. Stoichiometry of the purified endonuclease and the ratio of subunits produced in minicells. 2a shows a SDS-PAGE analysis of the proteins produced in minicells from the following plasmids: **Lane 1,** pBR322; **Lane 2,** pVMC3; **Lane 3,** pVM39. Size markers are drawn on the gel using radioactive ink. The histograms (2b) represent the relative ratios of subunits present in either the purified endonuclease (A) or the minicells (B). Both sets of data have been normalised such that the lowest value is equal to 1.

less effective on the unmethylated DNA [30] and the endonuclease can restrict the host chromosome [31]. This may explain the reduced frequency of transformation.

Sequence determination of pVM39

The plasmid pVM39 carries the following genes; *hsdR, hsdM* and $hsdS_{ts-1}$ and should, therefore, have a temperature-sensitive phenotype in *E.coli* C3-6. However, as described above, this plasmid was found to exhibit wild type R-M. In order to confirm that the *ts-1* mutation is still present on this plasmid the *Bgl*II-*Xmn*I fragment was purified from a preparative agarose gel and cloned into *Bam*HI+*Sma*I digested pGEM-7Zf(+). ssDNA for sequencing was isolated from this recombinant phagemid and the sequence determined. Presence of the base change associated with the temperature-sensitive phenotype [16] was confirmed.

Subunit production *in vivo* and stoichiometry of the *Eco*K endonuclease

Both pVM39 and pVMC3 plasmid were introduced into *E.coli* P678-54 and minicells isolated, newly synthesised proteins were labelled using ^{35}S-methionine. The proteins were separated by 10% SDS-PAGE and analysed by autoradiography (Figure 2a). The autoradiograph was analysed by densitometry, the area under each trace was calculated and the values corrected for the number of methionines in each protein. This experiment was repeated several times and the average ratio of the subunits present in each band plotted (Figure 2b).

The wild type R·*Eco*K endonuclease was purified from a freshly transformed *E.coli* C3-6[pVMC3] strain as described in materials and methods. The R·$EcoK_{ts-1}$ and R·$EcoK_{ts-1}^{+}$ endonucleases were similarly purified from a freshly transformed *E.coli* C3-6[pVM39] strain grown at 30° and 42°C respectively. The ratio of subunits in the endonuclease was determined by separation on SDS-PAGE and staining with Coomassie blue. It is known that Coomassie blue staining can vary from protein to protein [32] and consequently the ratios of subunits obtained here are not necessarily the absolute values for the stoichiometry, but serve as internal comparisons between wild type and mutant endonucleases. The ratio between the integrated peak areas for each subunit was determined and corrected for the molecular mass

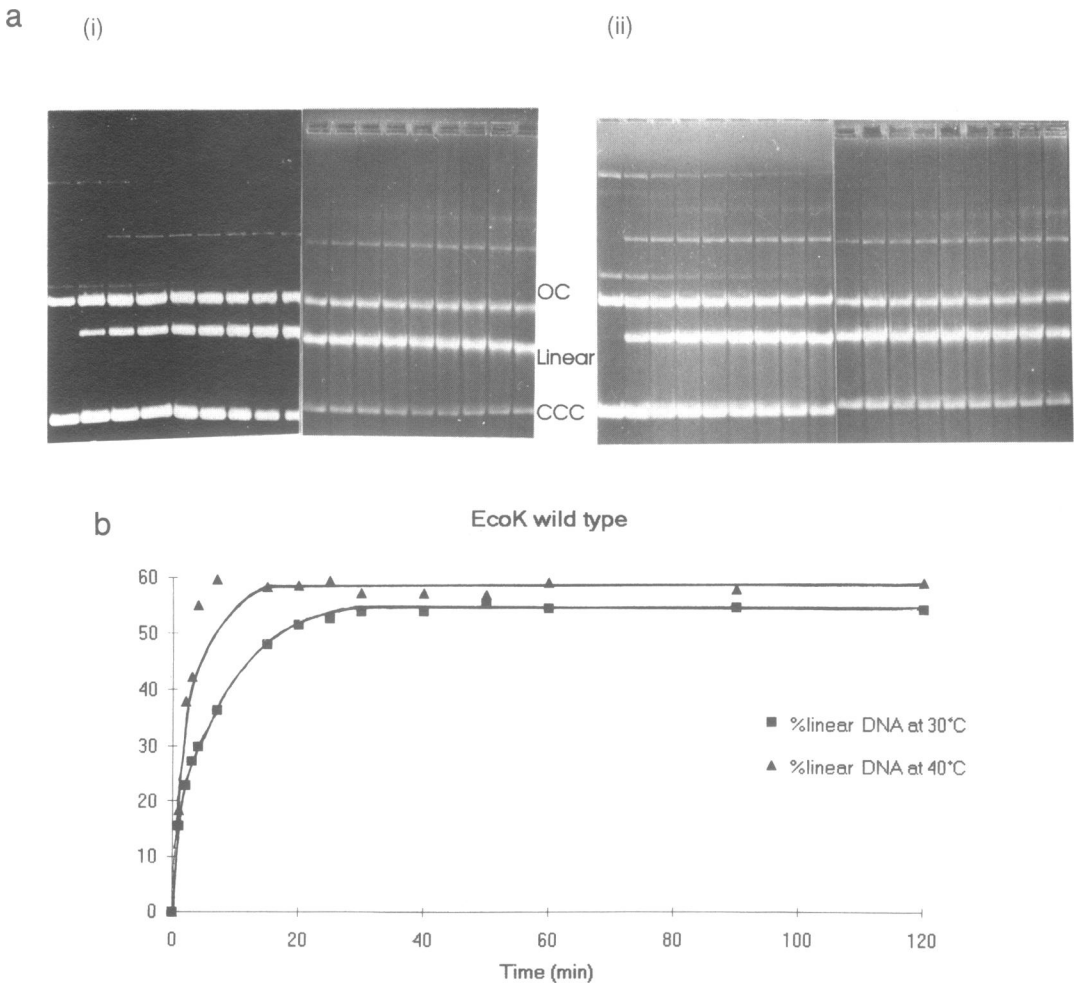

Figure 3. Restriction assay of wild type endonuclease. 3a(i) and 3a(ii) show the agarose gels used to determine the restriction activity of the wild-type endonuclease at 30°C and 42°C respectively. Lane 1 of each gel contains DNA only, the remaining lanes contain samples incubated for the following time intervals: 1,2,3,4,5,7,9,11,15,20,25,30,40,50,60,90,120 min. The bands are labelled: OC = open circular DNA, CCC = covalently closed circular DNA and linear DNA, DNA bands above the open circular are due to the presence of dimers and higher molecular weight multimers present in the preparation. 3b is a plot of the amount of linear DNA produced as a percentage of the total DNA present.

of each subunit. This gave a stoichiometry of R_2M_2S, which is in agreement with previous data for the wild type endonuclease [33]. The mutant enzymes were found to give the same ratio (data not shown). This indicates that the *ts-1* mutation does not prevent subunit assembly at the non-permissive temperature. Figure 2b shows that the ratio of subunits present in the minicell extraction is significantly different from that present in the endonuclease. The HsdR subunit appears to be the limiting component and may limit the production of the endonuclease.

In vitro endonuclease and methylase activities

Unmodified plasmid pBR322 DNA (containing both covalently closed, cccDNA, and open circular, ocDNA, forms) was used to estimate the endonuclease activity of the mutant and wild type endonucleases. DNA and protein were mixed at an approximate ratio of 2:1. ATP was used to start the reactions and the amount of linear DNA produced at various time intervals detected by separation of the products on agarose gels (Figures 3 & 4). Photographic negatives of the gels were scanned by densitometry, the integrated peak areas for each form of DNA were measured, and the amounts of linear DNA produced were expressed as a percentage of the total amount of DNA. The experiment was repeated at 30°C and 42°C for wild type *Eco*K, *Eco*K_{ts-1}^+(data not shown) and *Eco*K_{ts-1}. Figures 3b & 4b shows a plot of these percentage ratios of DNA against time. As can be seen for wild-type *Eco*K approximately 50% of the DNA is cleaved after 30 min and no further reaction appears to take place after this time. The mutant form of the endonuclease R·*Eco*K_{ts-1} produced only 20% linear DNA at 30°C and about 10% at 42°C. Again after approximately 30 min no further reaction is observed. Interestingly, the results obtained with enzyme *Eco*K^+_{ts-1} shows that its activity is lower than the activity of *Eco*K_{ts-1} at the permissive temperature. Only 10% of the total DNA is cleaved (data not shown).

Methylase activity of wild type and mutant endonucleases was monitored by transfer of a tritiated methyl group from *S*-adenosyl methionine to random heteroduplex linear pBR322 DNA. A molar ratio of 1:4 (DNA:protein) was chosen to ensure one endonuclease molecule per available S_K site. The number of incorporated counts was plotted against time for both mutant

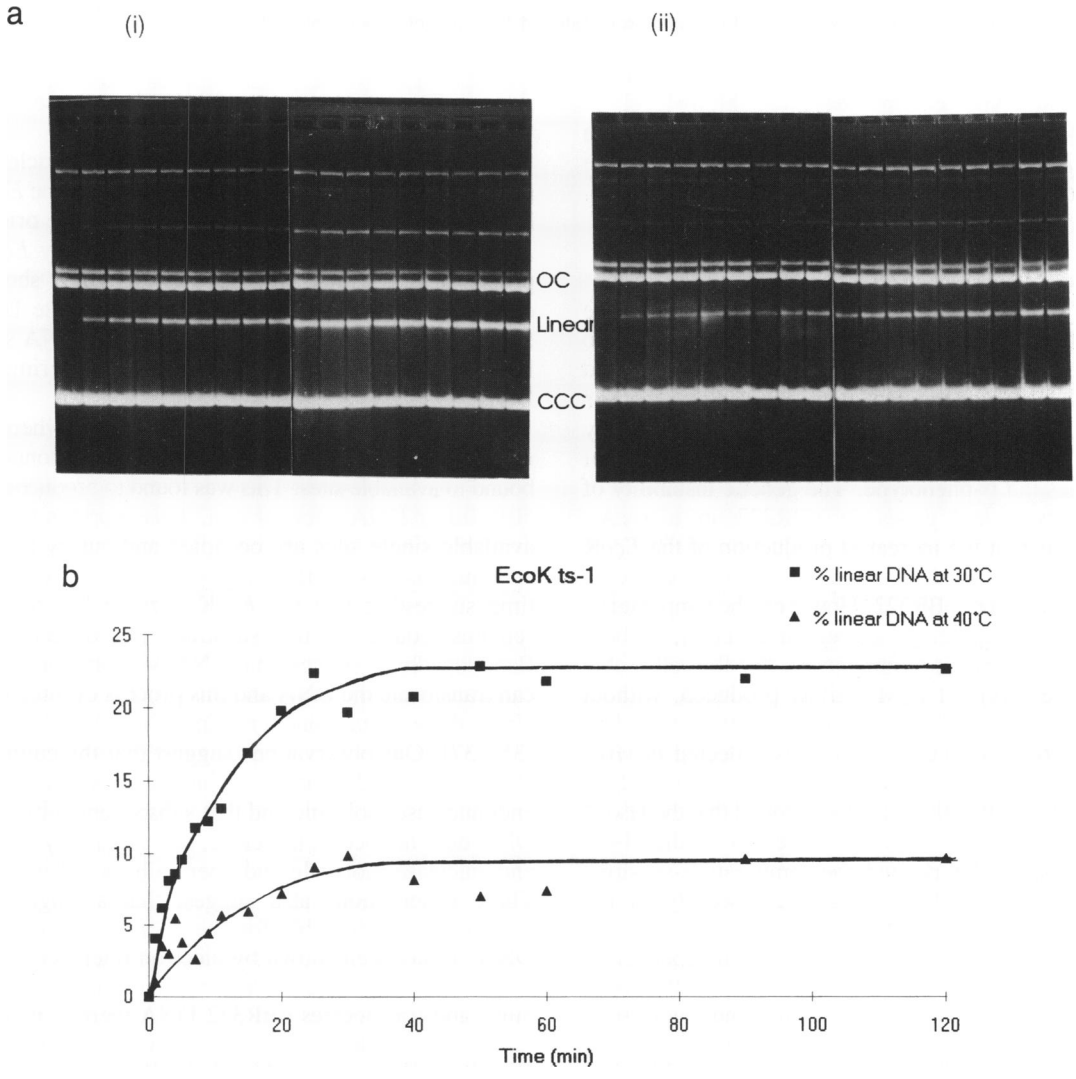

Figure 4. Restriction assay of the temperature-sensitive endonuclease. The gels were loaded as described for the wild-type endonuclease (figure 3). The DNA bands are labelled as described in figure 3.

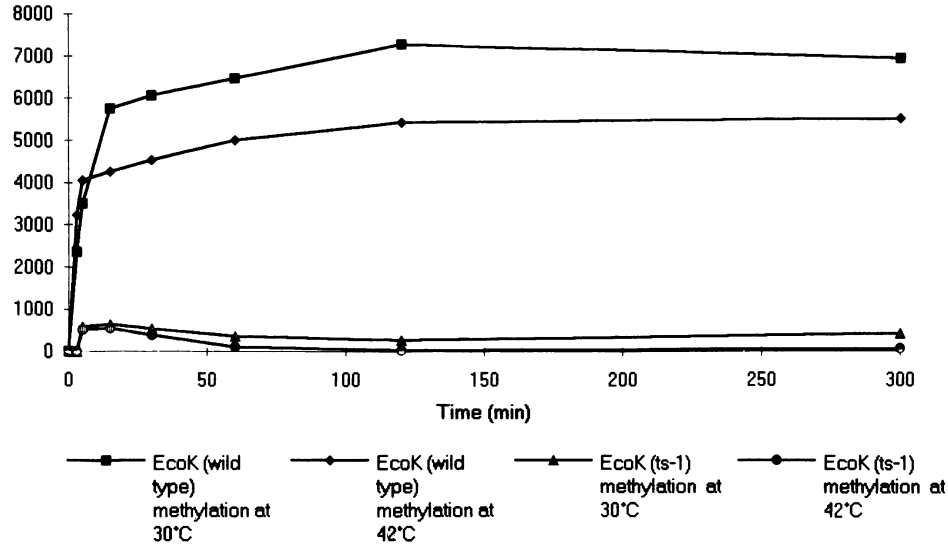

Figure 5. Methylase activity of wild-type and temperature-sensitive endonucleases at permissive and non-permissive temperatures. The methylase assay was performed as described in materials and methods. The amount of incorporated label was measured by scintillation counting. A control of no enzyme was used to determine the background level of tritium present in each sample, and this value was deducted from the total count obtained.

(*EcoK*$_{ts-1}$) and wild type endonuclease. A high level of methylase activity was shown by the wild type enzyme at both 30° and 42°. Little or no methylase activity was detectable with the temperature-sensitive enzyme at either 30° or 42° (Figure 5).

DISCUSSION

The inability of a multicopy plasmid, carrying the entire temperature-sensitive *hsd* operon from the chromosome, to express the temperature-sensitive phenotype was an unexpected result. Sequence analysis of the *hsdS* gene showed that the *ts-1* mutation was still present. It would appear that the increased copy number of pBR322, and, therefore, the increased production of *EcoK*$_{ts-1}$, influences the *ts*-phenotype. The genetic instability of the cloned wild type *hsd* genes in pBR322 in a *recA*$^+$ background suggests that the increased production of the *Eco*K endonuclease from pBR322 is detrimental to the cell and this leads to deletion of the genes from pBR322. However, the temperature-sensitive mutation is sufficiently altered in function, at both permissive and non-permissive temperatures, that the cell is able to function with the levels of R-M activity produced, without excision of the cloned *ts-hsd* genes. This suggests that the low level of enzyme activity observed *in vitro* is reflected *in vivo*.

The production of the three subunits in minicells should reflect the level of production within the cell. It was found that the HsdM and HsdS subunits were present in an excess over the HsdR subunit for both the wild type and the temperature-sensitive systems. This may reflect the pathway of assembly of the endonuclease; the HsdM,S methylase may be assembled in preference to the endonuclease and may function independently in the cytoplasm. It seems likely that the availability of the HsdR subunit may govern the final assembly of the endonuclease.

The purified endonucleases from both the wild type and the temperature-sensitive systems were found to have an identical ratio of subunits (2:2:1−HsdR:HsdM:HsdS). This shows that the temperature-sensitive mutation *ts-1* does not affect the subunit assembly of the endonuclease by preventing binding to the HsdR subunit. This stoichiometry of the endonuclease is in agreement with previous data [33].

The ability of the purified *EcoK*$_{ts-1}$ endonuclease to cleave pBR322 DNA was compared to that of wild type *Eco*K. pBR322 has two recognition sites for *Eco*K in opposite orientation [10]. A model describing the cleavage of DNA by *Eco*K has been proposed [12] which suggests that this DNA should bind two endonuclease molecules which will translocate DNA until the endonuclease molecules meet and then the DNA should be cut. However, this model was derived from experiments on linear DNA.

At a molar ratio of 2:1 (DNA:protein), where the ratio of recognition sites to protein is 4:1, all the endonuclease will be bound to available sites. This was found to produce approximately 50% linear DNA after 30 min. This supports the idea that the available single sites are occupied and cut by all the available endonuclease. No further digestion appears to occur after this time suggesting that the *Eco*K enzyme has no turnover and remains bound to the substrate [5, 6, 34]. Studier and Bandyopadhyay suggest that DNA with only one enzyme bound can translocate the DNA and this process eventually might stall. Under these circumstances nicking of the DNA has been observed [35−37]. Our observations suggest that the cutting of circular DNA is not dependent upon the presence of a second endonuclease molecule and that subsequent cutting of the linear DNA does not occur, therefore, this event may require a second endonuclease molecule and operate by a different mechanism. These observations also suggest that a single endonuclease molecule is responsible for the 50% cleavage of the circular DNA. It has been shown by electron microscopy that, even in the presence of excess enzyme, when one endonuclease molecule binds and translocates pBR322 DNA there is no evidence of a second endonuclease molecule binding to the same DNA [10]. Our data also supports this observation.

The *EcoK*$_{ts-1}$ endonuclease was found to digest plasmid DNA at 30°C in an analogous manner to the wild type enzyme, but only 20% of the DNA is fully cleaved (at which point no further

reaction occurs). This indicates that the $EcoK_{ts-1}$ enzyme is affected in its endonuclease activity even at the permissive temperature. At 42°C this digestion was reduced to approximately 10% of the total DNA showing only a two-fold temperature-sensitivity of the activity. However, methylation of plasmid DNA was at extremely low levels *in vitro* at both 30°C and 42°C. Such activity that could be measured was higher at 30°C than that at 42°C. Since the enzyme can function as a modification methylase at 30°C *in vivo* it must be assumed that this accomplished by the low level of *in vitro* activity that is observed. Similar results have been observed with a *ts*-mutation in the bacteriophage Mu *c* repressor [38].

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