Ph.D. Thesis

Analysis of certain aspects of restriction endonuclease function: DNA recognition and *in vivo* effects

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ABBREVIATIONS

AdoMet	S-adenosyl-L-methionine
ATP	adenosin 5′-triphosphate
bp	base pair
β-ME	β-mercaptoethanol
CTAB	hexadecyltrimethyl ammonium bromide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kiloDalton
LB	Luria Bertani medium
m^+	methyltranferase containing strain
MCS	multiple cloning site
M	modification
MW	molecular weight
PC	phosphocellulose
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
R	restriction
S	specificity
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

This PhD thesis comprises three related projects. The first study is a mutational analysis of DNA substrate recognition by the *Eco*RI endonuclease. The second deals with an *in vivo* effect of restriction enzymes. The third, as yet unfinished, project explores the possibility of constructing chimeric endonucleases and analyzes the phenotypes of one of the constructs. The common theme in all three projects is sequence-specificity of type II restriction endonucleases.

Discovery of restriction-modification systems

The phenomenon of restriction and modification was first encountered in the early 1950s. Luria and Human were the first to describe the observations on this phenomenon in 1952 (Luria and Human, 1952). Bertani and Weigle reported similar observation one year later (Bertani and Weigle, 1953). Certain strains of *E. coli* were found to "restrict" the propagation of viruses grown previously on a different strain. This effect was traced to sequence-specific endonucleases, of which some produced discrete DNA fragments upon cleavage (Arber, 1965; Meselson and Yuan, 1968; Hedgpeth *et al.*, 1972; Kelly and Smith, 1970; Old *et al.*, 1975). Since then over two hundred different specificities have been discovered and genes for over one hundred restriction-modification (R-M) systems have been cloned, approximately sixty of them have been sequenced (Roberts and Macelis, 2001). Restriction endonucleases became extremely useful in DNA manipulation and in studies of DNA-protein interaction.

Classes of restriction-modification systems

Restriction-modification systems comprise pairs of opposing intracellular enzyme activities: an endonuclease that recognizes and cleaves DNA at a specific sequence, and a methyltransferase that modifies the same sequence to protect the host chromosome from cleavage. R-M systems are thought of as a bacterial immune system with a role to destroy foreign DNA entering the cell. The recognition sequences usually consist of four to eight nucleotides; they can be continuous or interrupted, symmetric or asymmetric, defined or degenerated.

R-M systems are classified according to enzyme structure, cofactor requirements, recognition sequence symmetry, and cleavage position. Several types of R-M systems were discovered in the bacterial world: they were originally grouped as type I, II and III. Type I R-M systems consist of three subunits: R for restriction, M for modification, S for specificity. Modification requires the presence of AdoMet and occurs within the recognition sequence, while cleavage takes place at distant random sites and requires AdoMet as well as ATP, which is hydrolyzed following DNA cleavage. Type II endonucleases and methyltrasferases act independently and require Mg^{2+} for cleavage and AdoMet for methylation, respectively. Type III R-M systems consist of two subunits: R and M. While modification takes place in the presence of AdoMet, restriction requires the cooperation of R and M, and also the presence of ATP which, however, is not hydrolyzed during reaction. Modification occurs within the recognition sequence, and cleavage at an approximately 25 bp distance from this sequence (Wilson and Murray, 1991; Bickle and Krüger, 1993; Pingoud and Jeltsch, 1997).

Type II restriction endonucleases – enzymes with high specificity

Type II restriction endonucleases comprise one of the major families of endonucleases. These restriction enzymes have proven to be indispensable tools for genetic engineering and useful for studying sequence-specific protein-DNA interactions. The orthodox type II restriction enzymes are homodimers of typically ~2×30 kDa molecular mass, and recognize and cleave DNA in the presence of Mg^{2+} at palidromic sequences four to eight base pairs long. These enzymes catalyze the hydrolysis of phosphodiester bonds at precise positions within or adjacent to this sequence. Some R-M systems, originally defined as type II, turned out to have unusual properties and, therefore, form distinct subdivisions. A new nomenclature for type II restriction endonucleases has recently been proposed (Pingoud and Jeltsch, 2001).

On basis of a comparison of the available structures of type II enzymes two restriction endonuclease families were defined: *Eco*RI–like family and *Eco*RV–like family. The *Eco*RI–like family consist of the *Eco*RI, *Mun*I, *Bam*HI, *Bgl*II, *and Ngo*MIV and *Bso*BI restriction enzymes. The *Eco*RV, *Pvu*II and *Bgl*I enzymes are members of the *Eco*RV–like family. The distinction between the two restriction endonuclease families were made not only on basis of structural relatedness but also because of functional similarities: *Eco*RI, like *Bam*HI, binds the DNA from the major groove side

and produces 5′-overhanging sticky ends, whereas *Eco*RV, like *Pvu*II, approaches the DNA from the minor groove side and produces blunt ends.

Type II restriction endonucleases are among the most specific enzymes known. More than 3000 type II restriction enzymes representing more than 200 different specificities have been discovered (Roberts and Macelis, 2001). The co-crystal structures revealed that DNA recognition is achieved by direct and indirect readout, i.e. by base contacts, and backbone contacts, respectively. The protein makes a large number of contacts with the DNA, involving hydrogen bonds, ionic bonds, and hydrophobic interactions. Contacts to the bases are predominantly in the major groove and usually exhaust the hydrogen bonding potential in the major groove. This means that a hexanucleotide sequence is recognized by ~20 hydrogen bonds to the bases of the recognition sequence. Interactions with the backbone are often water-mediated. Although each individual contact is weak, the 20 or so contacts that are typically formed at the protein-DNA interface add together to ensure that the interaction is both highly specific and very strong. For example, *Eco*RI recognizes the sequence GAATTC and makes base contacts only in the major groove. The recognition interaction comprises 18 sequence-specific hydrogen bonds involving purines and pyrimidines, along with extensive van der Waals contacts with the pyrimidines (Rosenberg, 1991). Altogether these numerous contacts ensure high accuracy of DNA recognition by restriction endonucleases. Sequence specificity of restriction endonucleases is remarkable; the activity can be a million times lower as the result of a single base pair change within the recognition sequence. For example, the *Eco*RI endonuclease cleaves sites that differ from its recognition site by just one nucleotide at 10^5-10^9 times lower rates than the canonical site. This exquisite specificity of restriction endonucleases is crucial for the prevention of accidental cleavage at the many nonspecific sites in the host chromosome.

I. DNA substrate recognition by the *Eco*RI endonuclease

One of the best studied and most widely used enzymes is the *Eco*RI endonuclease, on which a variety of studies concerning the properties of the protein and the nature of its catalysis have been carried out (Thielking *et al.*, 1990; Lesser *et al.*, 1990; 1993;). The *Eco*RI endonuclease is a homodimer composed of two 277–residue polypeptide chains of MW = 31,063 (Modrich and Zabel, 1976; Newman *et al.*, 1981; Greene *et al.*, 1981). The *Eco*RI restriction endonuclease recognizes and cleaves double-stranded

DNA at GAATTC sites (Hedgpeth *et al.*, 1972; Heitman, 1992). The enzyme hydrolyzes the phosphodiester bond between the guanine and adenine residues resulting in 5'-phosphate end. The *Eco*RI endonuclease requires Mg^{2+} as cofactor for DNA cleavage, but can specifically bind to its cognate sequence in the absence of Mg^{2+}. *Eco*RI endonuclease was the first type II restriction enzyme for which a crystal structure of the complex between the protein and the cognate oligonucleotide became available (McClarin *et al.*, 1986; Kim *et al.*, 1990). Since that time the *Eco*RI endonuclease has become an attractive model system for investigating DNA recognition.

The *Eco*RI-DNA co-crystal structure revealed an intricate set of direct and water-mediated interactions between the protein and bases of the target sequence. In addition to protein-base contacts there is a buttressing network of interactions between amino acid side chains that is critical for recognition (Kim *et al.*, 1993). A thorough understanding of the molecular mechanism underlying sequence specificity requires the use of complementary approaches to test the predictions of the crystal structure. A number of these contacts, mainly those to purines of the recognition sequence, were previously tested by mutagenesis and biochemical studies (Wolfes *et al.*, 1986; Needels *et al.*, 1989; Heitman and Model, 1990; 1990a; Oelgeschlager *et al.*, 1990; Osuna *et al.*, 1991; Jeltsch *et al.*, 1993; Flores *et al.*, 1995). Less attention has been given to interactions with pyrimidines. The revised *Eco*RI-DNA cocrystal structure revealed the presence of an extended-chain motif (Met137-Ala142), which is a segment of extended polypeptide chain that runs through the major groove of the DNA. The segment of the gene that codes for the extended chain is a mutational hot spot (Yanofsky *et al.,* 1987). Many of the contacts formed by the extended-chain motif involve pyrimidine bases. Pyrimidine contacts had previously been implicated by base-analogue studies (Brennan *et al.,* 1986; McLaughlin *et al.,* 1987). Similarly, Heitman and Model suggested that the protein interacts with pyrimidines as well as purines (Heitman and Model, 1990). Multiple substitutions were introduced, by site-directed mutagenesis, at E144, R145 and R200, amino acids which were implicated in substrate recognition. Most of the generated mutants were inactive or retained partial endonuclease activity preserving specificity for the wild-type substrate. It has been proposed on basis of the crystal structure (Rosenberg, 1991), that the cytosine of the target sequence interacts with three residues: the amino group donates a hydrogen bond to the main chain carbonyl of A138 and H5 and H6 make van der Waals contacts with M137 and I197. The role of A138 in substrate recognition is also supported by genetic studies in which two replacements of

A138 (Val and Thr) were shown to confer relaxed recognition specificity (Heitman and Model, 1990a). Base-specific contacts suggested to play a role in substrate recognition by *Eco*RI are listed in Table 1.

Table 1. Recognition interactions of *Eco*RI endonuclease
(adapted from Rosenberg, 1991)

GC base pair:	
G	N7, O6 receive hydrogen bonds from one water molecule bound by Arg200 and Arg203.
C	N4 donates a hydrogen bond to the main-chain carbonyl of Ala138. H5, H6 make van der Waals contacts with Met137 and Ile197.
Outer AT base pair:	
A	N7 receives a hydrogen bond from Arg145. N7 also receives a weak hydrogen bond from the side chain of Asn141. N6 donates a hydrogen bond to the side-chain carbonyl of Asn141. N6 is also involved in a three-center hydrogen bond with O4 of both thymines.
T	The methyl group makes van der Waals contact with Gly 140 and the methyl group of the adjacent thymine.
Inner AT base pair:	
A	N7 receives a hydrogen bond from the side chain of Arg145. N6 donates a hydrogen bond to the side-chain carbonyl of Asn141.
T	O4 receives a hydrogen bond from the main-chain amide of Ala142. The methyl group makes van der Waals contacts with Ala142, Gln115 and the methyl group of the adjacent thymine.

II. DNA single-strand break repair in *E.coli*

One of the most common lesions that occur in the double-helical DNA structure is a single-strand break (nick) with neighbouring 5'-phosphate and 3'-hydroxyl ends. Nicks are constantly produced as normal intermediates of DNA metabolism. The main source of nicks, under physiological conditions, is probably discontinuous DNA replication. In this process, the newly synthesised lagging strand is assembled from

Okazaki fragments and the final step in joining these fragments is the closing of nicks by DNA ligase. Given the average size of Okazaki fragments (1500 nucleotides), perhaps as many as 3000 nicks are closed by ligase during replication of the *E. coli* chromosome (Kornberg and Baker, 1992).

Another source of nicks is DNA damage. Single strand breaks may appear as primary lesions or as intermediates of a repair process. In the latter case, a nick initiates an excision repair tract and repair synthesis ends with the religation of a nick that remains after DNA repair synthesis (Friedberg *et al.*, 1995). Of DNA lesions, single-strand breaks should be relatively benign because the intact DNA strand holds together the ends of the severed strand. Furthermore, because DNA ligase rapidly seals nicks between Okazaki fragments following DNA replication, one expects that ligase would also efficiently repair nicks incurred by DNA damage. *E. coli* mutants with a 100-fold reduction in DNA ligase activity are viable (Konrad *et al.*, 1973; Gottesman *et al.*, 1973), and thus wild-type *E. coli* may be especially well equipped to repair DNA nicks.

In the course of studies on DNA recognition by the *Eco*RI endonuclease, our collaborator Joseph Heitman isolated site-directed mutants of E144 and R200. R200 interacts with the guanine, and E144 receives hydrogen bonds from Asn141, Arg145 (from the opposite subunit), Arg203 and Lys148. The R200K and E144C mutants had reduced endonuclease activity and retained specificity for the wild-type *Eco*RI recognition site. When tested *in vitro*, both mutants were found to predominantly nick only one strand of the DNA substrate. The mutant enzymes displayed temperature-sensitive activity. Expression of these *Eco*RI endonuclease mutants in the absence of the *Eco*RI methyltransferase was found to induce the SOS DNA repair response and greatly reduce viability of *E. coli* hosts deficient in RecA and RecB functions. This was a surprising observation and suggested that DNA ligase by itself might not always be sufficient to repair single-strand breaks. It looked important to test how general this phenomenon was. To complement Heitman's studies, we chose another experimental system to generate nicks and test their effects in an *E. coli* host. The approach we used was based on the observations by Halford and coworkers. They found that the *Eco*RV endonuclease, when overproduced in an m+ host, makes nicks at noncognate sites (Taylor *et al.*, 1990).

III. *Eco*RI shares structural and functional similarities with its isochizomer *Rsr*I

*Rsr*I endonuclease is an isoschizomer of *Eco*RI, which recognizes the sequence GAATTC and cleaves it between the G and A residues. Methylation of the substrate sequence by M·*Eco*RI protects the site from cleavage by either enzyme (Greene *et al.*, 1988). Like the *Eco*RI endonuclease, the *Rsr*I endonuclease exists as a dimer in solution and requires Mg^{2+} cofactor for catalysis. There is a more than 50% amino acid sequence identity between *Eco*RI and *Rsr*I (Stephenson *et al.*, 1989). More importantly, all amino acids that are known to mediate sequence-specific recognition and catalysis in *Eco*RI are conserved in *Rsr*I (Siksnys *et al.*, 1994), suggesting that *Rsr*I uses the same mechanism as *Eco*RI to interact with its DNA substrate. The importance of this sequence conservation was further strengthened when the sequence of the *Mun*I endonuclease became available. *Mun*I recognizes the sequence **C**AATT**G** that partially overlaps with the recognition sequence of *Eco*RI (*Rsr*I), differing only in the external base-pairs (shown in bold). Comparison of the *Mun*I endonuclease amino acid sequence with that of *Eco*RI and *Rsr*I revealed only a low level of overall similarity (Fig.1). However, all residues forming the catalytic site and the residues responsible for recognizing the inner four nucleotides are conserved in *Mun*I (Siksnys *et al.*, 1994). The X-ray structure of the *Mun*I-DNA co-crystal has since confirmed the proposed role of these amino acids (Deibert *et al.*, 1999).

In substrate-enzyme complexes, both *Eco*RI and *Rsr*I were found to footprint 12 base pairs, bend DNA by 50°, and unwind the DNA helix by 25°. Based on these observations it was concluded that the two enzymes interact with their recognition sequences in similar way (Aiken *et al.*, 1991a). Other experiments performed by the same group identified differences in the DNA recognition mechanisms of *Eco*RI and *Rsr*I. The *Rsr*I endonuclease appeared to be more sensitive to alterations of the functional groups within its recognition sequence than *Eco*RI, suggesting that the *Rsr*I endonuclease exhibits a higher degree of discrimination against non-canonical sequences (Aiken *et al.*, 1991b). The sequence homology between the *Eco*RI and *Rsr*I endonucleases suggested to us that it could be possible to construct active hybrids of them. Making the hybrids of these two endonucleases we wished to test the structural and functional relatedness of *Eco*RI and *Rsr*I.

SPECIFIC AIMS

- To test the role of two amino acids (I197 and M137) which were predicted on basis of the crystal structure to mediate DNA recognition by the *Eco*RI endonuclease.
- To explore the repair pathway of DNA single strand breaks inflicted by restriction endonucleases.
- To test structural and functional relatedness between the *Eco*RI and *Rsr*I endonucleases by constructing hybrids between the two proteins.

MATERIALS AND METHODS

Bacterial strains and media

E. coli strains used in this work are listed in Table 2. Bacteria were grown routinely in LB liquid medium and on LB agar plates (Sambrook *et al.*, 1989). H-broth containing glucose (Vipond *et al.*, 1996) was used to test viability of strains expressing *Eco*RV endonuclease. Antibiotics were used at the following concentrations: ampicillin (Amp), 100 μg ml^{-1}; kanamycin (Kan), 50 μg ml^{-1}; chloramphenicol (Cam), 25 μg ml^{-1}. Thymidine (50 μg ml^{-1}) was added to media used to grow strains JH117, JH145, which carry Tn*10* insertion in the *thyA* gene linked to the *recB21* mutation. SOS induction was monitored by growing colonies on LB plates supplemented with 35 μg ml^{-1} X-gal and by assessing blue color intensity.

Table 2. *E. coli* strains

Strain	Relevant genotype	Reference
K91	K38 (λ^-)	Heitman and Model (1987)
JH20	K91 *lexA3*	Heitman *et al.* (1989)
JH27	K91 *recA56*	Heitman *et al.* (1989)
JH39	*dinD1*::Mu dI (Amp^r *lac*)	Heitman and Model (1987)
JH59	JH39 *recA56*	Heitman and Model (1987)
JH117	JH39 *recB21 thyA*::Tn*10*	Heitman *et al.* (1989)
JH137	K91 *dinD1*::Mu dI(Amp^r *lac*)	Heitman *et al.* (1989)
JH144	K91 *recN262 tyrA16*::Tn*10*	Heitman *et al.* (1989)
JH145	K91 *recB21 thyA*::Tn*10*	Heitman *et al.* (1989)
JH154	JH39 *lexA3 malE*::Tn*10*	Heitman *et al.* (1989)
JH158	JH39 *recN262 tyrA16*::Tn*10*	Heitman *et al.* (1989)
N2604	*lig ts7*	Gottesman *et al.* (1973)
K1053	(BW313) *Hfr dut1 ung1*	Kunkel *et al.* (1987)
HB101	*recA13*	Boyer, Roulland-Dussoix (1969)
RR1	HB101 $RecA^+$	Maniatis *et al.* (1989)
ER1398	*hsdR2* (r_k^- m_k^+) *mcrB1*	Raleigh (1987)

Plasmids

Plasmids used in mutational analysis of the EcoRI endonuclease

The pBR322-based phagemid pJH15b^{TS6} (Amp^R, Kan^R) carries an allele of the *ecoRIR* gene, which encodes a temperature-sensitive mutant (R56Q) of the *Eco*RI endonuclease (Heitman *et al.*, 1989). Plasmid pJC11 (Cam^R) carries the *Eco*RI methyltransferase gene cloned in the vector pACYC184. It was prepared from pJC1 (Heitman *et al.*, 1989) by *Bam*HI - *Pst*I double digestion, followed by treatment with Klenow polymerase and religation (Fig.2). Plasmids expressing the *Eco*RI endonuclease mutants R200S, R200V, R200C, R200K, A138V, A138T and H114Y have been described (Heitman and Model, 1990).

Plasmids used in the study of DNA single-strand break repair

pMetB (Kan^R) is a pACYC184-based plasmid that encodes the *Eco*RV methyltransferase and a heat-labile (*cI857*) lambda phage repressor (Taylor *et al.*, 1990). pBSKSRV (Amp^R) carries the *ecoRVR* gene, coupled to the λ P_L promoter, in the polylinker site of a derivative of pBlueScriptII KS$^-$ (Vipond and Halford, 1996). In this plasmid the *ecoRVR* gene is inactivated by a 30 bp *Pst*I "stuffer" fragment that had been inserted into the unique *Pst*I site in the coding sequence. It was necessary to maintain the *ecoRVR* gene in an inactive form because pBSKSRVD (see below) was genetically unstable even in the presence of the *Eco*RV methyltransferase (Vermote *et al.*, 1992). pBSKSRVD is the same as pBSKSRV but lacks the "stuffer" fragment, and thus expresses *Eco*RV endonuclease (Vipond and Halford, 1996). pBSKSRVD$^{Q69E+Y138K}$ is similar to pBSKSRVD but encodes a mutant *Eco*RV endonuclease that carries amino acid substitutions at positions 69 and 138. The mutant protein lacks nuclease activity (N. Stanford, pers. comm.). pMetB, pBSKSRV and pBSKSRVD$^{Q69E+Y138K}$ were kindly provided by S. Halford.

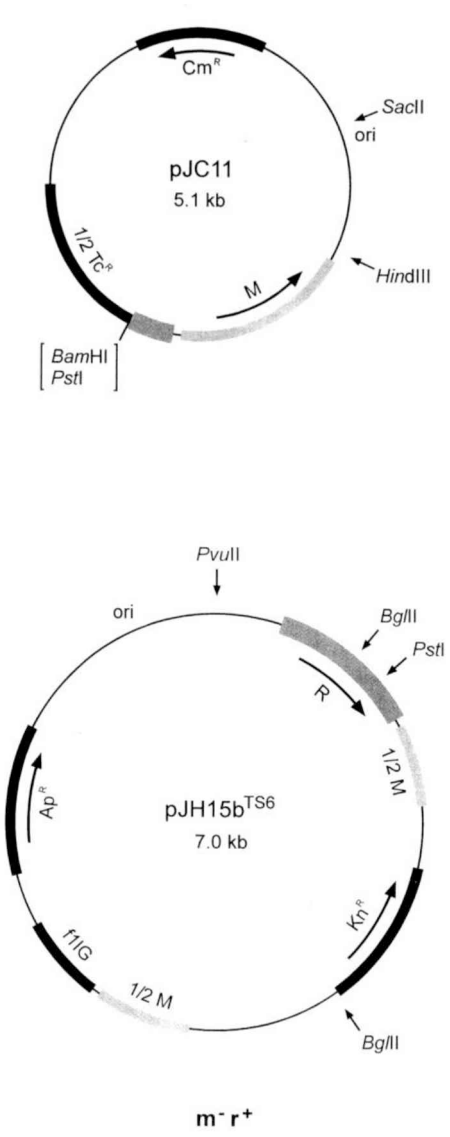

Fig. 2. Schematic map of pJC11 and pJH15b^{TS6}.

To obtain a derivative of pMetB that carries the *E. coli* DNA ligase gene, pLG2520, a plasmid containing the *lig* gene (Ishino *et al.*, 1986), was digested with *Bam*HI and *Nhe*I, the ends of the 2.6 kb fragment encompassing the *lig* gene and a short piece of pBR325 vector DNA, were filled in using Klenow polymerase, then the fragment was cloned in the unique *Bgl*II site of pMetB which had been made blunt ended by a filling-in reaction using Klenow polymerase. Ligase expression from the resulting plasmid (pMetBLG1) was verified by restoration of viability at 42°C of the *E. coli* strain N2604 *lig ts7* (Gottesman *et al.*, 1973).

Plasmids used to construct and analyze EcoRI-RsrI hybrids

To facilitate exchange of fragments between the genes encoding *Eco*RI^{TS6} (*ecoRIR*) and *Rsr*I (*rsrIR*), respectively, a derivative of pJH15b^{TS6} with unique *Hind*III, *Bgl*II and *Pst*I sites in the *ecoRIR* gene was constructed as follows. The *Bgl*II site close to the 5′ end of the kanamycin resistance gene was eliminated by digestion of pJH15b^{TS6} with *Ava*I and *Mun*I, filling-in the ends with Klenow polymerase and religating the large fragment to yield pTS6Δ10. The *Pst*I site in the *bla* gene was eliminated by replacing the *Pvu*II–*Bsu*15I fragment of pTS6Δ10 with the corresponding fragment of pBR322^{m}. pBR322^{m} was made by replacing the *Sca*I–*Eco*31I fragment of pBR322 with the equivalent fragment of pUC18, which lacks the *Pst*I site in the *bla* gene. The intermediate plasmid pTS6Δ11, with single recognition sites for *Bgl*II and *Pst*I, was further modified to have a single *Hind*III site. First pTS6Δ11 was linearized by *Bsu*15I digestion, then, to remove a few nucleotides from the ends, it was subjected to a very short BAL31 exonuclease treatment. The ends were repaired with *E. coli* DNA polymerase I large fragment, then the plasmid was recircularized by T4 DNA ligase to yield plasmid pTS6Δ16 (Fig.3).

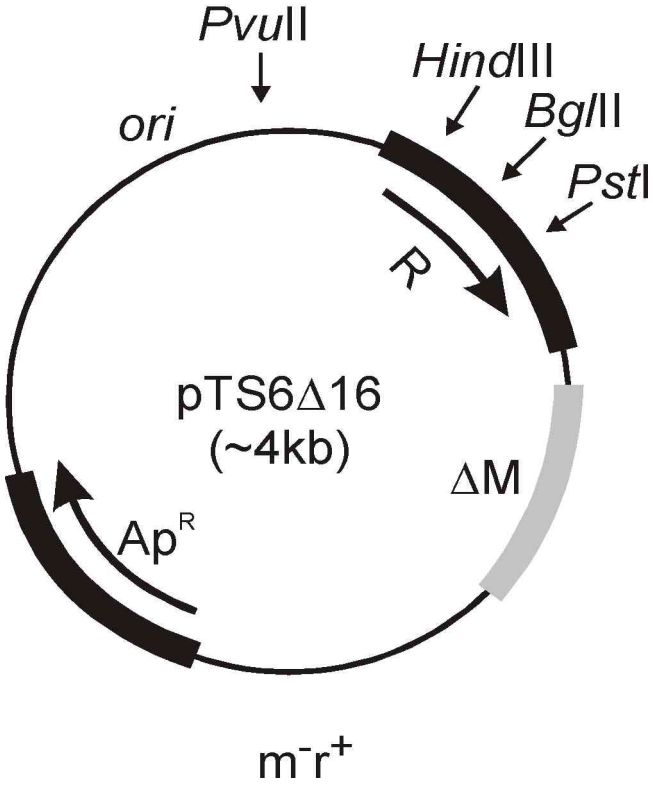

Fig. 3. Schematic map of pTS6Δ16.

Plasmid pTZ18U-*rsrIRM* containing the *rsrIRM* genes (Kaszubska *et al.*, 1992) was obtained from Richard Gumport. The *rsrIR* gene fragments used for construction of the hybrid *ecoRIR-rsrRI* genes were PCR-amplified using pTZ18U- *rsrIRM* as template and appropriate oligonucleotides as primers (Table 3, Fig. 5). The PCR-amplified fragments were cloned first in a pUC18 T-overhang vector prepared as described (Hadjeb and Berkowitz, 1996). Plasmid pTS6Δ17 carries a variant of the *ecoRIR* gene in which the 236bp *Hind*III-*Bgl*II fragment was replaced with the equivalent segment of the *rsrIR* gene. In pTS6Δ18 (Fig.4), the 188bp *Bgl*II-*Pst*I fragment of *ecoRIR* was replaced with *rsrIR* sequence. Plasmid pTS6Δ19 was constructed by inserting the *rsrIR*-derived *Bgl*II-*Pst*I fragment into the pTS6Δ17 backbone.

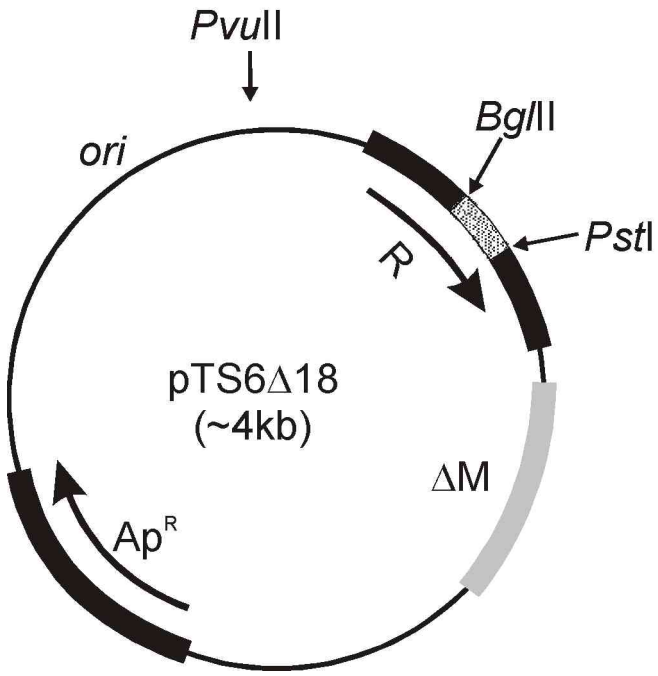

Fig. 4. Schematic map of pTS6Δ18. The segment between the *Bgl*II and *Pst*I sites is of *Rsr*I origin.

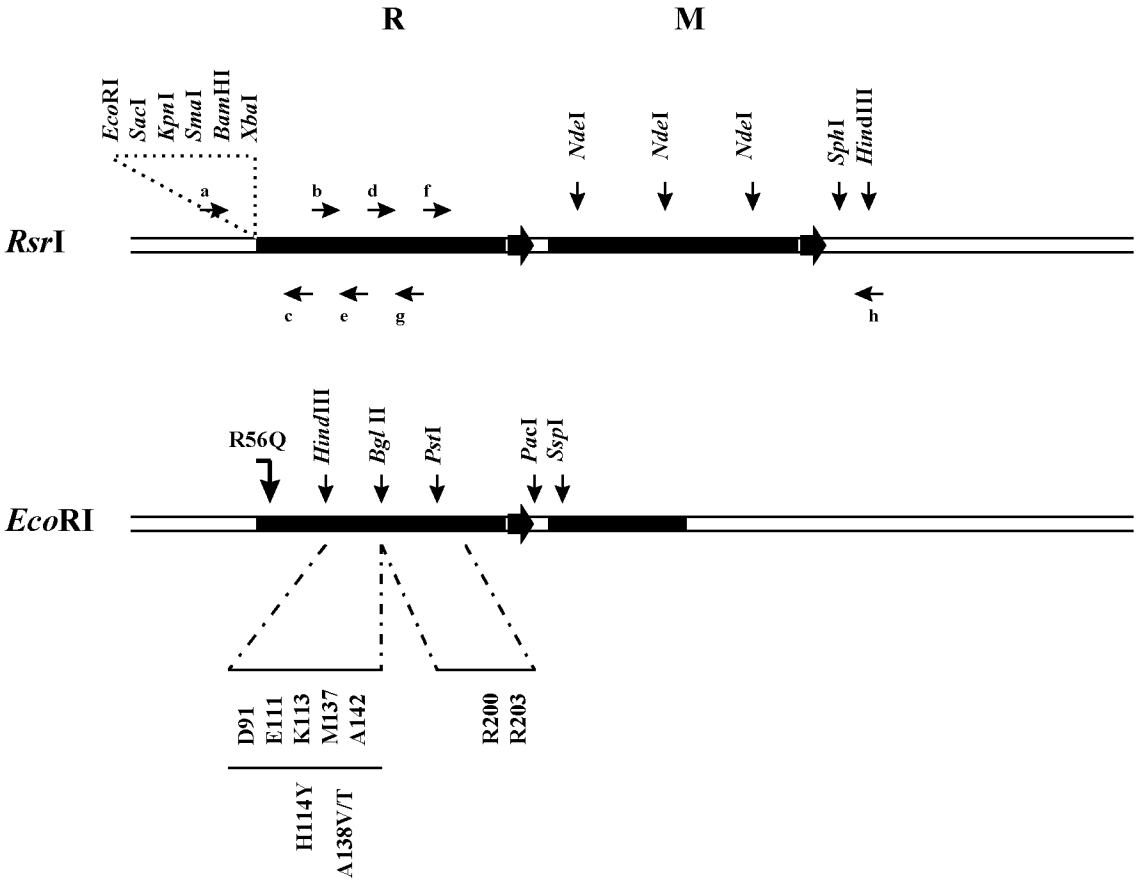

Fig.5. Schematic map of the *rsrIRM* and *ecoRIRM* genes in pTZ18U- *rsrIRM* and pTS6Δ16, respectively. Horizontal arrows indicate positions of PCR primers.

Plasmid pUC-TS6Δ20 carries a derivative of the *ecoRIR* gene in which the 5'-end was replaced with that of the *rsrIR* gene. The 5'-end of the *rsrIR* gene was PCR-amplified using primers **a** and **c** (Fig.5). Then the 413-bp PCR product was cloned into a pUC18*Hinc*II T-overhang vector. A 343-bp *Eco*RI-*Hind*III subfragment encompassing the 5′-end of the *rsrRI* coding region but not the T7 promoter was then excised from this plasmid and cloned into *Eco*RI-, and *Hind*III-digested pUC18 in the orientation allowing transcription from the P_{lac} promoter. The resulting plasmid was designated pUC18-N*rsrIR*. Then the 3'-segment of the *ecoRIR* gene starting at the *Hin*dIII site was added by cloning the *Hin*dIII-*Ssp*I fragment of pTS6Δ16 into this plasmid.

Table 3. Oligonucleotide primers used for PCR

a	agcggataacaatttcacacagga	(-48 primer NEB1#233)
b	aagctttgcgcagctttgatccg	(*Hind*III recognition site)
c	aagcttcgttgattgccttcttgg	(*Hind*III recognition site)
d	agatctcacaagaacgtcctcgaac	(*Bgl*II recognition site)
e	agatctctcaatcgcgttgccc	(*Bgl*II recognition site)
f	ctgcagctagcctgtcacgcgaaat	(*Pst*I recognition site)
g	ctgcagtcacgcggtcgatacgg	(*Pst*I recognition site)
h	gtaaaacgacggccagt	(-20 primer NEB#1211)
AK48	ctcgagatgtctaataaaaaacagtca	(*Xho*I recognition site)
AK49	ctcgagaaataggcgtatcacgag	(*Xho*I recognition site)

The 3'-end of the *rsrIR* gene was amplified using a deletion derivative of pTZ18U-*rsrIR* as template. In this plasmid the two *Nde*I fragments encompassing the internal part of the *rsrIM* gene are deleted. The resulting r^+ m^- plasmid pTZ18U-*rsrIR* was maintained in the cell in the presence of pJC11 expressing *Eco*RI methyltransferase. This deletion placed the hybridization site of primer **h** close to the *rsrIR* gene (Fig. 5). The 338-bp PCR product was cloned into a pUC18 T-overhang vector. Then the resulting plasmid was digested with *Hind*III to release the 333-bp fragment containing the 3'-end of *rsrIR*. The 333-bp *Hind*III fragment was treated with DNA polymerase I large fragment, digested with *Pst*I, then cloned into pTS6Δ16 plasmid, which had been digested with *Pac*I, treated with DNA polymerase I large fragment, and then digested with *Pst*I. The resulting plasmid was named pTS6Δ21. To protect the host DNA from potential nuclease digestion, plasmids encoding *Eco*RI-*Rsr*I hybrids were constructed and maintained in JH140 harboring pJC11. The sequence of PCR-amplified segments of the *rsrIR* gene was verified.

Plasmid pVH1(Kan^R) encodes the $lacI^Q$ repressor and is compatible with plasmids having ColE1 replicon (Haring *et al.*, 1985).

Plasmid pAN4 (Fig.6) carries the genes encoding the wild-type *Eco*RI R-M system (Newman *et al.*, 1981).

Plasmid pER23(-ATG)-EERE (Amp^R) carries the EERE (*eere*) and M·*Eco*RI (*ecoRIM*) genes coupled to the *rrnB* P2 promoter. To construct this plasmid, pAN4 was

digested with *Bgl*II and *Pst*I (there are two *Pst*I sites, Fig. 6). The two large fragments were purified from gel and ligated to the *rsrIR*-derived *Bgl*II-*Pst*I fragment. The resulting plasmid, designated pAN4(EERE) was digested with *Nde*I and *Cla*I. There are two *Nde*I sites, one of them is 33 bp upstream from the *ecoRIR* gene ATG start codon, the unique *Cla*I site is in the vector part, (pBR322), down stream of the *ecoRIM* gene. The ends of this *Nde*I-*Cla*I fragment, which carries the *eere* and *ecoRIM* genes, were filled-in using Klenow polymerase, then the fragment was cloned into the filled-in *Sal*I site of the pER23S (-ATG) expression plasmid. Plasmid pER23S(-ATG) is a derivative of pER23(-ATG) (Lukacsovich *et al*, 1981). It differs from the parental plasmid by a *Sal*I linker inserted into the *Pvu*II cloning site (T. Lukacsovich, unpublished). Genes cloned in this plasmid are transcribed from the *E. coli rrnB* P2 promoter and expression is controlled by the lac repressor.

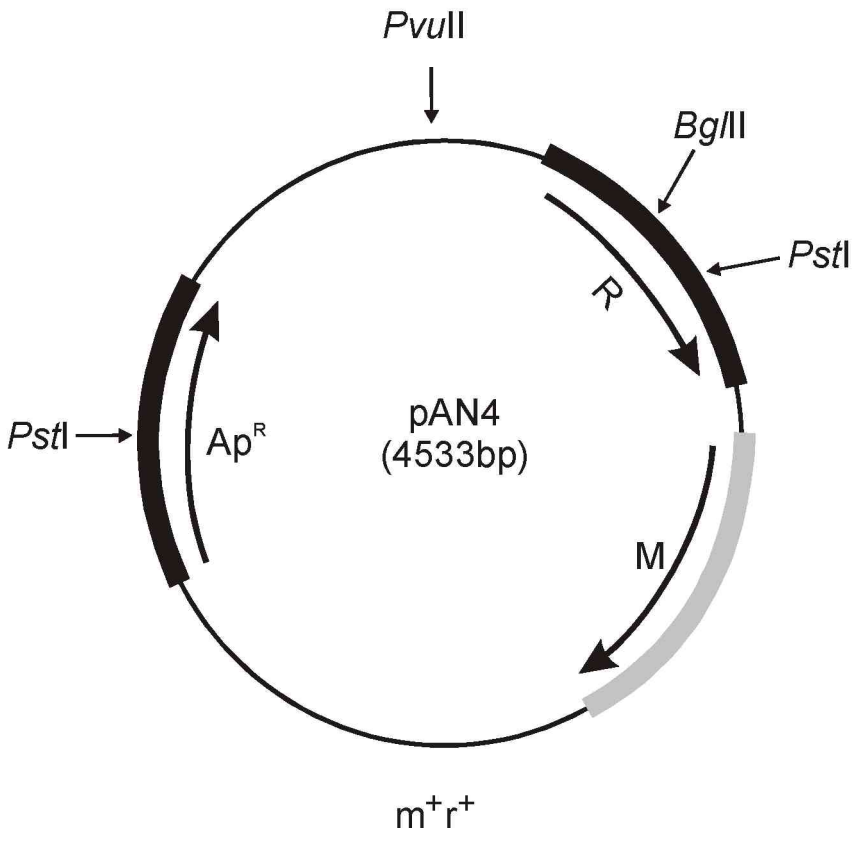

Fig. 6. Schematic map of pAN4.

Plasmid pUC18^{m} differs from pUC18 by the lack of the recognition site for *Eco*RI. First, pUC18 plasmid was linearized by *Eco*RI endonuclease, then the ends of the plasmid were filled-in using Klenow polymerase, then the fragment was recircularized by T4 DNA ligase.

To construct a derivative of EERE with an N–terminal 6×His affinity tail, the *eere* gene (together with the *ecoRIM* gene) was PCR–amplified using pAN4(EERE) template and AK48 and AK49 oligonucleotides as primers. Both primers contain a *Xho*I site (Table 3). The PCR product was cloned in a pBR322(*Eco*RV) T–overhang vector to yield plasmid pBR322-EERE. The structure of the *in vitro* synthesized EERE gene was verified by nucleotide sequencing. The fragment encoding the EERE protein was excised from the plasmid by *Xho*I digestion and cloned into the *Xho*I site of the expression plasmid vector pET3–His (Chen and Hai, 1994) to yield plasmid pET3-His-EERE.

Plasmid pFLAG-MAC-EERE carries the *eere* and *ecoRIM* genes under the control of the P_{tac} promoter. It was constructed by inserting the *Xho*I fragment (bearing *eere* and *ecoRIM* genes) of pET3-His-EERE into the *Xho*I site of the pFLAG-MAC expression plasmid vector (Sigma). The FLAG-*eere* gene junction was verified by sequencing.

DNA techniques

Restriction digestion, agarose gel elecrophoresis and transformation of *E. coli* were carried out using standard procedures (Sambrook *et al.*, 1989). Restriction endonucleases, DNA polymerase large fragment and T4 DNA ligase were purchased from Fermentas. DNA sequence was determined either manually by the chain termination method using a T7 Sequencing Kit (Pharmacia) or by an automated sequencer (ABI). PCR was done by standard procedures (Ausubel *et al.*, 1999). Taq DNA polymerase and Ex Taq^{TM} DNA polymerase were purchased from Fermentas and TaKaRa.

Site-directed mutagenesis of the *ecoRIR* gene

Site-directed mutagenesis of the *ecoRIR* gene was performed by the Kunkel-method (Kunkel *et al.*, 1987). The mutagenic oligonucleotides M137X: 5'- GGA GAT CAA GAT TTA **(AGCT)(AGCT)(GC)** GCT GCT GGT AAT GCT ATC G -3' and I197X: 5'-GAG TAT AAT TCT GGT **(AGCT)(AGCT)(GC)** TTA AAT AGG TTA GAT CG -3' were randomized at positions (in bold) that correspond to the M137 and I197 codons. Reversion oligonucleotide X137M was the same as M137X, but contained the wild-type sequence at the M137 codon. To prepare uracil containing single stranded DNA template, K1053 (pJH15b^{TS6} + pJC11) was infected with the helper phage R176 (Russel *et al.*, 1986). pJH15b^{TS6} template DNA was prepared by polyethylene glycol precipitation and phenol extraction. Synthesis of the second strand was performed using T4 DNA polymerase and the mutagenic oligonucleotides as described before (Heitman and Model, 1990). After the *in vitro* synthesis, the DNA was purified by phenol/chloroform extraction and ethanol precipitation, then it was introduced by electroporation into *E. coli* JH137 (pJC11) cells. Kan^R Cam^R transformants were selected. To prove that the site-directed mutations confer the observed phenotypes, the M137G/A/T/R/W/P/K mutations were reverted to WT by site-directed mutagenesis using the oligonucleotide X137M. The revertants showed the same phenotype as the WT clone. The entire coding sequence of the gene was determined for the following mutants: M137V/R and I197A/W/R. No extraneous mutation was found.

Construction of double mutants

Double-mutants were constructed by capitalizing upon two conveniently located *Bgl*II cleavage sites in pJH15b^{TS6}. One *Bgl*II site lies between the A138 and I197 codons in the *ecoRIR* gene, and the second is located close to the beginning of the gene encoding kanamycin resistance (Heitman *et al.*, 1989). There is no *Bgl*II site in plasmid pJC11. The large and small *Bgl*II fragments were isolated from different plasmids, ligated, and introduced by transformation into JH137 (pJC11) cells. Cam^R Kan^R double-resistant transformants were selected at 37°C. Clones that carried the desired combination of mutations were identified by DNA sequencing.

Phage restriction assay

Unmodified λ_{vir} bacteriophage was prepared by growing the phage on *E. coli* JH137. 5 µl aliquots of a 10-fold serial dilution of the phage stock were applied onto freshly prepared soft agar plates containing 0.1 ml saturated culture of the clones tested. The plates were then incubated overnight at 30 or 42°C. Efficiency of plating was calculated by dividing the phage titer determined on the test strain with the phage titer determined on a restrictionless strain.

Viability test with the strains expressing the R200K mutant *Eco*RI endonuclease

The isogenic host strains JH39 (wild-type), JH158 (*recN262*), JH59 (*recA56*), JH117 (*recB21*), and JH154 (*lexA3*) harbouring the plasmid pJH15a^{R200K} were grown at 42°C to stationary phase, serially diluted, then 20 µl portions were spread on a set of LB agar plates. Colony formation was challenged by incubation at 37°C that is permissive for enzyme activity for different lengths of time, then the plates were shifted to 42°C, and incubation was continued for 24 hrs to determine the fraction of surviving cells.

Viability test with strains expressing *Eco*RV endonuclease

To regenerate the active *ecoRVR* gene, pBSKSRV was cleaved with *Pst*I, recircularized using T4 DNA ligase, then digested with the *Eco*RV isoschizomer *Eco*32I (Fermentas). pBSKSRV has a single *Eco*RV site that is located in the "stuffer" fragment. The purpose of the *Eco*32I treatment was to linearize the plasmids from which the *Pst*I fragment had not been removed (Vermote *et al.*, 1992; Vipond and Halford, 1996). The digested DNA was used to transform *E. coli* cells harbouring pMetB. Amp^R Kan^R transformants were selected at 30°C. Because of potential problems due to the instability of pBSKSRVD (Vermote *et al.*, 1992), experiments were always started from fresh transformants. Single colonies were suspended in 0.5 ml H-broth, then serially diluted using cold H-broth. 0.1 ml aliquots were spread on the surface of H-broth plates, and the plates were then incubated for 18-20 hours at 30 or 42°C.

To test the effect of elevated levels of DNA ligase, JH20, JH27 and JH145 harbouring pMetBLG1 were used as hosts, and colony growth was scored after 18-20 hrs incubation at 30 or 42°C. If no colonies appeared on plates that were incubated at 42°C, the plates were shifted to 30°C for an 18-20 hr incubation to rescue survivors.

Viability test with strains expressing *Eco*RI^{TS6} and EERE hybrid endonuclease

The *E. coli* strains were transformed with pTS6Δ16 (*Eco*RI^{TS6}) and pTS6Δ18 (EERE) at 42°C. Transformants obtained at 42°C were resuspended in 500 µl of ice cold LB medium and plated at 10^4–fold dilution onto prewarmed LB/Amp plates and incubated at 30, 37 and 42°C overnight. The fraction of surviving cells was determined at all temperature tested.

Comparison of *in vivo* DNA degradation by *Eco*RI^{TS6} and EERE

E. coli JH140 harbouring the plasmid pTS6Δ16 (*EcoR*I^{TS6}) or pTS6Δ18 (EERE) was grown at 42°C overnight, then diluted 100 times into fresh LB medium supplemented with ampicillin. Cells were further grown at 30°C for different lengths of time, then harvested by centrifugation and genomic DNA was prepared (Ausubel *et al.*, 1999). Briefly, the cells were gently lysed using SDS, proteins were removed by digestion with proteinase K, cell wall debris, polysacharides, and remaining proteins were removed by selective precipitation with CTAB, then high-molecular weight DNA was recovered from the resulting supernatant by isopropanol precipitation. In parallel, cell viability was also monitored as follows. Aliquots of cell culture were taken at different time points, serially diluted, and 100 µl portions were spread on prewarmed (42°C) LB agar plates. The fraction of surviving cells was determined.

In vitro enzyme assay

Assay of EcoRI endonuclease in crude extracts

Cells from 20 ml overnight cultures were centrifuged then resuspended in 2 ml of extraction buffer containing 50 mM Tris-HCl pH 7.5, 10 mM NaCl and 10 mM $MgSO_4$. Cells were disrupted by sonication, then the extracts were centrifuged for 2 hours at 100,000 g. After adding sterile glycerol to 50 %, the supernatants were stored at –20°C. *Eco*RI endonuclease activity was estimated by digesting 0.5 μg λ phage DNA in *Eco*RI reaction buffer (100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 0.025 % Triton X-100) at 30°C for 1 hour followed by electrophoresis of the digestion products in 1 % agarose gels.

Assay of EcoRV endonuclease in crude extracts

10 ml cultures in LB supplemented with ampicillin and kanamycin were grown at 28 - 30°C to mid-logarithmic phase (OD_{550} ~0.4). Enzyme production was induced by adding an equal volume of fresh, prewarmed (55°C) medium to the cultures, then shaking was continued for 4 hours at 42°C. Crude extract was prepared and *Eco*RV endonuclease activity was titrated using λ phage DNA substrate (Bougueleret *et al.*, 1985).

Assays of EcoRI-RsrI hybrid proteins for endonuclease activity in crude extracts

Preparation of crude extracts was done as described for *Eco*RI endonuclease. Endonuclease activity was estimated by digesting 0.5 μg λ phage and/or pUC18 DNA in either *Eco*RI- or *Rsr*I reaction buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10 mM NaCl, 1 mM DTT) at 30 °C for 30 min or 1 hour.

Immunological detection of EERE by western blotting

Samples from different purification steps were run in SDS–polyacrylamid gels and transferred onto Protran^R Nitrocellulose Transfer BA85 membrane (Schleicher and Schuell) (Ausubel *et al.*, 1999). Western blot analysis was done with goat anti-rabbit IgG alkaline phosphatase conjugate antibody (Sigma) and polyclonal *Eco*RI antisera obtained from J.Heitman.

Protein purification

ER1398 cells harboring plasmids pVH1 and pER23S(-ATG)-EERE were grown at 30°C to OD_{550} ~ 0.5-0.7, then EERE production was induced by adding 1 mM IPTG. Shaking was continued for 5 hours at 30°C. The cells were resuspended in buffer A (20 mM K_2HPO_4, 10mM βME, 1mM EDTA, 0.2% Triton X-100, pH 7.7) containing 0.8 M NaCl and 20 μM PMSF, and disrupted by sonication. This, and all subsequent steps, were carried out at 0 - 4°C. The sonicated cell suspension was dialysed for three 12-h periods against buffer A with 0.4 M NaCl after which the cell debris, together with a precipitate that formed during the dialysis, were removed by centrifugation at 10 000 × g for 10 min. The supernatant left after dialysis and centrifugation was applied to a PC column that had been equilibrated with buffer A containing 0.4 M NaCl. After washing the column with the same buffer, proteins were eluted by a linear gradient of 0.4 M to 1.0 M NaCl in buffer A. Fractions were assayed for endonuclease activity.

RESULTS

I. DNA substrate recognition by the *Eco*RI endonuclease

Site-directed mutagenesis

To facilitate genetic manipulation of the *Eco*RI endonuclease, we used a two-plasmid-system in which the *Eco*RI endonuclease and methyltransferase genes (*ecoRIR* and *ecoRIM*) were cloned on two compatible plasmids. Plasmid pJC11 carries the *ecoRIM* gene (Fig. 2). Plasmid pJH15b^{TS6} encodes a temperature-sensitive variant (R56Q) of the *Eco*RI endonuclease (Heitman *et al.*, 1989; Fig. 2). At 30°C, the *Eco*RI endonuclease encoded by this allele has the same activity as the wild-type enzyme, whereas at 42°C it is inactive. For simplicity, we refer to this variant as wild-type (WT). The plasmid pJH15b^{TS6} can be maintained in the cell at 42°C even in the absence of *Eco*RI methyltransferase (Heitman *et al.*, 1989).

In vitro mutagenized DNA was introduced into *E. coli* JH137 cells harboring the methyltransferase plasmid pJC11. JH137 contains the *lacZ* gene fused to a DNA damage inducible promoter, and was previously used to identify *Eco*RI endonuclease mutants that cleave DNA even in the presence of the *Eco*RI DNA methyltransferase (Heitman and Model, 1990a). Mutants isolated using this assay were shown to cleave DNA at *Eco*RI* (star) sites, i.e. sites that differ from the canonical recognition site by one base pair. Some of the amino acids whose substitution led to relaxed sequence specificity were shown in the revised X-ray structure to be either in direct contact with the target sequence or to be part of the buttressing network of side chain interactions which stabilize the recognition complex (Rosenberg, 1991; Kim *et al.*, 1993). As M137 and I197 (Fig.7) were suggested by the revised X-ray structure to contact the cytosine of the recognition sequence (Rosenberg, 1991), we wished to test whether substitutions of these residues might relax specificity. Therefore, the transformants were plated on X-gal plates and grown at 42°C. After the colonies had become visible, the plates were transferred to 30°C and incubated further. Approximately 500 colonies were tested from both the M137 and the I197 mutagenesis experiments. All colonies tested remained white or light blue at 30°C, indicating that none of the clones encoded *Eco*RI endonuclease mutants with altered specificity. Plasmid DNA was prepared from randomly chosen colonies and the DNA sequence of the mutagenized region determined.

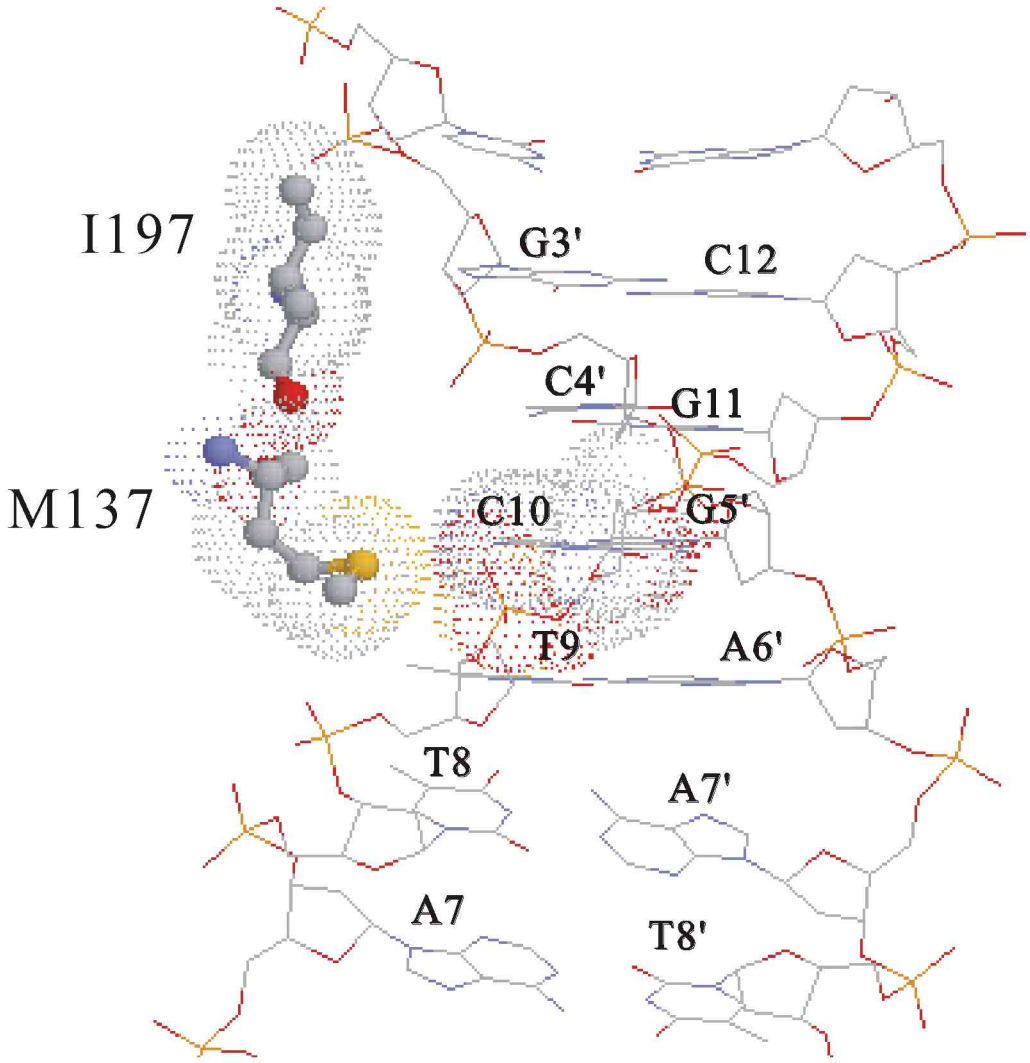

Fig. 7. Schematic view of a part of the *Eco*RI-DNA recognition complex (Brookhaven Data Bank Entry 1ERI). The residues M137 and I197 are shown in the 'ball and stick' representation. Numbers of bases refer to positions in the self-complementary deoxyoligonucleotide $T_1C_2G_3C_4G_5A_6A_7T_8T_9C_{10}G_{11}C_{12}G_{13}$. Van der Waals radii of M137, I197 and C10 are indicated by dotted surface. The figure was generated using the 'RasMol' program written by Roger Sayle.

Substitutions at Met137

A total of eight substitutions at M137 were identified (Table 4). To test whether the M137 substitutions affect the ability of the enzyme to restrict phage growth, the clones were infected with unmodified λ_{vir} bacteriophage. None of the clones with M137 mutant *Eco*RI endonucleases exhibited restriction (Table 4).

Table 4. Phenotypes of Met137 *Eco*RI endonuclease mutants

codon	amino acid	Viability[a]		SOS induction[a]		restriction[b]
		30°C	42°C	30°C	42°C	30°C
ATG	WT	–	+++		LB	10^{-4}
GGG	Gly	–	+++		LB	1
GCC	Ala	–	+++		LB	0.5
GTG	Val	–	+++		LB	1
ACG	Thr	–	+++		LB	0.5
AGG	Arg	+	+++	DB	W	0.5
TGG	Trp	+++	+++	LB	LB	1
CCG	Pro	+++	+++	LB	LB	1
AAG	Lys	+++	+++	W	W	0.5

[a]In the absence of the *ecoRIM* gene.

[b]In the presence of the *ecoRIM* gene.

Titer of an unmodified λ_{vir} phage stock divided by the titer measured on the restrictionless strain JH137(pJC11). Due to the semiquantitative nature of the restriction assay, these are approximate values.

–, no growth; + trough +++, poor to normal growth; DB, dark blue; LB, light blue; W, white;

To test whether these mutant alleles encode active endonucleases, plasmid DNA was purified from the clones, digested with *Sac*II, and reintroduced into the SOS::*lacZ* JH137 cells. Kan^R Cam^S transformants were selected at 42°C. Because the methyltransferase plasmid pJC11 has one *Sac*II site whereas the endonuclease plasmid lacks *Sac*II sites (Fig. 2), this procedure allows reintroduction of the endonuclease plasmid alone. All transformants developed healthy-looking colonies and had normal

growth rates at 42°C. When transferred to 30°C, mutants carrying M137 replacements G, A, V or T were inviable indicating that active *Eco*RI endonuclease was produced and damaged the host cell DNA. One mutant (M137R) grew very slowly and developed a dark blue color on X-gal medium. Three mutants (M137W, M137P and M137K) showed normal growth even at the permissive temperature (Table 4). The effect of M137 replacements was also tested by using extracts of the mutant clones to digest λ phage DNA *in vitro*. None of the extracts prepared from M137 mutants showed detectable *Eco*RI activity under the conditions described in Materials and Methods (not shown).

Substitutions at Ile197

By similar approaches, *Eco*RI endonuclease mutants with ten different substitutions at I197 were isolated (Table 5). When tested at 30 °C in the absence of the *Eco*RI methyltransferase, all of the I197 mutants were lethal. Some replacements (L, M) had no effect on the level of phage restriction compared to WT, whereas others (G, A, Q, R, W) led to a 10- to 100-fold reduction. Two mutations, I197D and I197P completely abolished restriction. Interestingly, the I197V substitution caused a tenfold increase in restriction (Table 5).

Table 5. Phenotypes of Ile197 *Eco*RI endonuclease mutants

codon	amino acid	viability[a]		restriction[b]
		30 °C	42 °C	30 °C
ATA	WT	–	+++	10^{-4}
GTG	Val	–	+++	10^{-5}
TTG	Leu	–	+++	10^{-4}
ATG	Met	–	+++	10^{-4}
GGG	Gly	–	+++	10^{-3}
CAG	Gln	–	+++	10^{-3}
GCG	Ala	–	+++	10^{-3}
TGG	Trp	–	+++	10^{-3}
AGG	Arg	–	+++	10^{-2}
GAC	Asp	–	+++	1
CCG	Pro	–	+++	1

[a]In the absence of the *ecoRIM* gene.

[b]In the presence of the *ecoRIM* gene. For further details see legend of Table 4.

The *Eco*RI activity detected *in vitro* in the extracts of I197 mutants largely paralleled data from the phage restriction assay. Extracts of mutants that exhibited WT or only moderately reduced levels of restriction, yielded complete digestion of λ DNA even after a tenfold dilution, whereas extracts of mutants with more severe reduction of *in vivo* restriction yielded only partial cleavage or did not digest at all (Fig. 8).

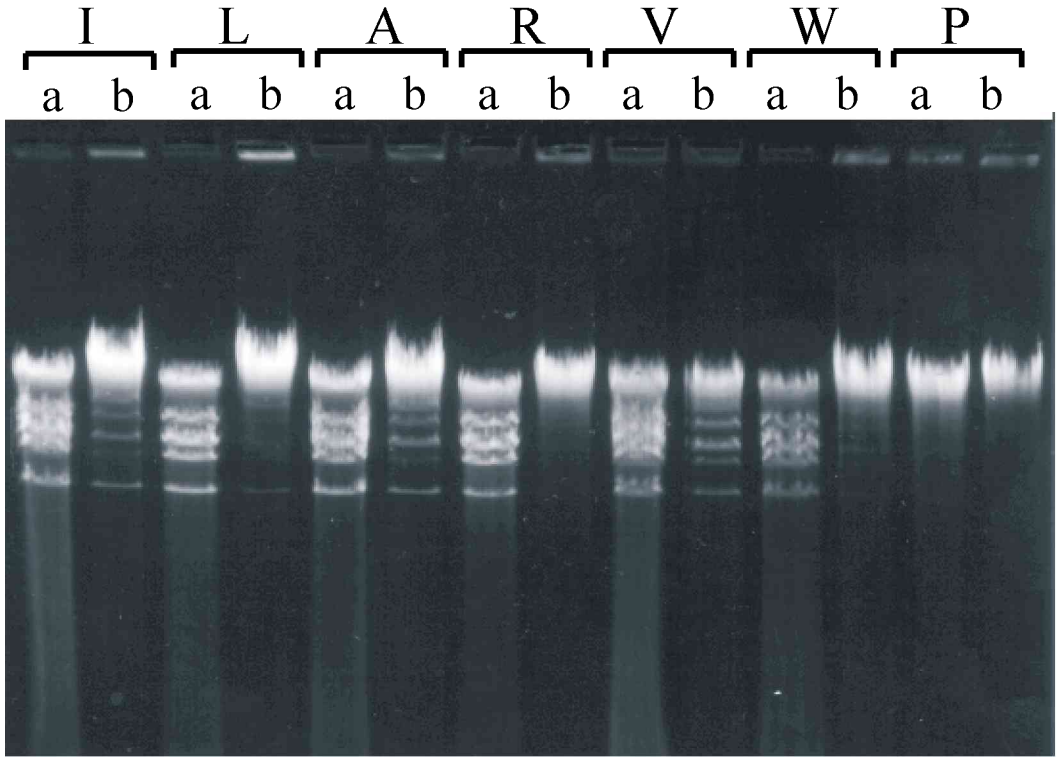

Fig.8. Restriction digest of λ phage DNA with I197 mutant *Eco*RI enzymes. Digestions were performed as described in Materials and Methods using 1.5µl of undiluted (a) or tenfold diluted (b) enzyme extracts. Capital letters above the lanes indicate amino acids at position 197.

Double Mutants

To test how simultaneous substitutions at M137 and I197 affect substrate recognition, two M137X mutations (M137G and M137A) were recombined with two I197X mutations (I197G and I197A). This was accomplished by replacing the smaller *Bgl*II fragment of the M137G/A mutants with that of the I197G/A mutants as described in Materials and Methods. The phenotype of double-mutants M137G + I197G, M137G + I197A and M137A + I197G was the same as that of the respective M137 single mutant: a) the mutant enzymes did not induce the SOS response when tested in the presence of the methyltransferase at 30°C ; b) clones harboring only the endonuclease plasmid were inviable when transferred to 30°C; c) the mutant enzymes did not restrict unmodified phage; d) no *Eco*RI activity could be detected by digestion of λ DNA *in vitro*. The phenotype of the double-mutant M137A + I197A was slightly different. The latter mutant displayed very poor growth and induced the SOS response at 30°C in the

absence of the methyltransferase indicating that substitution by Ala of both M137 and I197 is more deleterious to the *Eco*RI activity than the other three combinations (not shown).

In the crystal structure of the specific *Eco*RI-DNA complex, the guanine base of the substrate is recognized by R200 and R203, which bind a water molecule that donates two hydrogen bonds to N7 and O6 of the guanine (Rosenberg, 1991). To test the effect of simultaneous substitutions at M137 and R200, we constructed recombinants between two M137 mutations (M137G and M137A) and several R200 mutations. We chose R200 replacements which conferred a moderate (R200K) or severe (R200C, R200S, R200V) reduction in *Eco*RI endonuclease activity (Heitman and Model, 1990). The recombinants were constructed by replacing the smaller *Bgl*II fragment as described above. All possible combinations, except M137A+R200V, a total of seven double mutants, were obtained. When tested in the absence of the methyltransferase, these clones grew well and made white colonies on Xgal medium at 30°C, indicating that the *Eco*RI enzymes carrying these mutations were inactive (not shown).

Some replacements of A138 and H114 were shown to result in a "star" phenotype by reducing substrate specificity of *Eco*RI (Heitman and Model, 1990a). This was evident from the poor growth and induced SOS response displayed by the A138V and H114Y mutants even if the bacteria produced the *Eco*RI methyltransferase. The effect of the A138T replacement was even more severe: cells bearing this allele were inviable at 30°C where the R56Q mutant shows maximal activity (Heitman and Model, 1990a; Table 6). According to the crystal structure, the N4 nitrogen of the cytosine donates a hydrogen bond to the main-chain carbonyl of A138 (Rosenberg, 1991). The relaxed specificity displayed by the A138V/T mutants is presumably the result of the perturbation of this sequence-specific contact. H114 does not appear to make contact to any of the bases of the recognition sequence but its location in the structure of the recognition complex led to the hypothesis (Flores *et al.*, 1995) that the relaxed specificity displayed by some H114 replacements might be the result of interference with specific protein - DNA backbone contacts or perturbation of conformational changes needed to couple substrate recognition and cleavage.

Table 6. Effect of I197A substitution on the phenotype of *Eco*RI mutants exhibiting relaxed specificity

amino acid	viability		SOS induction		restriction
	30°C	42°C	30°C	42°C	30°C
WT	+++	+++	W	W	10^{-4}
A138V	++	++	MB	LB	
A138T	–	++		LB	
H114Y	+	++	DB	LB	
WT + I197A	+++	+++	LB	LB	10^{-3}
A138V + I197A	+++	+++	LB	LB	10^{-4}
A138T + I197A	+++	+++	W	W	10^{-4}
H114Y + I197A	++	+++	LB	LB	10^{-1}

All assays were performed in the presence of the *ecoRIM* gene.
For further details see legend of Table 4.

We tested the effect of the I197A mutation on the phenotype of these "star" mutants. Recombinants were constructed by replacing the smaller *Bgl*II fragment of the A138V, A138T and H114Y mutants with that of the I197A mutant. Transformants harboring both pJC11 and the endonuclease plasmid with the double mutations were selected at 42°C. When transferred to 30°C, cells expressing the H114Y + I197A double-mutant enzyme displayed slower growth and made colonies that were translucent and contained filamented cells indicative of DNA damage and SOS induction. Clones with the two other combinations displayed normal growth and colony morphology (Table 6). Phage restriction shown by the double mutants with the A138 substitutions was similar to WT whereas the H114Y+I197A mutant displayed a reduced level (Table 6). As expected, none of the clones was viable in the absence of the methyltransferase at 30°C (not shown).

II. DNA single-strand break repair in *E.coli*

Repair of EcoRV endonuclease-inflicted DNA single-strand breaks is RecA- and RecB-dependent

This project was the continuation of a work done by Joseph Heitman (Duke University, Durham, N.C., USA) with whom we collaborated in projects dealing with the *Eco*RI endonuclease. He found two *Eco*RI mutants (R200K and E144C), which predominantly cut only one strand of the *Eco*RI site. Both mutants exhibited a temperature–sensitive phenotype, cells expressing the mutant endonucleases were viable at 42 °C, but died at 30 °C. Interestingly, *recA*, *recB* and *lexA* hosts were more sensitive to these nicking enzymes than the isogenic wild-type strain. This was in sharp contrast to his previous observation which showed that *recA*, *recB* and *lexA* hosts were no more sensitive to *Eco*RI normal double-strand scissions than the isogenic wild-type strain (Heitman *et al.*, 1989). Because of the change of his research interest and changing funding conditions, Dr. Heitman could not complete the characterization of these mutants.

Therefore when we took over the project, it looked important to repeat some of the viability test to make the data statistically more reliable.

Viability tests were performed with the R200K mutant as described in Materials and Methods. The combined data (obtained by Dr. Heitman and by us) are shown on Fig. 9. The DNA repair and/or SOS induction defective strains were more sensitive to expression of *Eco*RI(R200K) than the wild-type host. To generalize observations made with the *Eco*RI mutants, we tested whether single-strand interruptions generated by another restriction endonuclease (*Eco*RV) require RecA and RecB for repair. It has been shown (Taylor and Halford, 1989) that the *Eco*RV endonuclease can cleave DNA at noncognate sites. Cleavage at noncognate sites is several orders of magnitude slower than cleavage at cognate sites.

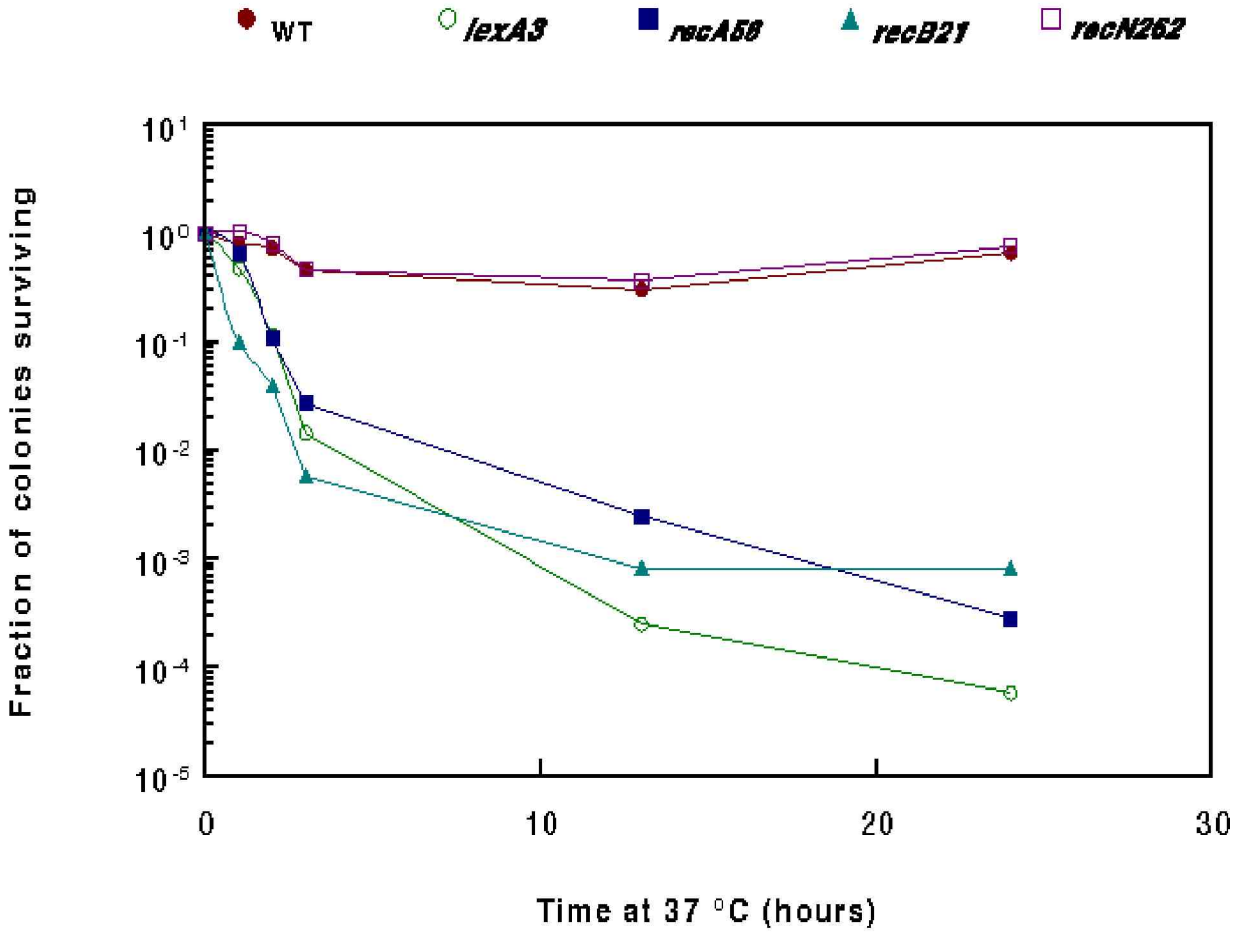

Fig. 9. Sensitivity of mutant *E. coli* strains to DNA scission by the R200K mutant *Eco*RI endonuclease. The isogenic strains JH39 (wild-type), JH158 (*recN262*), JH59 (*recA56*), JH117 (*recB21*), and JH154 (*lexA3*) producing *Eco*RI(R200K) were incubated at 37°C, a temperature partially permissive for enzyme action, then the plates were shifted to 42°C at each of the indicated times to determine the number of surviving cells.

Another difference is that at noncognate sites only one strand is cleaved and the enzyme dissociates from the substrate before it eventually binds to the same site and cleaves the opposite strand in a separate reaction (Taylor and Halford, 1989). *In vivo,* overproduction of the *Eco*RV endonuclease in cells also expressing the *Eco*RV methyltransferase leads to accumulation of single-strand breaks at noncognate sites which are not protected by methylation (Taylor *et al.*, 1990).

The *Eco*RV overexpresion system used in this work consists of two plasmids, pMetB and pBSKSRVD, which encode the *Eco*RV methyltransferase and endonuclease, respectively (Vipond and Halford, 1996). Expression of the *Eco*RV endonuclease in this system is controlled by the temperature-sensitive lambda repressor. To test the involvement of different mechanisms in the repair of *Eco*RV-inflicted nicks, the plasmids were introduced into a set of repair-defective strains. Because the selectable marker on the plasmid carrying the *Eco*RV endonuclease gene confers ampicillin resistance, we used an isogenic set of ampicillin-sensitive host strains: wild-type (K91), *lexA3* (JH20), *recA56* (JH27), *recN262* (JH144) and *recB21* (JH145). The *in vivo* effect of *Eco*RV endonuclease was assayed by determining the number of viable cells at 30 and 42°C, as described in Materials and Methods (Fig.10). A plasmid encoding an inactive mutant version of the *Eco*RV endonuclease (Q69E+Y138K) served as a control.

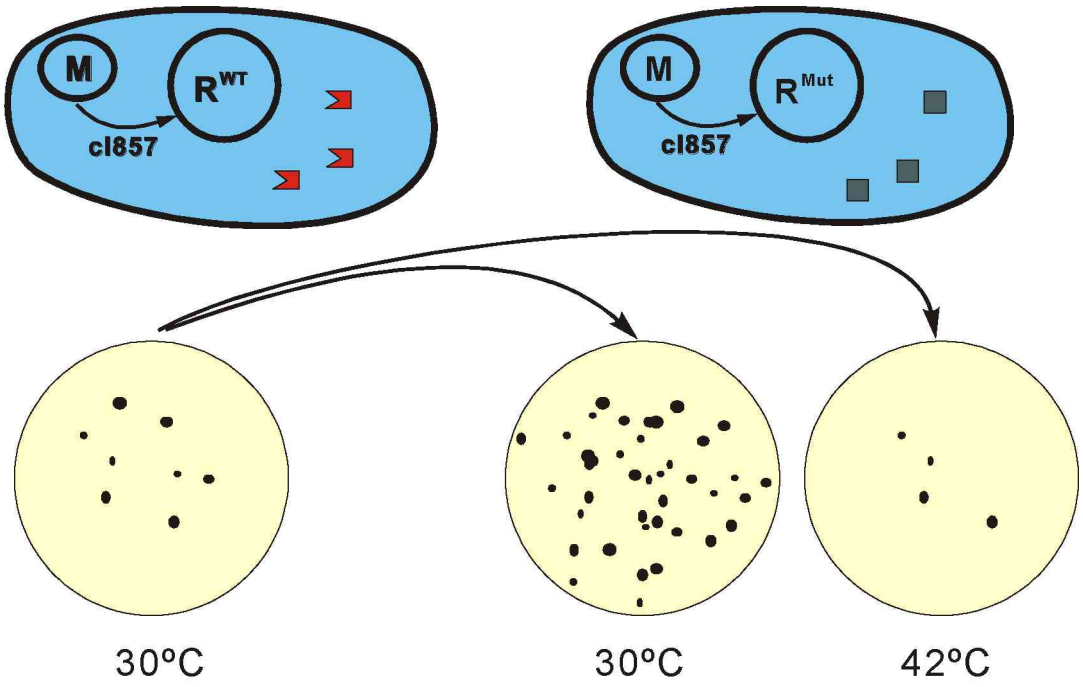

Fig. 10. Scheme of viability test with strains expressing the *Eco*RV endonuclease. M, plasmid expressing *Eco*RV methyltransferase; cI857, heat-labile lambda phage repressor; R^{WT}, plasmid expressing *Eco*RV endonuclease (WT); R^{Mut}, plasmid expressing (Q69K+Y138K) mutant *Eco*RV endonuclease.

The wild-type and the *recN262* mutant strains showed similar colony-forming efficiency at both temperatures indicating that they were not sensitive to increased *Eco*RV endonuclease activity. In contrast, the viability of the *recA56* and *recB21* strains was at least five orders of magnitude lower at 42°C than at 30°C (Table 7). Colonies of the *recA56* and *recB21* strains, which appeared at low frequency at 42°C, were of normal size and morphology. Crude extracts prepared from several such colonies did not digest λ DNA, and thus contained at least a thousandfold lower *Eco*RV endonuclease activity than the wild-type bacteria (not shown). This observation indicated that *Eco*RV endonuclease overexpression was toxic to *recA56* and *recB21* cells. It also suggested that, under the conditions used, *recA56* and *recB21* mutant cells that survived to form colonies were not synthesising active enzyme. Another observation to support this interpretation was that an 8 hr exposure to 42°C was enough to decrease viability by four orders of magnitude and no new colonies appeared if the

plates were further incubated at 30°C. The *lexA3* mutant strain displayed an intermediate phenotype. The number of colonies formed at 42°C was only slightly decreased compared to incubation at 30°C, but at 42°C the colonies exhibited slower growth and altered morphology (unequal size, irregular edges and shape), indicating that *Eco*RV endonuclease overexpression is deleterious to *lexA3* mutant cells.

Bacteria that carried pMetB alone, or pMetB plus the control plasmid expressing an inactive *Eco*RV endonuclease, showed the same colony-forming efficiency and morphology at both the repressing (30°C) and inducing (42°C) temperatures, indicating that the decreased viability was caused by *Eco*RV endonuclease activity rather than by protein overexpression *per se* (Table 7). It must be noted that expression of the wild-type *Eco*RV endonuclease did not cause substantial differences between the wild-type and the repair deficient hosts when viability was tested on LB solid medium, suggesting that faster growth in glucose-containing H-broth makes the cells more sensitive to DNA damage.

These experiments suggested that an increased level of nicks leads to DNA damage that requires a recA- and recB-dependent mechanism for repair. To test if the damage can be prevented by increasing DNA ligase activity in the cell, viability assays were performed with cells that contained pMetBLG1, a derivative of pMetB carrying the *E. coli lig* gene. After a 20 hr exposure to 42°C, *recA56* cells did not form visible colonies and only very small colonies appeared when the *recB21* host was used. However, if these plates were shifted to 30°C, healthy, well-growing colonies appeared and the number of the rescued colonies was similar to that observed on plates that were incubated permanently at 30°C (Table 7). These experiments showed that *Eco*RV overexpression was not lethal to *recA56* and *recB21* cells carrying pMetBLG1, it only arrested or slowed growth and essentially all cells could be rescued by subsequent incubation at the non-inducing temperature. This is in sharp contrast with the observation obtained with cells that contained pMetB, where a much shorter induction (8 hrs) led to a drastic drop in viability (see above). The beneficial effect of the extra copies of the *lig* gene was also evident from the phenotype of the *lexA3* host, in which the colonies exhibited normal morphology at 42°C (Table 7). The reason why the growth rate of the *recA56* and *recB21* hosts was not restored to wild-type level could be that because of uncertainty in the *lig* promoter sequence assignment (Ishino *et al.*, 1986), the fragment that we inserted into pMetB may not contain all sequences necessary for the optimal expression of the *lig* gene.

Table 7. Effect of *Eco*RV endonuclease overproduction on the viability of strains deficient in DNA repair

host	*Eco*RV active	*Eco*RV inactive
K91 WT	0.89	0.9
JH27 *recA56*	$<10^{-5}$	1.1
JH145 *recB21*	$<10^{-5}$	0.87
JH 20 *lexA3*	0.78[a]	0.92
JH144 *recN262*	0.92	0.93
JH27 *recA56* (+*lig*)	0.7[b]	N.D.
JH145 *recB21* (+*lig*)	0.65[c]	N.D.
JH20 *lexA3* (+*lig*)	0.72	N.D.

Viability is defined as the ratio between the numbers of colony-forming units at 42 and 30°C as described in Materials and Methods. The values are the average of 7 to 14 experiments.

a. Colonies of different size and with irregular shape at 42°C.

b. Colonies appeared if subsequent to exposure to 42°C, the plates were incubated at 30°C.

c. Tiny colonies.

N.D. not determined.

III. Construction of *Eco*RI-*Rsr*I recombinant endonucleases

We have constructed *Eco*RI-*Rsr*I hybrid proteins by replacing segments of the *ecoRIR* gene with the corresponding, PCR-amplified segments of the *rsrIR* gene. To construct the chimeric proteins, we took advantage of three restriction sites (*Hin*dIII, *Bgl*II and *Pst*I), which divide the *ecoRIR* gene in four segments (Fig. 3). All three sites are located in regions characterized by high level of amino acid sequence similarity between *Eco*RI and *Rsr*I. By selecting these conserved regions for recombining the two proteins we wanted to minimize the structural perturbation caused by the fusion. Oligonucleotide primers containing *Hind*III, *Bgl*II and *Pst*I sites as a 5'-extension were designed for PCR-amplification of the *rsrIR* gene segments (Table 3). The PCR-products were first cloned in a T-overhang vector, then the fragment was excised and inserted into plasmids carrying the rest of the *ecoRIR* gene as described in Materials and

Methods. Five *Eco*RI-*Rsr*I hybrids were constructed (Table 8). The chimeric proteins were named according to their structure with regard to the origin of the fused parts (E, *Eco*RI segment, R, *Rsr*I segment).

Table 8. Plasmids encoding *Eco*RI-*Rsr*I hybrid endonucleases

plasmid	substituted fragment	structure of the protein
pTS6Δ16	none	EEEE
pTS6Δ17	*Hind*III - *Bgl*II	EREE
pTS6Δ18	*Bgl*II - *Pst*I	EERE
pTS6Δ19	*Hind*III - *Pst*I	ERRE
pUC-TS6Δ20	N- terminus	REEE
pTS6Δ21	C - terminus	EEER

In vivo characterisation of the *Eco*RI-*Rsr*I recombinant proteins

First, we determined whether the hybrids possessed nuclease activity. To protect host DNA from *Eco*RI-specific endonuclease activity, plasmids encoding the hybrid endonucleases were initially maintained in JH140 harboring pJC11 (m+ host). To test the effect of the hybrids on viability of host cells whose DNA was not protected by *Eco*RI-specific methylation, plasmid DNA preparations were digested with SacII, then transformed into JH140. Plasmid pJC11 has one *Sac*II site, whereas the plasmids encoding the hybrids do not have *Sac*II site. Amp^R, Cam^S transformants were selected at 42°C. All hybrids except REEE contained the substitution R56Q, a mutation which made the parental *Eco*RI mutant (TS6) inactive at 42°C, thus it seemed conceivable that the hybrids would also be inactive at this temperature. Growth of the transformants was then tested on agar plates at 37 and 30°C. Recombinant endonucleases REEE, EREE, EEER and ERRE proved inactive at all temperatures tested by the criteria that JH140 cells, which contained the plasmid with the hybrid endonuclease gene, but did not produce M·*Eco*RI, were viable. Cells with the EERE construct (pTS6Δ18) were viable only when *Eco*RI methyltransferase was present, indicating that the EERE recombinant endonuclease was active.

Fortunately, EERE exhibited TS phenotype which facilitated study of its properties *in vivo*. The JH140 strain carries an SOS::*lacZ* fusion that enabled us to monitor SOS induction as an indication of DNA damage caused by EERE. JH140 carrying either pTS6Δ18 (EERE) or pTS6Δ16 (*Eco*RI^{TS6}) was transferred on X-gal indicator plates, then growth, colony morphology and color was assessed at three different temperatures. In an m^- host at 42°C, expression of neither *Eco*RI^{TS6}, nor EERE led to SOS induction or affected growth. At 30°C, both *Eco*RI^{TS6} and EERE were lethal. At 37°C, production of EERE was slightly more deleterious than that of *Eco*RI ^{TS6}. This was evident from the size as well as from the appearance of the colonies (Table 9). In an m^+ host, *i.e.* a host expressing *Eco*RI methyltransferase, EERE production did not affect growth, nor did it induce the SOS response even at 30°C, suggesting that the recombinant endonuclease had canonical *Eco*RI specificity.

Table 9. Effect of EERE and *Eco*RI^{TS6} expression on viability and SOS induction in JH140

	30°C		37°C		42°C	
	growth	SOS	growth	SOS	growth	SOS
(m^-) *Eco*RI^{TS6}	-		+++	MB	++++	LB/W
(m^-) EERE	-		++ (*)	MB	++++	LB/W
(m^+) *Eco*RI^{TS6}	++++	LB	++++	LB/W	++++	LB/W
(m^+) EERE	++++	LB	++++	LB/W	++++	LB/W

++++, healthy; - , inviable; *, flat, translucent colonies
W, white; LB, light blue; MB, medium blue;
The m^+ host contained pJC11.

To see whether the observed *in vivo* effect was dependent on a particular genetic background, we tested other host strains. RR1, a K12/B hybrid strain was known to be tolerant to expression of some restriction endonucleases when the corresponding methyltransferase was absent (G. Wilson, pers. comm.). We compared the effect of *Eco*RI^{TS6} and EERE on viability of RR1 and of its *recA* derivative HB101 (Table 10). EERE was found to be more toxic to either strain than *Eco*RI^{TS6}. Taken together, viability tests performed with different hosts strains suggested that, at permissive

temperatures, the EERE protein might have similar or even slightly higher nuclease activity than *Eco*RI^{TS6}.

Table 10. Effect of EERE and *Eco*RI^{TS6} expression on viability of RR1 and HB101

	30°C	34°C	37°C	42°C
RR1 (*Eco*RI^{TS6})	++	N.D.	++++	++++
RR1 (EERE)	-	N.D.	++++	++++
HB101 (*Eco*RI^{TS6})	-	++	+++	+++
HB101 (EERE)	-	-	+	+++

-, no growth; ++, poor growth; +++, normal to good growth; N.D., not determined

Table 11. Effect of EERE expression on viability of strains defective in DNA repair and/or SOS induction

	37°C	42°C
K91 (wt)	+	++++
JH20 (*lexA*)	-	+++
JH27 (*recA*)	-	+++
JH145 (*recB*)	-	+++

Cells contained pTS6Δ18.

-, no growth; +, poor growth; +++, normal to good growth; ++++, very good growth;

HB101 was found to be more sensitive to EERE than RR1, which suggested that DNA damage caused by EERE requires DNA recombination for repair. To address this question in a more systematic fashion, we tested the effect of EERE on K91 and its isogenic, repair-defective derivatives. The DNA repair-, and/or SOS induction-defective strains were more sensitive to EERE than the WT parental strain (Table 11). This was a surprising observation because, in an earlier study, the repair-defective strains JH27, JH20 and JH145 were no more sensitive to DNA scission by *Eco*RI^{TS6}, than the isogenic parent K91 (Heitman *et al.*, 1989). The greater sensitivity of repair-defective strains to EERE suggested that the DNA lesions caused by this protein might be different from normal *Eco*RI double-strand cuts.

We tested the *in vivo* function of EERE by measuring restriction of unmodified λ_{vir} phage. JH140 harboring pJC11 and pTS6Δ18 did not show phage restriction at 30°C. Under the same conditions JH140 (pJC11 + pTS6Δ16) had a restriction ratio of 10^{-4} . Later we found that no restriction could be achieved even if EERE was overproduced (pER23S(-ATG)-EERE).

***In vitro* characterization of EERE**

Crude extracts prepared from JH140 (pJC11+pTS6Δ18) and JH140 (pJC11+pTS6Δ16) grown at 30°C were assayed for restriction enzyme activity using *Eco*RI and *Rsr*I reaction buffers. Under the conditions of the assay (see Materials and Methods), a tenfold diluted extract prepared from JH140 (pJC11+pTS6Δ16) yielded almost complete digestion of λ DNA, whereas no specific endonuclease activity was detected in the JH140 (pJC11+pTS6Δ18) extract even if it was used undiluted (not shown). Taking into account the sensitivity of the method, we can conclude that the *Eco*RI-specific activity in JH140 (pJC11+pTS6Δ18) extract was at least 1000 times lower than in extracts of control cells producing $Eco\text{RI}^{TS6}$.

To purify EERE for *in vitro* characterization, we constructed the overexpression plasmid pER23S(-ATG)-EERE, in which the *eere* gene is transcribed from the *E. coli rrnB* P2 promoter. SDS-polyacrylamide gel electrophoresis of samples from IPTG-induced cultures revealed the appearance of a band with a mobility corresponding to the calculated molecular mass of EERE (not shown).

Purification of EERE turned out to be a very difficult task. Inspite of considerable effort, we could not purify the protein using conventional techniques. We think that the most likely reason for the failure is the low solubility of EERE. This conclusion was drawn from a comparison, by SDS polyacrylamide gel electrophoresis, of samples prepared from whole cells lysed by SDS, or from supernatants of centrifuged extracts: the large majority of EERE was in the fraction soluble only in SDS (data not shown). Several purification techniques were tried. The method, which gave the best results and yielded a partially purified EERE preparation, was a modified version of one of the procedures described for the *Eco*RI endonuclease (Luke *et al.*, 1985). A key factor in this procedure was the use of high salt concentration in the extraction buffer, and in the dialysis step. The salt probably increased solubility of the protein. The dialysed extract was fractionated by phosphocellulose chromatography as described in Materials and

Methods. The fractions eluted from the PC column were assayed for endonuclease activity using pUC18 DNA. Several digestion conditions were tried (*Eco*RI buffer, *Rsr*I buffer, 37°C, 30°C). No specific endonuclease activity was observed in any of the PC fractions tested. However, in some fractions (eluting around ~ 0.6-0.7 M NaCl), the plasmid migrated more slowly than in other fractions, and had a blurred appearance (Fig. 11). This effect was especially evident if *Rsr*I buffer was used in the reaction. Because, in lack of cleavage activity, the EERE protein could not be detected, we tried to identify EERE-containing fractions using an immunological method. Samples of the PC fractions were analyzed by Western blotting using antibody raised against *Eco*RI endonuclease. This analysis showed that fractions causing the gel shift contained immunologically reactive material which, in an SDS-polyacrylamide gel, had electrophoretic mobility consistent with the calculated molecular mass of EERE (not shown).

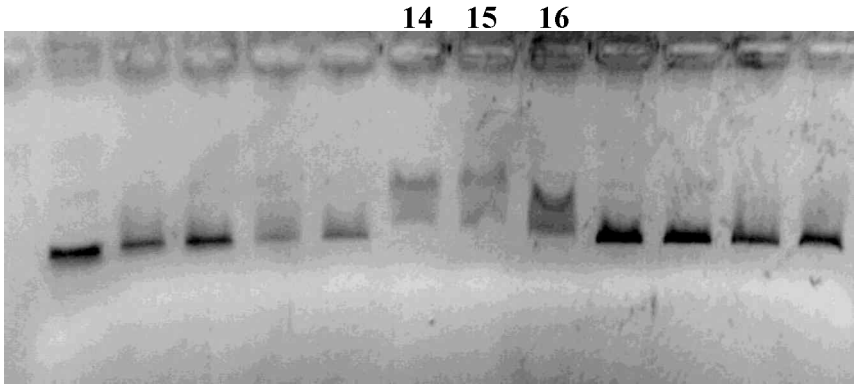

Fig.11. Electrophoretical DNA mobility shift in agarose gel caused by EERE (PC fractions 14,15,16). Reactions were performed in *Rsr*I buffer with Mg^{2+}. pUC18 (one *Eco*RI site) was used as substrate.

We tested whether the observed electrophoretic retardation was dependent on the presence of the *Eco*RI site in the plasmid. Plasmid $pUC18^{m}$, in which the *Eco*RI site had been destroyed, was tested under similar conditions, and was found to be shifted to a much lesser extent than the parental plasmid (Fig. 12), indicating that the protein has higher affinity to *Eco*RI sites, than to other sequences. Because the plasmid DNA used

in these experiments was mostly supercoiled, we also tested if the observed retardation was dependent on supercoiled conformation. Electrophoretic mobility of pUC18 and pUC19 linearized by *Ssp*I digestion was retarded in the same way as that of the supercoiled forms (Fig. 13). The retardation effect was evident with a larger, 13 kb plasmid (pES1, 7 *Eco*RI sites, A. Kiss pers. comm.), but it was hardly detectable with λ DNA (~50 kb).

All these *in vitro* experiments indicated that EERE has no nuclease activity. It is, however, possible that the reaction conditions used were far from optimal. Alternatively, the hybrid endonuclease might be active only on *Eco*RI sites flanked by particular nucleotides. We have preliminary data indicating that supercoiled pUC18 and pUC19 are cleaved at a very low rate. This cleavage was detected when proteins were removed by phenol extraction before loading the samples on agarose gels (not shown). In this experiment removal of the proteins allowed all plasmid forms to be resolved on the gel without the obscuring effect of bound protein(s). No cleavage was detected when linear plasmid DNA was used.

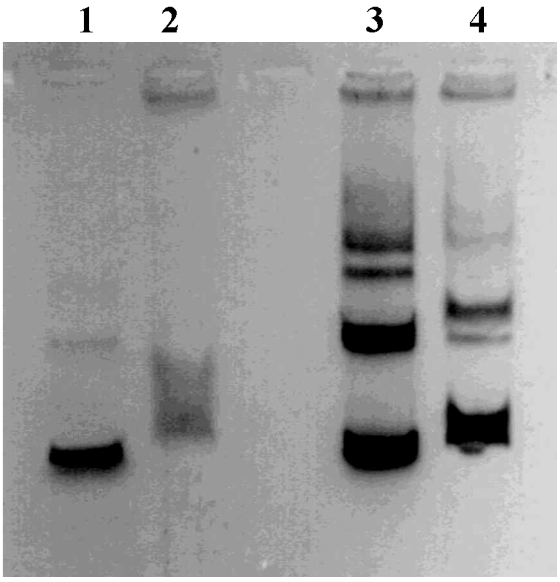

Fig.12. Electrophoretical mobility shift of DNA in agarose gel caused by a partially purified EERE preparation (phosphocellulose fraction). pUC18^{m} lacks *Eco*RI site
1. pUC18; 2. pUC18+EERE, 3. pUC18^{m} ; 4. pUC18^{m}+EERE;

Although these *in vitro* experiments provided some useful data about EERE, it became clear that proper biochemical characterization of the protein and understanding of its phenotypes will require preparations of much higher purity. Attempts to further purify the PC fractions using other chromatographic methods have failed. After the failure with conventional chromatographic methods, we decided to try to purify EERE by affinity chromatography. Two plasmids were constructed (see Materials and Methods). Plasmid pET3-His-EERE carries the *eere* gene fused to a sequence encoding an N-terminal Hisx6 affinity tag. The other plasmid (pFLAG-MAC-EERE) encodes an EERE variant, which carries the FLAG octapeptide (Sigma) as an N-terminal fusion. Experiments to purify the affinity-tailed variants are in progress. The FLAG fusion seems to be especially promising: preliminary observations indicate that the fusion protein has higher solubility, than EERE. We hope that the fusion variants will facilitate purification and characterization of the protein.

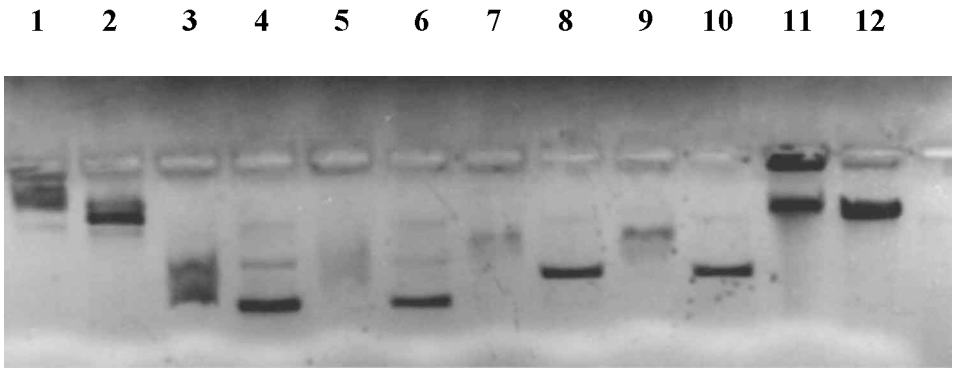

Fig.13. Electrophoretical mobility shift of DNA in agarose gel. Reactions were performed in *Rsr*I buffer (Mg^{2+}).

1. pES1 + EERE; 2. pES1; 3. pUC19 + EERE; 4. pUC19;

5. pUC18 + EERE; 6. pUC18; 7. pUC19*Ssp*I + EERE;

8. pUC19*Ssp*I; 9. pUC18*Ssp*I + EERE; 10. pUC18*Ssp*I;

11. λ DNA + EERE; 12. λ DNA;

Degradation of host DNA by EERE *in vivo*

There seemed to be a contradiction between the observed properties of EERE. Viability studies suggested that it had strong nuclease activity. This was not confirmed by phage restriction experiments and by *in vitro* observations. We hypothesized that the toxic effect of EERE *in vivo* was due to very strong binding of the protein to *Eco*RI sites rather than to cleavage of *Eco*RI sites in the host DNA. To address this question, we compared the state of the host DNA in cells expressing EERE or *Eco*RI^{TS6}. Cultures of JH140 harboring either pTS6Δ16 or pTS6Δ18 were grown at 42°C overnight, then diluted 1:100 into 50 ml of LB containing 100 µl/ml ampicillin and grown further for 3 and 5 hours at 30°C. As expected, the viable cell number dropped sharply in both cultures after the transfer to the lower temperature (Table 12). Total DNA purified from pTS6Δ16-containing cells showed extensive degradation, whereas DNA extracted from pTS6Δ18-containing cells was much less degraded (Fig. 14), suggesting that it is not the nuclease activity that accounts for the observed high toxicity of EERE.

Table 12. Dependence of JH140 viability on the length of exposure to a temperature (30°C) permissive for EERE or *Eco*RI^{TS6} action

	0 h	3 h	5 h
pTS6Δ16	1.29	1.09×10^{-4}	5.4×10^{-7}
pTS6Δ18	1.18	5.04×10^{-5}	6.7×10^{-7}

Viability is defined as the ratio between the number of colony-forming units at 30°C and 42°C as described in Materials and Methods. The values are the avarage of 3-4 experiments.

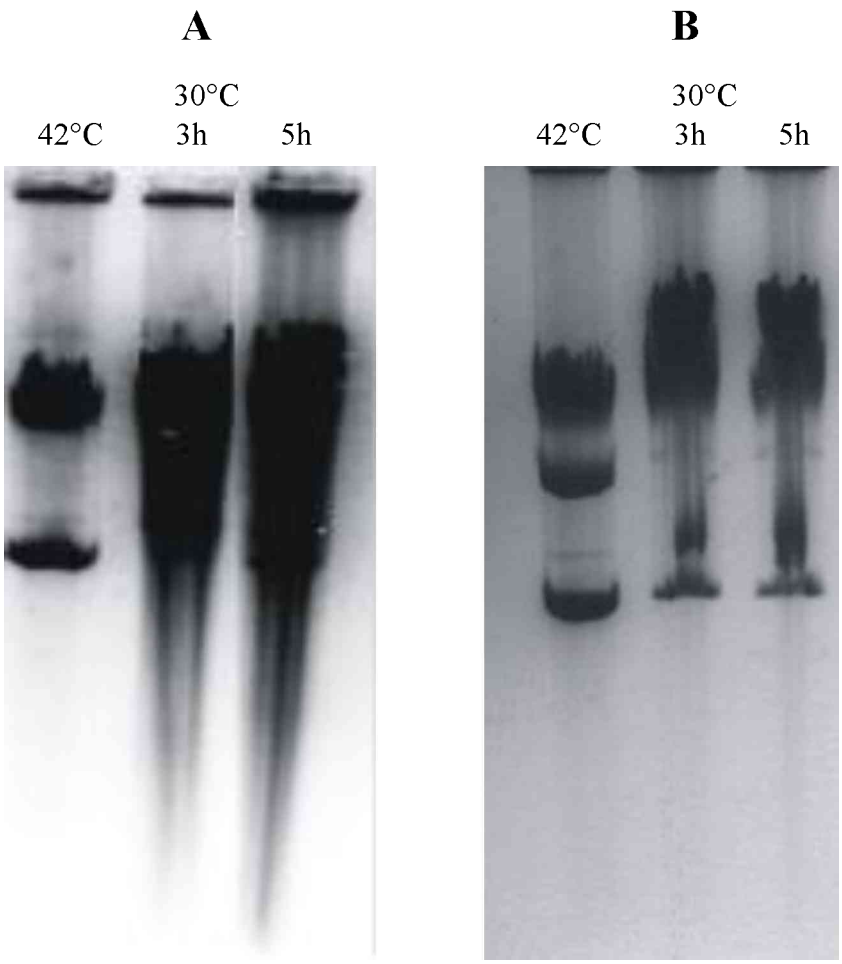

Fig. 14. Comparison of *in vivo* DNA degradation by *Eco*RI^{TS6} (**A**)and EERE (**B**). Genomic DNA was prepared and resolved in agarose gel as described in Materials and Methods.

DISCUSSION

I. DNA substrate recognition by the *Eco*RI endonuclease

Analysis of the crystal structure of the *Eco*RI-DNA complex led to a molecular model in which the GC base pair is recognized by residues R200, R203, M137, A138 and I197 (Kim *et al.*, 1990; Rosenberg, 1991). Results obtained from previous mutagenesis studies support the role of R200, R203 and A138. R200 substitutions decreased enzymatic activity but did not alter the specificity (Needels *et al.*, 1989; Heitman and Model, 1990). Replacement of R203 with Gln or Lys inactivated *Eco*RI (Yanofsky *et al.*, 1987; Heitman, 1992). *Eco*RI variants with A138V and A138T replacements displayed relaxed recognition specificity (Heitman and Model, 1990a).

In this study we used site-directed mutagenesis to introduce multiple substitutions at M137 and I197. *Eco*RI activity in the mutants was assessed using three *in vivo* assays: phage restriction, cell viability, and SOS induction as an indication of *Eco*RI-inflicted DNA damage. These tests measure different ranges of enzyme activity. Because viability and the SOS test monitor effects of DNA scission that occur in the cellular DNA, they can detect levels of *Eco*RI endonuclease activity that are too low to restrict phage infection. When performed in the presence of the methyltransferase, the viability and SOS tests can detect *Eco*RI variants with altered recognition specificity (Heitman and Model, 1990a). To confirm *in vivo* data, cell-free extracts were prepared from the mutants and used for digestion *in vitro* to estimate *Eco*RI activity.

In general, M137 mutations had a more drastic effect than I197 mutations. Whereas all M137 replacements abolished phage restriction, only the I197D and I197P mutations resulted in a restrictionless phenotype. Three M137 mutants (W, P and K) were viable even in the absence of the methyltransferase indicating that the mutant *Eco*RI enzymes bearing the replacements M137W/P/K were inactive. In contrast, all I197 mutants were inviable under the same conditions. The effect of I197 substitutions on restriction capacity largely reflected the chemical nature of the respective amino acid side chains, i. e. conservative replacements tended to have weaker effects than non-conservative ones.

As substitution of A138 led to relaxed sequence specificity (Heitman and Model, 1990a), we expected that substitutions of M137 and/or I197 might also alter recognition specificity. However, none of the single mutants isolated in this study induced the SOS

response in the presence of the *Eco*RI methyltransferase, indicating that all retained canonical recognition specificity. The phenotype of double-mutants that were designed to perturb both postulated van der Waals contacts to the cytosine base (M137A/G+I197A/G) also did not show altered specificity. It must be noted, however, that in this case, as well as in the case of M137X single mutants, the low overall activity may have prevented detection of relaxed specificity. The phenotype of the M137A/G+R200K/C/V/S double-mutants indicates that disruption of contacts to both members of the GC base pair inactivates the enzyme rather than alters its specificity. The lack of change in substrate specificity was not surprising. It has been demonstrated for several residues, whose function in sequence-specific recognition by the *Eco*RI endonuclease is well-established, that replacements did not result in relaxed-specificity phenotype (Needels *et al.*, 1989; Heitman and Model, 1990; Osuna *et al.*, 1991). The general picture emerging is that, for restriction enzymes - unlike most DNA binding proteins - sequence specificity is overdetermined due to redundant contacts to the bases and the DNA backbone. Therefore, elimination of a specific contact does not necessarily lead to altered specificity (Jeltsch *et al.*, 1996).

Combination of the A138V/T mutations with the I197A mutation resulted in the mutual suppression of the phenotypes of the parental single-mutants, that is both the decreased restriction capacity of the I197A mutant and the decreased specificity of the A138V/T mutants were restored to WT levels. Mutual suppression of phenotypes between binding site and "star" mutants has been observed before (Heitman and Model, 1990a). An important difference is that while both the A138V/T and the H114Y "star" mutations exhibited mutual suppression in combination with some R200X mutants, only the A138V/T mutations suppressed the phenotype of the I197A mutant. On the other hand, I197A suppressed - albeit to different extents - "star" activity of the A138V/T and H114Y mutants. This finding suggests a difference in the molecular mechanisms underlying the suppression observed with different "star" mutations.

In conclusion, our observation that substitution of M137 leads to a severe loss of activity of the *Eco*RI endonuclease is consistent with the role predicted for M137 (Rosenberg, 1991; Kim *et al.*, 1993). Interpretation of the experiments with the I197 mutations is less straightforward. Although the mutual suppression between the A138V/T binding site mutations and the I197A mutation suggests the involvement of I197 in substrate recognition, the fact that even some non-conservative replacements of I197 were compatible with substantial levels of *Eco*RI activity strongly argues against

such a model. Based on these findings, we conclude that I197 is not directly involved in substrate recognition by the *Eco*RI endonuclease. This notion is also supported by the fact that M137 is conserved in the highly homologous isoschizomer *Rsr*I endonuclease whereas I197 is not (Stephenson *et al.*, 1989). Further refinements of the *Eco*RI endonuclease-substrate structure may be required to resolve this discrepancy.

II. DNA single-strand break repair in *E.coli*

During studies of substrate recognition by the *Eco*RI endonuclease, Heitman and Model isolated *Eco*RI mutants with alterations in the substrate binding pocket (Heitman and Model, 1990). *In vitro* the purified mutant enzymes are greatly reduced in specific activity and, unlike the wild-type enzyme, produce mainly nicked DNA. They tested how the DNA lesion inflicted by these mutants is repaired *in vivo*. Plasmids encoding the mutant *Eco*RI endonucleases were introduced into a set of repair defective strains that did not produce *Eco*RI methyltransferase. This approach was possible because the temperature-sensitive phenotype of the mutants allowed to control the expression of the *Eco*RI endonuclease activity by shifting the temperature of culture growth. The effect of *Eco*RI endonuclease mutants was investigated by two methods: assaying the SOS response as an indication of DNA damage in a repair proficient host and determining the viability of repair-defective strains under conditions partially or fully permissive for enzyme activity.

These experiments demonstrated that expression of the R200K and E144C mutant enzymes results in DNA damage. SOS induction could be blocked by *Eco*RI-specific methylation indicating that the DNA damage inflicted by the mutant enzymes was due to cleavage at cognate *Eco*RI recognition sites. Viability tests showed that expression of the R200K and E144C mutant endonucleases was toxic to strains defective in RecA, RecB, or LexA functions. Because the purified mutant enzymes predominantly nick DNA *in vitro*, it was suggested that the toxic effect on *recA56*, *recB21* or *lexA3* repair-defective strains can be attributed to DNA nicks. Another conclusion drawn from these experiments was that nicks, or more likely the DNA lesions they are converted to, require a RecA-, RecB- and LexA-dependent pathway for repair. It was considered an alternative model in which the mutant enzymes slowly release the nicked DNA, blocking repair by simple ligation and necessitating repair by recombination. However,

this model was not supported by *in vitro* experiments in which the purified R200K mutant enzyme did not inhibit ligation of nicks by *E. coli* DNA ligase.

The conclusions drawn from the experiments with the *Eco*RI R200K and E144C mutants are supported by our observations made with a different experimental system. We tested the viability of repair defective strains that overproduce the *Eco*RV endonuclease. In these experiments the host cells contained *Eco*RV methyltransferase that protects cognate *Eco*RV sites. In this approach we capitalized on the observations by Halford and colleagues who had shown that *Eco*RV endonuclease, if present at high concentration in the cell, can nick noncognate sites (Taylor and Halford, 1989; Taylor *et al.*, 1990). We found that *Eco*RV endonuclease overexpression was lethal in *recA56* and *recB21* mutant strains and moderately toxic in a *lexA3* mutant strain. Because of the lack of a SOS-inducible indicator gene in the K91 strain, we could not directly test whether the *Eco*RV endonuclease-inflicted DNA damage induced the SOS response. However, as the LexA protein specified by the *lexA3* allele is defective in SOS induction (Walker, 1996), the impaired growth and altered colony morphology displayed by the JH20 (*lexA3*) strain upon induction of *Eco*RV endonuclease synthesis suggest that repair of the DNA damage was at least partially dependent on SOS repair. Although the *Eco*RI and *Eco*RV experiments employed different *lexA3* strains and thus the results are not directly comparable, the less severe phenotype observed with the *Eco*RV system probably indicates a difference in the level of nicking activity in the two experimental systems. We interpret this finding to mean that whereas the basal level of RecA protein was enough to repair DNA lesions inflicted by the *Eco*RV endonuclease, repair of a larger number of nicks generated by the *Eco*RI endonuclease mutants was only possible if, due to SOS-induction, more RecA protein was available.

DNA ligase is the only enzyme that is known to be required to repair DNA nicks. Given the abundance of ligase in *E. coli* (Lehman, 1974), the lethal effect of nicks in strains deficient in recombinational repair looks surprising at first glance. We suggest that the key to this phenomenon is the balance between the cellular ligation capacity and the level of single-strand breaks in the cellular DNA. Nicks are normal intermediates of DNA replication and are constantly generated and sealed in the process of joining Okazaki fragments. In our interpretation, this balance between nicks and ligase capacity was shifted due to the nicking activity of the *Eco*RI or the *Eco*RV endonucleases. By overwhelming the ligation capacity, some of the single-strand breaks escaped repair by

ligation and were converted to more severe DNA lesions, which require a RecA and RecB-dependent pathway for repair.

A number of *E. coli* mutations are known which increase the endogenous level of DNA nicks. These include: DNA ligase mutations (*lig*), DNA polymerase I mutations (*polA*), Dam methyltransferase mutations (*dam*), and dUTPase enzyme mutations (*dut*). In *lig* mutants the rate at which nicks are sealed is decreased (Gottesman *et al.*, 1973; Konrad *et al.*, 1973; Pauling *et al.*, 1976). In *polA* mutants joining of Okazaki fragments is inefficient due to the lack of nick translation activity of DNA polymerase I (Lehman and Uyemura, 1976). Dam methyltransferase mutations (*dam*) prevent strand discrimination by mismatch repair enzymes and may increase their activity since an increased level of DNA single strand breaks occur (Marinus and Morris, 1974). Mutations of dUTPase (*dut*) result in increased cellular levels of dUTP, leading to more frequent incorporation of uracil into the DNA that is subsequently removed by uracil-N-glycosylase (Tye *et al.*, 1977). Some of these mutations were tested and found to induce the SOS response (Condra and Pauling, 1982; Peterson *et al.*, 1985). The *lig*, *polA* and *dam* mutants are inviable in combination with *recA* and *recB* mutations (Gross *et al.*, 1971; Monk and Kinross, 1972; Marinus and Morris, 1974; Morse and Pauling, 1975; Wang and Smith, 1986; Cao and Kogoma, 1995).

These observations further suggest that, under conditions leading to an elevated number of nicks or gaps, cell survival depends on RecA and RecB functions. We could show that the drastic drop in viability caused by *Eco*RV-inflicted nicks in *recA* and *recB* cells could be reversed by increasing the level of DNA ligase activity in the cell. This finding suggests that, for the cell, it is the relative amount of DNA nicks vs. ligase activity rather than the absolute amount of nicks that is of primary importance.

A model (Kuzminov *et al.*, 1994; Kuzminov, 1995) incorporating elements of an earlier model (Skalka, 1974) offers an economical interpretation of the RecA- and RecB-dependent phenotype of mutations leading to increased levels of nicks. The model suggests that if the replication fork runs into a single-strand break in the template DNA, its corresponding arm falls off and the framework of the whole structure collapses creating a double-strand end. It further proposes that the detached arm can be reassembled into a replication fork framework by a repair process requiring RecBCD nuclease/helicase and RecA protein. In this process the RecBCD nuclease unwinds and degrades the detached arm starting from the double-strand end. The degradation capacity of RecBCD is reduced and the polarity of degradation switched to 5'→3' when

it reaches a properly oriented Chi site (Stahl *et al.*, 1990; Anderson and Kowalczykowski, 1998). Degradation of the 5'-terminal strand creates single-strand DNA with free 3' end, which subsequently engages in RecA-dependent homologous recombination with the intact DNA arm to restore the replication fork framework. An attractive feature of the model is that it offers an explanation for the biased orientation of the Chi sites in the *E. coli* genome (Burland *et al.*, 1993; Medigue *et al.*, 1993).

Our findings presented here can be best explained in the framework of the Kuzminov model. First, we have shown that repair of DNA lesions caused by the accumulation of single-strand breaks in *E. coli* DNA is dependent on RecA and RecB. Second, we have demonstrated that an elevated number of single-strand breaks leads to SOS induction, which is indicative of the appearance of single-strand DNA (Walker, 1996; Anderson and Kowalczykowski, 1998), an intermediate of the recombinational repair process. Third, we found that strains that carried the *recA56*, *recB21,* or the *lexA3* mutation were more sensitive to *Eco*RV-inflicted nicks in the glucose-containing H-broth than in LB. Although we cannot exclude that the concentration of *Eco*RV endonuclease may be higher in cells growing in H-broth than in LB, this observation suggests that at faster growth rates repair of nicks is less efficient. This in turn implies that it is secondary lesions arising during replication of nicked template DNA, and not nicks themselves that are lethal in *recA56* or *recB21* mutant strains. The enhanced toxicity of *Eco*RV-inflicted nicks in glucose-containing medium was also observed under conditions when ligase was limiting (Taylor *et al.*, 1990). This interpretation is supported by data in other systems. Deletion of the *polA* gene or combination of *xth* mutation with *recA200*, conditions that lead to an increased number of nicks and gaps, are more deleterious in rich than in minimal medium (Joyce and Grindley, 1984; Wang and Chang, 1991).

Finally, the Kuzminov model suggests a key to the apparent contradiction between data presented here and our earlier observations. Previously we found that repair of double-strand breaks inflicted by *Eco*RI temperature sensitive mutants that, like the wild-type enzyme, produce double-strand breaks, was dependent on ligase but not on RecA, RecB and LexA functions (Heitman *et al.*, 1989). Why are *Eco*RI-inflicted single-strand breaks more deleterious than *Eco*RI-inflicted double-strand breaks? We suggest the following explanation. When a replication fork collapses because of a single-strand interruption in the template DNA strand, a single end is created which, in lack of a partner double-strand end, cannot be re-attached by simple

ligation, necessitating recombinational repair (Skalka, 1974; Kuzminov, 1995). On the other hand, a double-strand scission by *Eco*RI produces two cohesive ends, which are good substrates for *E. coli* DNA ligase. It would be interesting to test how double-strand breaks inflicted by restriction enzymes that produce blunt ends are repaired. The substrate preference of *E. coli* DNA ligase suggests that such breaks would require recombinational repair.

III. Construction of *Eco*RI-*Rsr*I recombinant endonucleases

Unlike DNA methyltransferases, most type II restriction enzymes, even isoschizomers, i.e. enzymes recognizing the same sequence, do not show sequence homology. This raises the question concerning the evolution of these functionally related enzymes. It was suggested that restriction enzymes showing no homology have evolved independently (Wilson and Murray, 1991). Later, when more X-ray structures became available, common features in the three-dimensional structures of restriction enzymes, which display no sequence homology, were recognized (Venclovas *et al.,* 1994; Kovall and Matthews, 1998). Some of these features, e.g. the common core structure and the **PD**...**D/EXK** motif, which forms the active site, seem to characterize all type II restriction endonucleases, others only apply to a group of enzymes, e.g to the *Eco*RI-, or to the *Eco*RV family (Pingoud and Jeltsch, 2001).

*Eco*RI and *Rsr*I represent a rare example of isoschizomers, which share amino acid sequence homology. Importantly, all amino acids, known to play a role in substrate recognition and catalysis by *Eco*RI, are conserved in *Rsr*I, suggesting that these two enzymes recognize and cut the GAATTC sequence using the same mechanism (Stephenson *et al.,* 1989; Fig. 1). Some biochemical studies supported this notion (Aiken *et al.*, 1991a), other observations from the same laboratory indicated differences between *Eco*RI and *Rsr*I (Aiken *et al.,* 1991b). To test the structural and functional relatedness of these two enzymes, we constructed chimeric *Eco*RI-*Rsr*I endonucleases by splicing segments of the two genes. Of the five hybrids constructed, four proved inactive. The fifth hybrid (EERE) displayed a puzzling combination of phenotypes. The plasmid encoding EERE could only be maintained in hosts whose DNA was protected by *Eco*RI-specific methylation. Moreover, in some assays, EERE seemed to be even more toxic to m- hosts, than the parental *Eco*RI^{TS6}. In sharp contrast to this, cells expressing EERE did not restrict λvir phage and we could not find significant specific

endonuclease activity in crude extracts of the clone or in partially purified preparations of EERE. Although we cannot exclude the possibility that the conditions of the *in vitro* assay were far from optimal, the lack of phage restriction and the modest level of DNA degradation *in vivo* under conditons where viability dropped by several orders of magnitude, support the conclusion that the EERE protein has much lower endonuclease activity than *Eco*RI[TS6].

If EERE has such low nuclease activity, why is it so toxic to *E. coli* cells? One of the possibilities that we consider is that EERE is a nicking enzyme, that is, instead of cutting both strands, it makes only single-strand cuts at *Eco*RI sites. This explanation is consistent with the finding that *recA*, *recB* and *lexA* strains were more sensitive to EERE than the wild-type strain. However, our attempts to demonstrate nicking activity *in vitro* using the partially purified EERE preparation were not successful.

An alternative explanation for the toxic phenotype is that EERE binds very tightly to *Eco*RI sites and kills the cell by interfering with transcription and/or replication.

In bacterial cells, progression of the replication fork can be impeded by a DNA lesion, a template strand break, or a bound protein complex (Bierne and Michel, 1994; Kuzminov, 1995; Kuzminov, 1999; Hyrien, 2000; Cox *et al.,* 2000). There are several observations in the literature supporting the notion that tightly bound proteins can lead to replication arrest. The best studied case is that of the Tus protein of *E. coli*. In *E. coli*, chromosome replication is arrested in the terminus region at six *Ter* sites. The *Ter* sites are located opposite to *oriC,* the site where the bidirectional replication of the *E. coli* chromosome starts and they serve to limit encounter of the replication forks to a defined region of the chromosome (Hill, 1996). The *Ter* sites function as replication pause sites only when occupied by the Tus protein. *In vitro* studies have shown that Tus binds to *TerB* with a very high affinity ($K_D = 3.4 \times 10^{-13}$ M) resulting in a complex that inhibits unwinding of DNA by helicase (Gottlieb *et al.,* 1992). The function of the *Ter*-Tus system becomes important when arrival of one of the replication forks is delayed. To study the effect of replication fork arrest, Horiuchi and Fujimura constructed strains, which contained a *Ter* site at an ectopic location. Such cells displayed hyper-Rec phenotype, RecA-, and RecB-dependent growth and SOS induction (Horiuchi and Fujimura, 1995). The SOS response was observed even if the *Ter* site was on a plasmid (Taki and Horiuchi, 1999). To account for the observed phenotypes, a model was proposed (Horiuchi and Fujimura, 1995), which incorporated several elements of earlier models explaining repair of double-strand breaks by the homologous recombination

system. In this model, a double-strand break occurs at the stalled replication fork. The exonucleolytic activity of the RecBCD enzyme degrades the broken arm from the end until a properly oriented Chi site converts RecBCD into a recombinase. After the Chi site RecBCD continues to degrade DNA, but only the 5′-ending strand, generating a 3′ single-stranded overhang, which engages in homologous recombination with the intact arm. After resolving the Holiday structure, the replication fork is restored (Horiuchi and Fujimura, 1995). A few years later Michel and colleagues provided experimental evidence for the occurrence of the ds breaks upon arrest of the replication fork (Michel *et al.*, 1997; Seigneur *et al.*, 1998).

Although no other system is as thoroughly characterized as the *Ter*-Tus system, several data indicate that other bound proteins can also interfere with progression of the replication fork (Bedinger *et al.*, 1983; Yancey-Wrona and Matson, 1992). An indirect, yet very convincing evidence for the potential interference with replication by RNA polymerase was the recognition that in the *E. coli* genome practically all frequently transcribed genes have their 5'-ends proximal to *oriC* (reviewed in Brewer, 1988).

The Horiuchi-Fujimura model would explain some of the phenotypes displayed by the EERE protein, such as induction of the SOS response, greater sensitivity of *recA* and *recB* mutants. The observed strong gel-shift, which suggests tight binding of EERE to *Eco*RI sites, supports this model.

A combination of the two proposed mechanisms is also possible: the enzyme may nick *Eco*RI sites and then stay attached to the substrate site „frozen" half-way on the normal reaction pathway.

It is obvious that understanding the mechanism by which EERE interacts with DNA should involve a quantitative characterization, *in vitro*, of both the DNA-binding and the cleavage properties of the protein. Such experiments will require highly purified EERE preparations. So far, all attempts to prepare purified EERE have failed. We think that the most likely reasons for this failure were instability and low solubility of the protein. We hope that the new overexpression plasmids, which code for variants with His(6x) or FLAG affinity tag will facilitate purification of the protein.

In EERE, a segment consisting of 60 amino acids (from His147 to Ala206) was replaced by the corresponding *Rsr*I segement. Of the 60 amino acids 38 remained unchanged, the numbers of conservative (chemically similar) and non-conservative substitutions are 13 and 9, respectively. The replaced segment comprised part of α-helix α^4, β-strands β^4, β^{III}, β^{IV} and part of α-helix α^5 (Rosenberg, 1991). In lack of an X-ray

structure for *Rsr*I, it is not easy to offer even a tentative hypothesis for explaining why the replacement of this segment resulted in the observed phenotypes. We have initiated a collaboration with M. Fuxreiter (Inst. of Enzymology, BRC) to perform molecular modeling with EERE. We plan to create a 3D model structure of EERE using molecular dynamics methods and hope that the model will help explain the behavior of the protein.

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LIST OF PUBLICATIONS

1. Ivanenko, T., Heitman, J. and Kiss, A. (1998). Mutational analysis of the function of Met137 and Ile197, two amino acids implicated in eequence–specific DNA recognition by the *Eco*RI endonuclease. Biological Chemistry, *379*: 459 – 465.
2. Heitman, J., Ivanenko, T. and Kiss, A. (1999). DNA nicks inflicted by restriction endonucleases are repaired by a RecA– and RecB–dependent pathway in *Escherichia coli*. Mol. Microbiol. *33*, 1141 – 1151.

PRESENTATIONS AT SCIENTIFIC MEETINGS

1. Ivanenko, T., Heitman, J. and Kiss, A.: "Role of Met137 and Ile197 in sequence specific recognition by the EcoRI endonuclease" Second Molecular Biology Workshop of the Hungarian Biochemical Society, May, 1997, Lillafured, Hungary (talk).
2. Ivanenko, T., Heitman, J. and Kiss, A.: "Role of Met137 and Ile197 in sequence specific recognition by the EcoRI endonuclease" 4th New England Biolabs Workshop on Biological DNA Modification, September 2 – 7, Igls, Austria. (poster).
3. Ivanenko, T., Heitman, J., Kiss,A.:"Repair of ssDNA breaks inflicted by restriction endonucleases in *E.coli*", Straub Days, January 20-22, 1999, BRC, Szeged, Hungary (talk).
4. Heitman, J., Ivanenko, T. and Kiss, A.:" DNA nicks inflicted by restriction endonucleases are repaired by a RecA – and RecB – dependent pathway in *Escherichia coli*" Howard Hunges Medical Institute Meeting of International Research Scholars, June 22 – 25, 1999, Moscow, Russia, (talk).
5. Ivanenko, T., Heitman, J. and Kiss, A.: "Construction of hybrids between the EcoRI and RsrI restriction endonucleases" Howard Hunges Medical Institute Meeting of International Research Scholars, June 20 – 23, 2000, Chevy Chase, Maryland, USA (talk).