The mechanism of regulation of the restriction activity of the

*Eco*KI, a type I restriction enzyme .

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DECLARATION

This thesis and all the work herein was composed by myself, unless otherwise stated. Many ideas on the project were devised in collaboration with my supervisor, Professor Noreen. E. Murray

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February, 2001

Abbreviations

AdoMet	S-adenosylmethyonine
Amp	Ampicillin
2AP	2-aminopurine
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
$^{\circ}C$	degrees celsius
dH_2O	de-ionised water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	ethylene diaminetetra-acetic acid
e.o.p.	efficiency of plating
kb	kilobase
kD	kiloDalton
l	litre
LB	Luria-Bertany Broth
M	molar (moles per litre)
mol	mole
min	minute
MW	molecular weight
μ	micro ($\times 10^{-6}$)
n	nano ($\times 10^{-9}$)
NADH	β-nicotinamide adenine dinucleotide, reduced form
Nal	nalidixic acid
O.D.	optical density
ORF	open reading frame
Ω	ohms
p	pico ($\times 10^{-12}$)
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

PEG	polyethlene glycol
POD	peroxidase
PMSF	phenylmethylsulphonyl fluoride
rpm	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
TEMED	N,N,N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
T_m	melting temperature
UV	ultraviolet
V	volt

AMINO ACIDS

Amino acids	Three letter abbreviation	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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CHAPTER 1 General Introduction

Bacteria have evolved mechanisms that allow them to distinguish their own DNA from foreign DNA. Modified bases within the resident DNA provide an imprint that identifies the DNA as "self", whereas DNA that lacks the appropriate imprint is designated foreign and is subject to attack by endonucleases. The fragmentation of foreign DNA restricts its acquisition or propagation, hence these endonucleases were designated restriction endonucleases. It has usually been assumed that should modification of a bacterial chromosome fail, then DNA would resemble foreign DNA and be a substrate for the resident endonuclease. Unrepaired breaks in the bacterial chromosome would lead to cell death. However, evidence is emerging that the regulation of restriction activity can prevent the occurrence of breaks in the resident DNA (O'Neill *et al.*, 1997; Makovets *et al.*, 1999). This evidence is the topic of this thesis.

A restriction enzyme that recognises unmodified DNA must coexist with a modification enzyme and the two enzymes, or activities, are commonly referred to as a restriction and modification (R-M) system. The modification enzyme catalyses the transfer of a methyl group from a methyl-donor, S-adenosyl methionine (AdoMet) to specific bases within a target sequence, and the restriction endonuclease hydrolyses phosphodiester bonds within the DNA backbone if the target sequences lack the appropriately modified bases. The restriction and modification activities may reside in separate enzymes or in different subunits of an oligomeric protein. R-M systems have been classified according to the target they recognise, their subunit composition and their cofactor requirements. The traditional classification distinguishes 3 major types of R-M systems – type I, II and III (for reviews see (Wilson and Murray, 1991; Bickle and Kruger, 1993; King and Murray 1994)

Type I restriction enzymes recognise an asymmetric target, which consists of two parts divided by a nonspecific spacer sequence. The oligomeric modification complex comprises a specificity subunit combined with two modification subunits. The addition of two restriction subunits generates a restriction complex. If this

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complex binds to a target sequence that is unmodified it translocates DNA in a process dependent on the hydrolysis of ATP, but should an obstacle block the translocation process, a double-strand break (DSB) is introduced . The restriction reaction requires S-adenosylmethyonine (AdoMet), ATP and Mg^{2+}.

Typical type II R-M systems recognise palindromic targets. These R-M systems have two separate components – restriction and modification enzymes that recognise the same target. Unmodified targets are modified by the modification enzyme or cut by the restriction enzyme at a specific position. The restriction reaction requires only Mg^{2+}.

Type III R-M systems recognise asymmetric targets. The modification component consists of only one type of polypeptide, which in combination with restriction subunits produces the restriction complex that has recently been shown to require AdoMet, ATP and Mg^{2+} for the restriction process (Saha and Rao, 1995).

However, the accumulating data from new systems merge the borders between different types of restriction systems. For example, Bcg-like enzymes combine characteristics of type I and type II R-M systems. A restriction enzyme is an oligomeric complex that recognises a symmetric target with a spacer and requires AdoMet and Mg^{2+} for the restriction reaction. A restriction enzyme of a provisional type IV R-M system recognises a non-symmetric target and can either modify it or introduce a DSB; AdoMet stimulates cleavage (Janulaitis *et al.*, 1992).

There are also restriction systems that attack modified DNA. These modification-dependent restriction systems (MDRS) can be divided into two groups: multisubunit nucleoside triphosphate-dependent systems (Bickle and Kruger, 1993) and type II-like systems based on a single polypetide (Lacks and Greenberg, 1975). Both types of MDRS lack cognate modification enzymes.

In the present account restriction systems will be divided into the nucleoside triphosphate dependent (NTD) restriction systems and nucleoside triphosphate

independent systems. The restriction reaction of NTD systems requires the hydrolysis of either ATP or GTP. This requirement correlates with the oligomeric composition of the enzymes, hypothesised to be subjects of posttranscriptional regulation (Murray,2000).

1.1 Restriction and modification systems

Nucleoside triphosphate-independent R-M systems

These restriction enzymes cut double-stranded DNA within or close to a recognition sequence, producing a distinct pattern of restriction fragments. Their wide use for genetic engineering stimulated intensive searches for these endonucleases and several thousand have been described (see for review (Roberts and Macelis, 2001); **http://rebase.neb.com/rebase/rebase.html**).

The members of the broad group of nucleoside triphosphate-independent R-M systems include type II restriction systems, where the restriction and modification components are separate enzymes (Lunnen *et al.*, 1988; Heidmann, *et al.,* 1989; Brooks *et al.*, 1991). Most type II restriction enzymes require only Mg^{2+} for the cleavage of their palindromic target sequence in double stranded DNA.

The typical R-M operon of type II R-M systems includes an ORF additional to two that code Res and Mod genes, called [the name of restriction nuclease] + C, for example bamHIC (Ives *et al.* 1992). This ORF encodes a small polypeptide which acts at the level of transcription to stimulate expression of restriction enzyme and to repress DNA methyltransferase synthesis (Tao *et al.*, 1991).

The analysis of nucleotide sequences of type II R-M systems revealed a consensus sequence between the genes encoding the R-M system. This sequence, named C-box, may play the role of an operator sequence (Rimseliene, *et al.,* 1995).

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Nucleoside triphosphate-dependent restriction (NTDR) systems

a) Type I R-M systems

The first R-M system to be discovered, hence type I, was the *Eco*KI system of *E.coli* K-12 (Bertani and Weigle, 1953). Type I restriction enzymes are the most complicated in their mechanism of action (for recent extensive review of type I systems see (Murray, 2000).

Genetic organisation

The genes encoding *Eco*KI, *hsd* (for *h*ost *s*pecificity of *D*NA) are located at 98.5 min of the genetic map of *E.coli* K-12 close to the *serB* locus (Arber and Wauters-Willems, 1970). The region including this locus is sometimes referred to as the "immigration control region" because not only the type I restriction and modification genes are located here but the *hsd* genes are flanked by *mrr* on one side and by *mcrBC* on the other. These genes encode the systems that restrict methylated DNA (Raleigh, 1992). A characteristic of the *hsd* region is its hypervariability. More than twenty different type I systems encoded by allelic genes have been identified in isolates of *E.coli, Salmonella and Citrobacter* (Barcus *et al.*, 1995).

hsd genes are organised into two transcription units: p_{res} controls the *hsdR* gene (for *r*estriction) and p_{mod} the *hsdMS* operon (for *m*odification and *s*pecificity, correspondingly), transcribed in the same direction (Loenen *et al.*, 1987; Titheradge et al., 1996).

An *hsd* locus with an unusual structure was reported for *Mycoplasma pulmonis* (Sitaraman and Dybvig 1997; Dybvig *et al.* 1998). In this system the *hsdM* and *hsdR* genes are flanked by *hsdS* genes. Within the two *hsdS* genes there are at least 3 sites of similar sequence and inversions of *hsd* genes during growth of bacterial cultures leads to clones with restriction systems of different specificities. As there is only one promoter, some inversions lead to clones which have the restriction system silenced.

A gene switch similar to one reported for *Mycoplasma* has been described for a type I restriction system of *Lactococcus lactis* (O'Sullivan & Klaenhammer, 1998).

Sequence analysis of *Haemophilus influenzae* and *Neisseria meningitidis* detected the presence of inversion-prone repeat sequences near the genes for their predicted R-M systems (Hood *et al.* 1996; Saunders *et al.*, 2000). Similarly, repeated sequences were discovered near the surface antigen genes, where divergence is known to be important for the pathogenicity. Thus occurrence of inversion-prone sequences near genes of some R-M systems might suggest a selective advantage for changing specificity of a resident restriction system for a pathogen.

Type I systems found in Enteric bacteria have been sub-divided into four families (IA, IB, IC, ID) according to their sequence similarity, genetic complementation, cross-reactivity of antibodies and biochemical characteristics of enzymes (Murray *et al.*,1982; Dryden *et al.*, 1993; Barcus *et al.*, 1995; Titheradge *et al.*,1996). The systems that belong to one family, for example, *Eco*KI and *Eco*BI have a high degree of sequence similarity and their subunits are interchangeable in genetic complementation analysis. On the other hand, systems from different families, e.g. *Eco*KI (IA family) and *Eco*AI (IB) have a very limited sequence similarity and, for example, HsdR of *Eco*KI system cannot interact with the methylase of *Eco*AI system. Some examples of representatives of different families are shown in Table 1.1.

Type IA, IB, ID are chromosomally encoded (Barcus *et al.*, 1995) type IC are found on conjugative plasmids, except for the *Eco*prrI system, which is located in the chromosome at a site different from allelic type I systems (Kaufmann, *et al.*, 1986; Tyndall *et al.*, 1994).

The best genetic and biochemical descriptions of type I restriction systems are available for representatives of the Enterobacteriaceae and particularly *E.coli*, but this is a reflection of history of the research in the field of bacterial genetics and the availability of tools for genetic analysis, rather than an indication of the distribution of type I restriction systems among bacteria.

Table 1.1 Some representatives of type I families.

Family	Enzyme	Target
IA	*Eco*KI	AAC(N$_6$)GTGC
	*Eco*BI	TGA(N$_8$)TGCT
	*Sty*LTIII	GAG(N$_6$)TAYG
	*Sty*SPI	AAC(N$_6$)GTRC
IB	*Eco*AI	GAG(N$_7$)GTCA
	*Eco*EI	GAG(N$_7$)ATGC
IC	*Eco*R124I	GAA(N$_6$)RTCG
	*Eco*DXXI	TCA(N$_7$)RTTC
ID	*Sty*SBLI	CGA(N$_6$)TACC

Genes of type I systems that belong to different families have very little sequence similarity. Nevertheless, analysis of the predicted amino acid sequences of their subunits reveals motifs common to DNA adeninemethylases (in HsdM) (Dryden *et al.*, 1993; Willcock, *et al.*, 1994); ATPases, helicases and endonucleases (in HsdR) (Gorbalenya and Koonin, 1991; Titheradge *et al.*, 1996; Davies *et al.*, 1998; Davies *et al.* 1999b).

Contemporary computer analysis takes advantage of the operon organisation of *hsdMS* genes and their close association with *hsdR* using it in so called "guilt by association" method for the prediction of the presence of type I systems (Bult *et al.*, 1996; Nelson *et al.*, 1999; Tettelin *et al.*, 2000). For example, during detection of putative R-M systems specified by the genomes of prokaryotic organisms, searches for ORFs with methyltranferase motifs closely associated with an unknown ORF and subsequent BLASTX search against known sequences of R-M systems detected among all other types of R-M systems, presence of type I-like sequences in 15 out of 28 species of prokaryotes (Kong *et al.*, 2000). This search identified a type I R-M system in *E.coli* which may be considered as a positive control of the efficiency of the search.

Thus, representatives of type I R-M systems are described, or at least predicted, for all three divisions of Bacteria kingdom:

- Gram-negative (Gracilicutes), for example, *Klebsiella* (Lee *et al.*,1997) *Pasteurella* (Highlander and Garza, 1996), *Pseudomonas* (Droge *et al.*, 2000).

- Gram-positive (Tenericutes), for example, *Bacillus* (Xu et al., 1995), *Lactococcus* (Schouler *et al.*, 1998; Seegers *et al.*, 2000);

- Mycoplasma (Mollicutes), for example, *Mycoplasma* (Sitaraman and Dybvig, 1997; Kong *et al.*, 2000)

- Additionally, type I restriction systems were found by genome sequence analysis in Archaea (Bult *et al.*, 1996; Roberts, 1998; Nelson *et al.*,1999).

Type I enzymes are large oligomeric complexes of three types of subunits: S – for *s*pecificity, M – for *m*odification and R for *r*estriction, which form two complexes: M_2S_1 capable of methylation of the adenine residues in the non-palindromic target of 7-8 residues (Table 1) and $R_2M_2S_1$ which methylates hemimethylated DNA and cuts DNA that contains unmethylated targets (Taylor *et al.*, 1992; Dryden *et al.*, 1993).

hsdS gene and the HsdS polypeptide.

In the early work of Murray *et al.* (1982) sequences of three type IA *hsdS* genes were compared and the two regions (~450 b.p.) that showed no similarity at the level of nucleotide sequence were named "variable" in contrast with regions that have a high degree of homology and were defined as "conserved". It was suggested that the polypeptide sequences encoded by the variable regions take part in target recognition and these regions of HsdS have been named "target recognition domains (TRDs)". One of the TRDs proved to be responsible for the recognition of the three-nucleotide component of a target and the other one for the recognition of the second part of the sequence.

The extensive random mutagenesis of the amino-TRD of *Eco*KI was performed in order to find amino acids of a TRD involved in interactions with the target (O'Neill *et al.,* 1998) It has been shown that out of 101 substitutions affecting 79 residues only seven caused the loss of restriction and modification activity, the phenotype expected for mutations that lead to loss of specificity. The authors proposed an explanation that the corresponding amino acids lie on the protein-DNA interface and subsequent site-directed mutagenesis supported this suggestion. The fluorescence anisotropy and DNA binding experiments showed that there was a strong correlation between the r$^-$ m$^-$ phenotype and failure to bind DNA target.

A new type I system *Sty*SQI was isolated from a P1 transduction between *Sty*SPI and *Sty*LTIII (previously called *Sty*SBI) strains (Table 1.1). (Bullas and Colson, 1975). The sequence of the central conserved region of the *hsd*S of *Sty*SQI has the sequence

identical to the 5' part of *Sty*SPI joined together with 3'part of *Sty*LTIII. As a result the new system recognises a three-nucleotide sequence identical to that of the target of *Sty*SPI, has the same 6-nucleotide spacer and recognises the same target tetranucleotide as the *Sty*SBI (Fuller-Pace & Murray, 1986).

The study of *Eco*R124I showed that, at a low frequency, cells gain the ability to restrict phage modified by the original enzyme. The target of the new system of restriction-modification (*Eco*R124/II) differs only in the length of the spacer (Hughes, 1977; Price *et al.*, 1989). The sequences of the corresponding genes are the same except for a 12bp sequence in the central conserved region that is present twice in the initial *hsdS* gene and three times in the *Eco*R124/II gene.

The transposition of Tn5 that led to the formation of a truncated HsdS subunit of the type IC system *Eco*DXXI resulted in an enzyme with a new specificity - TCA(N$_6$)TGA. This enzyme proved to be the result of an interaction between two "half-subunits" of HsdS (Meister *et al.*, 1993). It was shown later that the 3'end of the same gene, fused to the transcriptional and translational signals produces a peptide that can interact with HsdM and HsdR and confers specificity for the interrupted palindromic DNA sequence GAAY(N$_5$)RTTC (MacWilliams and Bickle, 1996).

A symmetrical model for the organisation of a type I methylases has been proposed by (Kneale, 1994). On the basis of homology between the central conserved domain and sequences near N and C termini of the protein a circular organisation of the S-subunit have been postulated, where one M subunit interacts with a conserved domain and the other holds together the "split domain" (Figure 1.1).

Even though the HsdS subunit determines the specificity of both the methylation and restriction complexes of type I R-M systems, the frequent appearance of hybrid *hsdS* genes and therefore new type I systems raises the question of the regulation of restriction, particularily since the methylase of type IA systems have a pronounced preference for hemimethylated DNA, and new systems could restrict unmodified chromosomal DNA.

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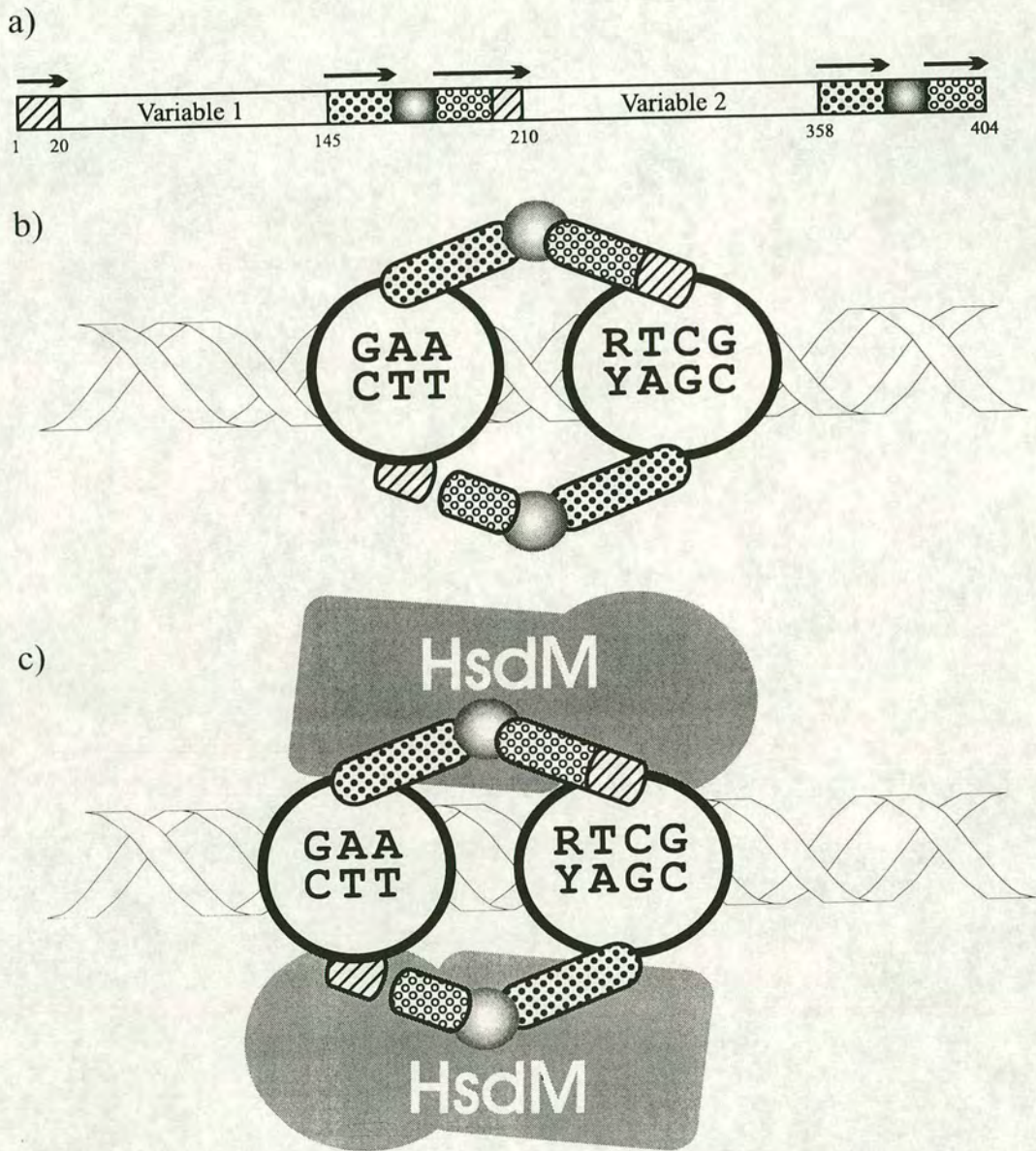
Figure 1.1 HsdS subunit of *EcoR* 124I enzyme.

(a) Organisation indicating the two TRDs and conserved sequences. (b) Model of
Kneale, in which the repeated sequences form linkers joining the TRDs. (c) Model
of the *EcoR*.124I methyltranferase, in which the two HsdM subunits bind to the
linker region. From reference Murray (2000).

hsdM gene and polypeptide. The modification complex.

Early analysis of the predicted amino acid sequence of the HsdM subunit of *Eco*KI revealed motifs common to methyltransferases (Loenen *et al.*,1987).Comparisons of the amino acid sequences of many methyltransferases (Mtases) identify 9 or 10 motifs, two of which are highly conserved. These motifs are common to all methyltransferases, from the simplest acting on organic compounds such as catechol methyltransferase to those active on bases within polynucleotides (Schluckebier, *et al.*, 1995). All these enzymes, which share the ability to catalyse the transfer of a CH_3 group to a substrate molecule, are believed to contain a characteristic alpha/beta structural fold (Figure 1.2) (Tran *et al.*, 1998).

The methyltransferases active on DNA are divided into 3 classes according to which residue is methylated and the position to which the methyl group is transferred: N6-adenine methyltransferases, C5-cytosine Mtases and N4-cytosine DNA methyltransferases (for reviews see (Cheng 1995; Dryden, 1999). C5-cytosine Mtases are the best studied because the presence of a covalent intermediate in the process of methylation (Wu and Santi, 1987) permitted the determination of the high-resolution three dimensional (3D) structure of the *Hha*I-DNA complex (Klimasauskas *et al.*,1994).

All type I R-M enzymes transfer a methyl group from the cofactor AdoMet to the N6 position of adenine. Malone et al. (1995) made a structure-guided sequence comparison of N6-adenine and N4-cytosine DNA Mtases, identifying nine conserved sequence motifs, which correspond to the motifs I to VIII and X previously found in C5-cytosine DNA Mtases (Posfai *et al.*,1988; Posfai *et al.*, 1989).

DNA N6-adenine methylases have been subdivided into 3 groups: α,β and γ according to the order and spacing between conserved motives (Wilson, 1992). The arrangement of the conserved motifs places the HsdM subunits of type I restriction
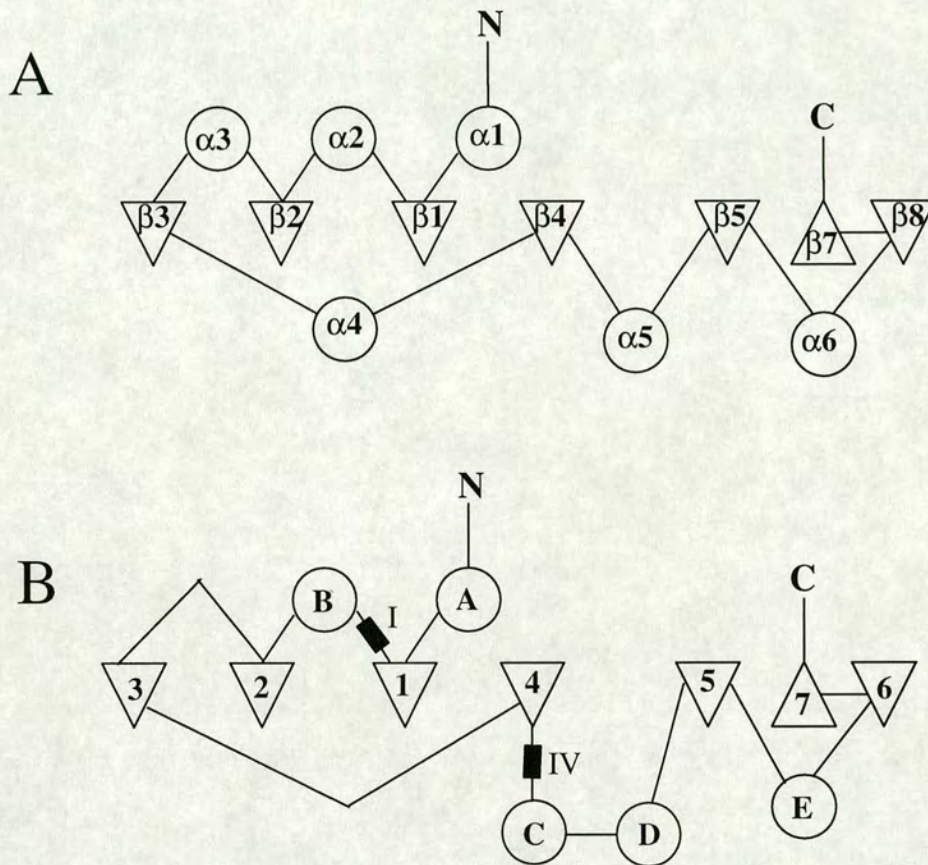
Figure 1.2 Consensus fold of methylases (Tran *et al.*, 1998)

Circles represent α-helices and triangles represent β-strands; the N and C

termini are indicated; motifs conserved in adenine methylases shown as black rectangles.

A Fold of M.*Taq*I

B Predicted fold of HsdM of *Eco*KI (Dryden D., unpublished)

enzyme in the γ class of methyltransferases. Unfortunately, no 3D-structure of a type I restriction or modification enzyme has been determined, but the structure of M.*Taq*I, a representative of the γ class of methyltransferases has been solved (Figure 1.2) (Labahn *et al.,* 1994).

The mechanism of modification reaction was discovered for *Hha*I, a C5-methyltranferase but there is supporting evidence that a similar mechanism is applicable to all DNA Mtases. For *Hha*I the methyl group of AdoMet, which is bound to a conserved glycine of motif I is transferred to the target base that is flipped out of the double helix through the minor groove into the enzyme pocket by the amino acids of motif IV (Klimasauskas *et al.,* 1994). A similar 3D-structure was obtained for M.*Hae*III, another type II methyltransferase (Reinisch *et al.,* 1995).

Amino acid substitutions within the two most conserved motifs of *Eco*KI (motif I, consensus PAxAxGP and motif IV, consensus N/D/S P/I P Y/F/W) produced enzymes incapable of methylating the target DNA either *in vivo* or *in vitro*. The change G177D in motif I led to an enzyme unable to bind the cofactor AdoMet, which supports the idea that this motif is responsible for binding the cofactor as was shown for *Hha*I. The amino acid substitutions in motif IV apparently led to enzymes that retained the conformation of the wild-type enzyme and were able to bind AdoMet, but unable to transfer the methyl-group to DNA, except when F was replaced by an aromatic residue: the F269W substitution resulted in enzyme with partial activity and F269Y resulted in an enzyme with wild-type activity (Willcock *et al.,* 1994).

Site-directed mutagenesis to change the Y residue of motif IV of M.*Taq*I led to an enzyme that according to measurement of changes in the fluorescence of 2-aminopurine (2AP) incorporated into an oligonucleotide duplex was capable of flipping of the target base but had a reduced catalytic constant (Pues *et al.* 1999). The authors proposed that the aromatic residue Y/F/W of the motif IV is involved in

placing the extrahelical target base in an optimal position for transfer of the methyl group.

Indirect evidence supports the current model of base flipping as an intermediate in the mehylation reaction performed by type I R-M enzymes. Hydroxyl radical footprinting studies of DNA complexed with M.$EcoR124$ revealed an increased accessibility of the bases that are the substrates for the methylation, and a marked change in the structure of the sugar-phosphate backbone of the DNA including these bases, after binding of the enzyme (Mernagh and Kneale, 1996). Additionally, like M.HhaI, the constant of binding of M.EcoR124 to the oligonucleotide duplex containing the recognition sequence where an abasic pair or mismatch substitute for the target adenine is higher than that of binding to an oligonucleotide duplex that includes the target sequence (Mernagh $et\ al.$, 1998).

The type I complex, capable of methylation, is a trimer consisting of one DNA recognition subunit (S) and two modification subunits (M): M_2S_1. The partly assembled dimer form, M_1S_1, binds DNA with weaker affinity. The addition of a second modification subunit to form M_2S_1 also confers Mtase activity (Taylor $et\ al.$ 1992; Dryden $et\ al.$, 1993)

The M_1S_1 complex of EcoKI has a footprint on the DNA substrate of the same length as M_2S_1 implying that the modification subunits are located on either side of the DNA helical axis (Powell $et\ al.$, 1998a). The M.EcoKI has two non-interacting AdoMet binding sites and it was shown by gel-retardation analysis that binding of AdoMet to the methyltransferase enhances binding to both specific and non-specific DNAs, but the enhancement is greater for the specific DNA (Powell $et\ al.$, 1993).

The tertiary structure prediction of the M subunit of EcoKI reveals three major domains: an N-terminal m* region, relevant to the higher methylation activity of type IA enzymes on hemimethylated DNA (Kelleher $et\ al.$, 1991), the catalytic domain and a C-terminal tail.

Using the similarity between the type I enzymes and the type II γ class of methylases a model for the type I methylase bound to DNA was proposed. According to this model the two M-subunits bind to the conserved regions of HsdS in the opposite orientation. The HsdM-HsdS interface includes both the catalytic region and joined m*- tail region of HsdM (Dryden *et al.*, 1995).

hsdR gene and polypeptide. Restriction complex.

The early genetic experiments showed that the product of *hsdR* gene is responsible for the restriction of unmodified DNA although the products of all three *hsd* genes are required for it (Colson *et al.*, 1965; Wood, 1966; Boyer and Roulland-Dussoix, 1969).

The *hsdR* genes for all four families of type I systems encode polypeptides containing motifs characteristic of ATP binding proteins (Walker *et al.*, 1982). However, the "A" component of a classical "Walker motif" GAXXXGKST is in the form GXGKS, a variant characteristic of members of the "DEAD box" family of helicases, so called because prototypic members shared the motif "Asp-Glu-Ala-Asp" (DEAD). The HsdR subunit includes all the motifs common to ATP-dependent helicases (Gorbalenya and Koonin, 1991; Murray *et al.*, 1993; Titheradge *et al.*, 1996; Davies *et al.*, 1998).

Limited proteolysis and structural modelling have shown that HsdR can be functionally subdivided into 3 domains: an aminoterminal domain containing an endonuclease motif, a central domain containing DEAD-box motifs, which is predicted to have a similar structure to the catalytic domain of a DNA helicase such as PcrA, and a carboxyterminal part that is critical for binding HsdR to the methylase (Davies *et al.*, 1999).

Substitutions in the endonuclease motif, reminiscent of the P-D (D/E)-X-K the catalytic motif of many type II restriction enzymes, led to proteins that were unable to cut DNA, while still retaining the translocation activity (Davies *et al.*, 1999a). Conserved changes in DEAD-box motifs resulted in proteins that were able to bind specifically to the target in the presence of ATP and AdoMet and undergo an ATP-

dependent conformational change. However, these enzymes were unable to hydrolyse ATP and translocate DNA and they failed to cut DNA (Davies *et al.*, 1999a).

The stoichiometry of the restriction complex of type I restriction systems is $R_2M_2S_1$ (Dryden et al., 1997). The report that R.*Eco*R124I complex is $R_1M_2S_1$ by Janscak *et al.* (1996) was explained (Mernagh *et al.*, 1998) by the finding that M.*Eco*R124I has two non-equivalent sites for binding HsdR. While binding of the first HsdR subunit to the corresponding methylase is very tight the second HsdR binds weakly and is easily dissociated to yield $R_1M_2S_1$ (Janscak *et al.*,1998).

In the presence of both cofactors, and either a hemimethylated or unmethylated target sequence, *Eco*KI undergoes a conformational change (Bickle *et al.*, 1978; Burckhardt *et al.* 1981; Powell *et al.*, 1998b). In the case of hemimethylated target the change leads to the methylation of the other strand of DNA followed by the release of the enzyme (Burckhardt *et al.* 1981).

Gel retardation experiments showed that, irrespective of the presence of AdoMet, the *Eco*KI restriction enzyme could bind to DNA, even without a target, in the absence of ATP. Following the addition of both AdoMet and ATP, the size of the footprint changes to that of the methyltransferase although restriction subunits remain bound to the complex (Powell *et al.*, 1998b). The authors interpretation of these findings is that restriction subunits can make unspecific contacts with DNA but these interactions are weakened if the methylase core of the enzyme recognises the target allowing the enzyme to undergo the conformation change necessary for the restriction reaction (Powell *et al.*, 1998b)(Figure 1.3B).

The restriction complex requires AdoMet both as an allosteric factor and methyl donor. In the absence of ATP, *Eco*KI and *Eco*BI methylate the target DNA but at a very slow rate (Burckhardt *et al.* 1981). Thus, theoretically, the restriction reaction will always lead to the DSB before the modification reaction will be completed.

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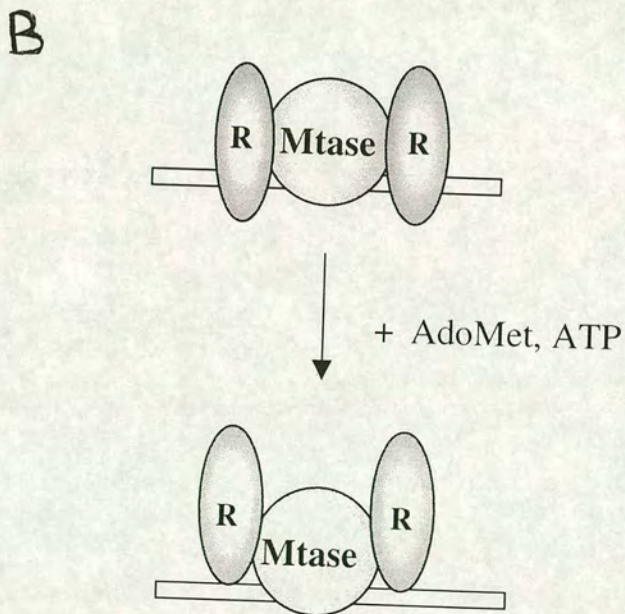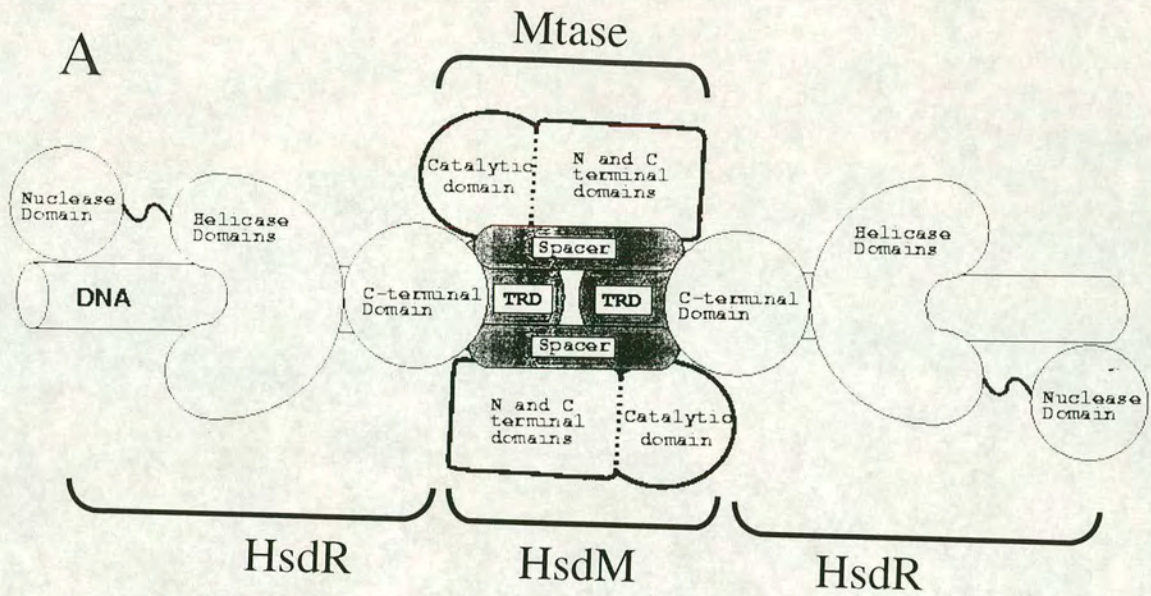
Figure 1.3 A model of *Eco*KI and the effect of cofactor binding on the *Eco*KI complexes with specific DNA

A. Model of *Eco*KI (Davies *et al.*, 1999b). HsdS is shown in grey; HsdR, HsdM and the methylase core (Mtase) are defined by brackets.

B. Effect of cofactors (Powell *et al.*, 1998).

With unmodified DNA the enzyme starts to hydrolyse ATP and uses this energy to translocate DNA in both directions, simultaneously, while remaining bound to the target. (Studier and Bandyopadhyay, 1988; Firman and Szczelkun 2000).

The requirement for ATP hydrolysis for DNA translocation was confirmed by an assay of T7 DNA entrance into a bacterial cell. When bacterial and T7 RNA-polymerases, which are usually responsible for internalisation of phage T7 genome are inhibited, *Eco*KI can pull the phage genome into the cell in the absence of the product of the 0.3 gene (see Chapter 2. Regulation of the restriction activity) (Garcia and Molineux ,1999). Mutations in each of the DEAD-box motifs were shown to block the translocation activity of *Eco*KI and this correlated with loss of the ability to hydrolyse ATP (Davies *et al.*, 1999a).

Atomic force microscopy has shown the dimerisation of two *Eco*KI molecules, bound to two unmodified targets on the plasmid DNA, in the absence of ATP. A rapid collapse of DNA molecules was observed after ATP was added, consistent with ATP-dependent translocation (Ellis *et al.*,1999).

DNA translocation by *Eco*AI along the helical path of a plasmid simultaneously generates positive supercoils ahead and negative supercoils behind the moving complex in the contracting and expanding DNA loops respectively (Janscak and Bickle, 2000). When the translocation is impeded by an encounter with a fixed Holliday junction, translocating complexes introduce a DSB within the DNA molecule (MacWilliams *et al.*, 1996; Janscak *et al.*, 1999; Studier and Bandyopadhyay, 1988). Encountering another type I restriction enzyme, but not any other protein bound to DNA, e.g. Lac-repressor, triggers cutting of DNA(Janscak *et al.*, 1999). After cleavage the enzyme remains tightly bound to the DNA (Eskin and Linn, 1972; Ellis *et al.*, 1999).

Despite the presence in HsdR of DEAD-box motifs common to DNA and RNA helicases, there is no direct evidence for strand-separating activity for any type I restriction enzyme. However, some proteins such as phage DNA packaging enzymes that are able to translocate DNA do not employ a strand separating mechanism (for review see (Fujisawa and Morita, 1997). Additionally, it is possible to uncouple the translocation and strand-separation activities of a helicase (Graves-Woodward *et al.*, 1997; Soultanas *et al.*, 2000). Therefore, type I restriction enzymes may just track along double-stranded DNA without strand separation.

b) Type III R-M systems

Type III systems are highly reminiscent of type I R-M systems (see Bickle and Kruger, 1993; King and Murray, 1994 for review). Putative type III enzymes are mostly chromosomally located (Roberts, 1998; Roberts and Macelis, 2001) and references therein), although the most detailed studies of type III restriction systems were done on the *Eco*PI system of phage P1 and EcoP15I of plasmid P15B.

The modification enzyme of type III systems has motifs characteristic of adenine-methyltransferases. Substitutions in motif I of the Mod subunit of EcoP15I led to an enzyme unable to bind AdoMet. The mutants in motif IV bind AdoMet but do not perform the methylation reaction (Saha *et al.*, 1998).

The restriction subunit is active only in a complex with the modification subunit (Hadi *et al.,* 1975). The Res polypeptide has DEAD-box motifs (Gorbalenya and Koonin, 1991; Saha and Rao, 1997). Substitutions in motif I (GxGKS) resulted in a mutant enzyme unable to hydrolyse ATP and cut DNA, mutagenesis of motif II (DEPH) resulted in uncoupling of ATP hydrolysis and cleavage reaction (Saha and Rao, 1997).

Type III enzymes recognise asymmetric sequences without a spacer and methylate adenine at the N6 position, or restrict DNA in a reaction dependent on Mg^{2+} and ATP (De Backer and Colson, 1991). For successful cleavage two unmethylated sites must be inversely oriented (Kunz *et al.*, 1998) as co-operation between two enzymes is required for the restriction reaction (Saha and Rao, 1995)

c) Modification-dependent restriction systems (MDRS)

Three such systems have been found in *E.coli* K-12: McrA (for *m*ethyl*c*ytosine *r*estricting) (RglA), McrBC (RglB) and *mrr* (Bickle and Kruger, 1993). They were discovered as the factor responsible for the restriction of non-glycosylated T-even phages (Rgl) and were the main barrier to cloning C5-methylated DNA from eukaryotes in *E.coli* (Whittaker *et al.*, 1988; Raleigh *et al.*, 1988).

McrA is a single 31kDa polypeptide that restricts only cytosine methylated DNA (Shivapriya *et al.*, 1995).

mcrB specifies two polypeptides – full length, $McrB_L$ and a truncated, $McrB_S$ (Beary *et al.*, 1997 ; Panne *et al.*, 1998). The McrB component of the McrBC restriction system has a GTP-binding motif. McrB is able to restrict 5meC preceded by a purine in a reaction dependent on GTP, Mg^{2+} and McrC. McrC regulates the activity of McrB and the optimal enzyme activity is obtained at a ratio of three to five McrB per McrC, suggesting that DNA is cleaved by a multisubunit complex (Panne *et al.*, 1999). $McrB_S$, which lacks 161 amino acids at the N-terminal part of McrB, regulates the activity of McrBC complex by sequestering McrC (Panne *et al.*, 1998)

McrBC recognises and cleaves DNA containing modified cytosine residues (mC): 5-hydroxymethylcytosine (restriction of T4 phage is the $RglB^+$ phenotype), N4-methylcytosine, 5-methylcytosine (Dila *et al.*, 1990). For DNA containing 5-methylcytosine it has been shown that the cleavage requires two mC sequences, usually separated by at least 22 bp and no more than 2 kb, where only one of the strands has to be methylated (Stewart and Raleigh, 1998). A linear substrate requires 2 mC for cleavage, but a circular substrate containing only one mC is cleaved successfully (Panne *et al.*, 1999). Additionally, a mutant unable to hydrolyse GTP cuts DNA close to the target, resembling DEAD-box mutants of *Eco*KI that are able to introduce nicks into the substrate despite absence of the ability to hydrolyse ATP. McrBC translocates DNA and its behaviour is quite similar to type I restriction enzymes. However, unlike type I restriction enzymes, an encounter with Lac repressor bound to the linear substrate triggers the restriction reaction (Panne *et*

al., 1999). Mrr (for *m*ethylated adenine *r*ecognition and *r*estriction) is an endonuclease that restricts DNA containing N6mA and C5mC (Heitman and Model, 1987).

1.2 The regulation of restriction activity of nucleoside triphosphate-dependent restriction systems.

Introduction

Regulation of restriction activity of ATP or GTP-dependent R-M systems

Two situations that arise in bacterial cells are the subjects for the regulation of nucleoside triphosphate-dependent restriction systems: acquisition of new restriction systems and regulation of the restriction activity of a resident R-M system under stress conditions.

The latter phenomenon is often referred to as restriction alleviation (RA). However, some other processes, which lead to inhibition of restriction activity, are referred to in the literature using the same term. These processes include the inhibition of type I restriction systems by the antirestriction proteins of plasmids (Belogurov *et al.,* 1985) or phages (Spoerel *et al.,* 1979). However, antirestriction proteins often inhibit both restriction and modification reactions of type I restriction enzymes (Bandyopadhyay *et al.,* 1985; Moffatt and Studier, 1988) and the Ral protein of phage λ enhances the modification of unmodified DNA (Zabeau *et al.,* 1980; Toothman, 1981).

In this thesis restriction alleviation (RA) is considered to be a phenomenon in which the level of restriction but not modification of foreign DNA is reduced in genetically restriction-proficient cells under some physiological conditions. RA occurs after bacterial cells are exposed to DNA- damaging agents or as a result of certain mutations that are predicted to lead to DNA damage. The phenomenon of restriction alleviation is characteristic of type I and some modification-dependent but not of type II systems.

For example, the normal level of restriction of λ with 5 targets by *E.coli* K-12 cells is 10^4 and in the case of RA the level of restriction may be as low 10 fold (Bertani and Weigle, 1953 ; Day, 1977). This remaining restriction activity will be referred to as residual restriction activity.

The acquisition of type I *hsd* genes and their regulation.

One might expect that the acquisition of type I *hsd* genes conferring a new specificity would lead to cell death because the chromosome of the recipient bacterium has unmodified targets for the new specificity. However, *hsd* genes can be easily transferred from one strain to another by transfection, conjugation or transformation (Sain and Murray, 1980; Suri and Bickle, 1985; Skrzypek & Piekarowicz 1989; O'Neill *et al.,* 1997). But when an F'plasmid, carrying the *Eco*AI genes was used in matings with a recipient that expressed *hsdR* on a multicopy plasmid, this conjugation was lethal for the recipient cell due to the restriction of its genome (Suri and Bickle, 1985). Similarly, when λ phage encoding *hsdS* of *Eco*AI or *Eco*EI infected bacteria that had a plasmid with *hsdR* and *hsdM* of *Eco*AI the recipient bacteria were killed (Fuller-Pace *et al.,* 1985). These facts imply the existence of mechanisms for regulation of the expression of *hsd* genes during their establishment in a bacterium that lacks the appropriate protective modification.

The *hsdR* gene of the *Eco*KI system has a separate promoter but no evidence for transcriptional regulation has been found (Loenen *et al.,*1987, Daniel *et al.,* 1987). Prakash-Cheng *et al.* (1993) using *hsd::lacZ* fusion found no evidence for transcription regulation of expression of the *hsd* genes of *Eco*KI following transfer by conjugation of F'-plasmids. Both the fusion with the promoter of *hsdR* and that with *hsdMS* genes had approximately the same level of β-galactosidase activity. Additionally, a mutant, *hsdC* (for *c*ontrol), has been isolated that cannot survive transfer of an F'factor that includes *hsdR, M* and *S* while the corresponding methylase genes can be transferred readily (Prakash-Cheng *et al.,* 1993).

A reduction in the level of restriction was also noted following conjugation experiments from m_k^- Hfr donors (Glover and Colson, 1966). This RA was not dependent on the origin of transfer of the chromosomal DNA and a saturation of the restriction enzyme by targets on the unmodifued chromosomal DNA was proposed as an explanation of the observed effect. However, RA was also noticed in conjugation experiments with F' and F$^+$ donors, although to a lesser degree.

Many years later it was shown (Makovets *et al.*, 1998) that *clpX* and *clpP*, which encode the ClpXP protease, are critical for the establishment of type IA and type IB systems. Mutants deficient in this protease either do not survive (*Eco*KI) or show a lower rate of survival (*Eco*AI) after transfer of *hsd* genes by conjugation, transformation and transduction. The drop in the titre of the recipient in the conjugation experiments is more severe using the *hsdC* recipient than that detected for a *clpX* recipient and the presence of an additional mutation in the *hsdC* recipient was suggested (Makovets *et al.*, 1998). Makovets et al.(1998) proposed a hypothesis that ClpXP degrades HsdR, but not HsdM or HsdS, and thus allows the methylase to modify the host chromosome in a naïve cell.

The 'missing link' between the reduced level of restriction during the establishment of type I R-M systems, restriction alleviation after exposure of the cells to DNA-damaging agents and the constitutive RA found in some mutants was provided by the work of Makovets and colleagues (1999). It was shown that RA is ClpXP-dependent in mutants that show constitutive RA (*dam*, *mut*) and in *hsd*$^+$ cells grown in the presence of 2-aminopurine (2AP).

The authors proposed a hypothesis that the degradation of the restriction subunit by ClpXP protease was caused by unmodified targets appearing on the bacterial chromosome and required the presence of a functional translocation complex. An amino acid substitution in one of the DEAD-box motifs in HsdR of *Eco*KI, which led to an enzyme unable to translocate and cut DNA, prevented degradation of HsdR even in the presence of functional *Eco*AI complex, e.g. a *cis* signal is required for the degradation of a type I restriction complex.

Inducible Restriction Alleviation

The first report of restriction alleviation of a type I restriction enzyme followed the exposure of E.coli K-12 cells to UV radiation (Bertani and Weigle, 1953). Day (1977) proposed a blockage of restriction enzyme by inhibition of its action as one of the possible causes of RA.

UV-induced RA affects infection with unmodified phages, e.g. λ, unglycosylated T4 and T2 (Dharmalingam and Goldberg, 1980) and transformation by unmodified plasmids, although in the case of transformation the residual restriction activity is much higher (Hiom and Sedgwick, 1992). The maximum level of RA of type I restriction enzyme is reached at 60-90 minutes after treatment and declines later. RA requires protein synthesis (Thoms and Wackernagel, 1982).

The induction of restriction alleviation *Eco*KI is abolished in mutants in which the RecA protein is defective (*recA13, recA56*) or its protease activity is altered (*recA430*) (Thoms and Wackernagel, 1984). On the other hand constitutive derepression of the SOS regulon by the *recA730* allele did not lead to RA and UV irradiation is still required (Hiom and Sedgwick, 1992). Additionally, UV-induced RA does not occur in *lex* (Day 1977), *recBC* (Thoms and Wackernagel, 1984) and *recF* (Thoms and Wackernagel, 1984) strains.

An additional unidentified protein necessary for the induction of RA was later found to be functional UmuD'C: RA is one hundred times lower in an *umuD* mutant and is not activated by overexpression of UmuDC if UmuD cannot be processed to yield UmuD' (Hiom and Sedgwick, 1992).

Summarising the genetic requirements for the UV-inducible RA, there is a parallel between it and the requirements for SOS mutagenesis (reviewed in Humayun, 1998). During SOS mutagenesis proteolysis of LexA repressor by the activated RecA-DNA complex is necessary for the activation of the SOS response and for proteolysis of UmuD. Proteolysis of UmuD leads to the production of an active form, UmuD', and
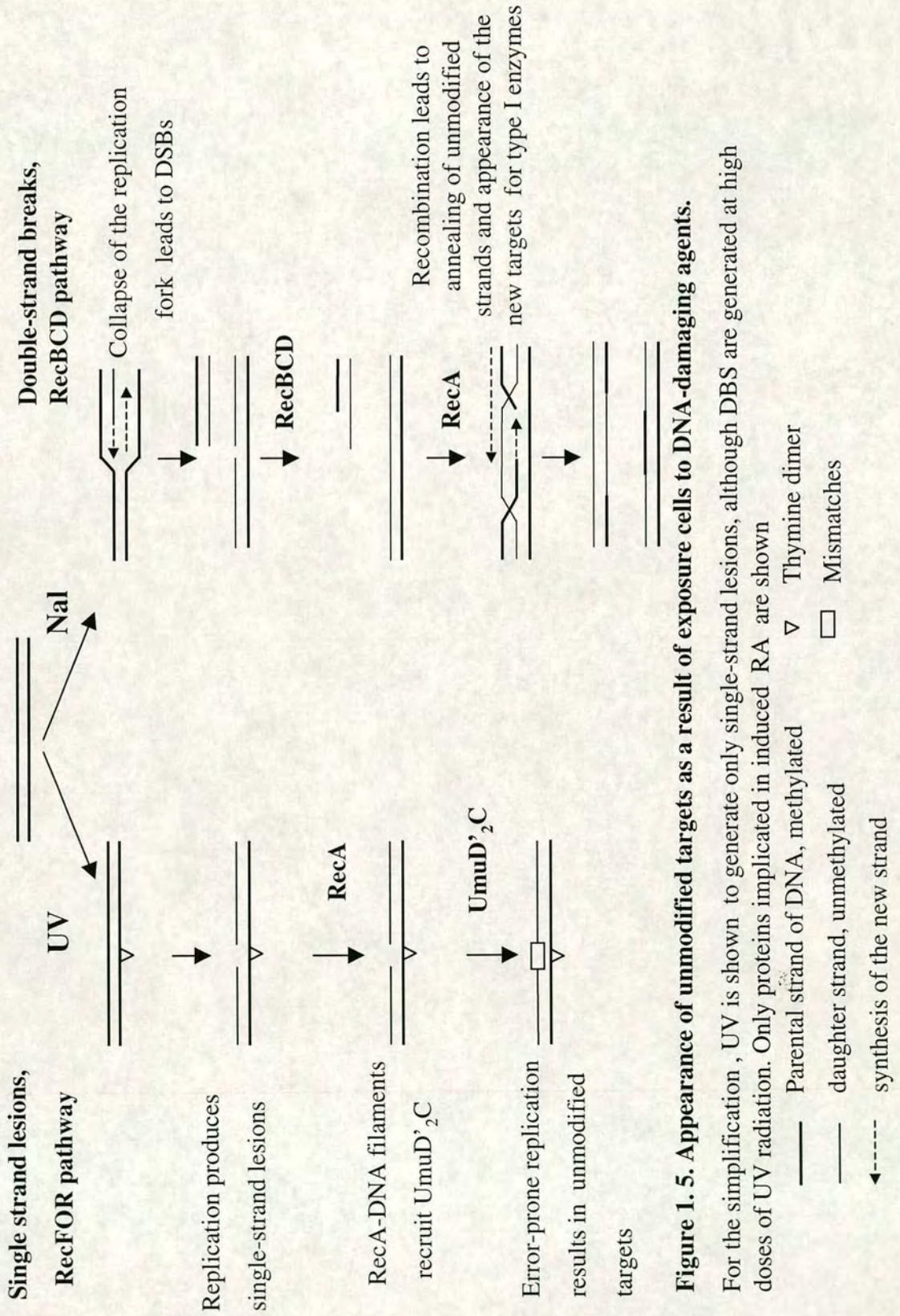
consequent formation of UmuD'$_2$C. UmuD'$_2$C is an error-prone DNA polymerase and it has an ability to stimulate both nucleotide misincorporation and mismatch extension (Tang *et al.*, 1998).

Nalidixic acid (Nal)-induced RA occurs when rapidly growing cultures are exposed to a sublethal concentration of the antibiotic for 90-120 minutes; Nal inhibits DNA gyrase. Nal-induced RA is dependent on *recBC*, but not on *lex* or *recF*. It can be pointed out that UV differs from nalidixic acid as a DNA-damaging agent: Nal produces DSB as a result of stalling of replication forks and UV leads primarily to lesions in the DNA as a result of the replication fork by-passing thymine dimers. The single-strand gaps can be repaired by recombinational repair – hence the requirement for *rec* genes or, if the error free repair systems are saturated, for the UmuD'$_2$C pathway. Accordingly, the RecFOR pathway repairs single-strand gaps in the daughter strand and RecBCD takes part in the repair of double-strand breaks (Kuzminov, 1999).The difference in time of occurrence of UV and Nal-inducible RA can be explained by the repair of daughter strand occurring earlier than the repair of DSBs (Kuzminov, 1999).

Efimova et al (1988b) reported that the level of restriction by *Eco*KI, *Eco*BI and *Eco*DI but not type II systems (*Eco*RI) was found to be reduced after treatment of the restriction-proficient cells with either 2AP or 5-bromouracil (5BU). The relief of restriction was the biggest for *Eco*KI and smallest for *Eco*AI and was independent of *recA* or *lexA* genes. Both 2AP and 5BU are strong inducers of mismatches (Bebenek and Janion, 1983).

Makovets and colleagues (1999) compared all three inducers of RA of type I R-M enzyme (UV, Nal and 2AP) and found that inducible RA is completely abolished in *clpX* and *clpP* mutants. Direct evidence for the degradation of the HsdR subunit during the growth of restriction-proficient cells in the presence of 2AP was obtained by pulse-chase experiments. The authors suggested that the new, unmodified, targets that appear on the chromosome as a result of any DNA-damaging agent induce RA (Figure 1.5).

Single strand lesions, RecFOR pathway

Double-strand breaks, RecBCD pathway

UV

Nal

Collapse of the replication fork leads to DSBs

Replication produces single-strand lesions

RecA

RecBCD

RecA-DNA filaments recruit UmuD'$_2$C

RecA

Recombination leads to annealing of unmodified strands and appearance of the new targets for type I enzymes

UmuD'$_2$C

Error-prone replication results in unmodified targets

Figure 1.5. Appearance of unmodified targets as a result of exposure cells to DNA-damaging agents.

For the simplification , UV is shown to generate only single-strand lesions, although DBS are generated at high doses of UV radiation. Only proteins implicated in induced RA are shown

▽ Thymine dimer

□ Mismatches

—— Parental strand of DNA, methylated

— daughter strand, unmethylated

----▶ synthesis of the new strand

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A deficiency in ClpXP protease has been shown to be lethal in the presence of functional *Eco*KI when bacteria are grown in the presence of 2AP (Cromie and Leach, 2001). The levels of sensitivity to 2AP in *rec* mutants are consistent with *Eco*KI introducing DSB into the bacterial chromosome.

Some type IC and type ID systems show RA under conditions of treatment with 2AP, although the RA for a type IC system is not dependent on Lon or Clp proteases (S.Makovets and N.E.Murray, unpublished observations).

Constitutive Restriction Alleviation

This thesis describes mutations in the *hsdM* gene of *E. coli* K-12 restriction enzyme, which lead to a permanent low level of restriction by EcoKI independent of the growth conditions, i.e. restriction is alleviated constitutively.

A number of amino acid substitutions in the N-terminal TRD of the HsdS gene of the same system lead to constitutive restriction alleviation. The mutants have impaired modification activity and require ClpX for viability (O'Neill *et al.*, 2001).).

The previous section (Inducible restriction alleviation) has shown that recombination associated with DNA repair and error-prone DNA synthesis, which lead to accumulation of new targets for type I R-M enzymes, result in RA. One might expect that mutations that result in the increased recombination and/or accumulation of replication errors would result in RA.

Indeed, some mutations, which are not located in the *hsd* region, lead to constitutive restriction alleviation, which, in contrast to inducible RA, is not transient. First, a mutation in *dam,* the gene specifying the maintenance methyltransferase, was shown to result in constitutive RA, (Efimova *et al.,* 1988a). The characteristic traits of the

Dam phenotype are hypermutability (Arraj and Marinus, 1983; Marinus *et al.*, 1984) and hyperrecombination (Peterson and Mount, 1993; Marinus 2000) presumably because of the constitutive SOS response in *dam* strains. It has been shown that *dam* mutants are deficient in the correct repair of mispaired bases (Glickman *et al.*, 1978).

The hypothesis of mismatches as inducing agents of RA in the case of type I R-M systems was checked by Efimova et al.(1988b). Mutants with defects in genes responsible for the mismatch repair function (*mutH, mutL, mutS*) were shown to be deficient in restriction by type IA systems. The same phenotype of constitutive RA was conferred by a mutation in *dnaQ*. This mutation compromises the fidelity of DNA polymerase (Makovets *et al.*, 1999).

The effect of *topA* mutations is presumably similar to that obtained with Nal, leading to the disturbance of normal topology of the nucleoid, stalling of the replication forks and, finally, to DSBs although it might be pointed out that this mutation has a pleiotropic effect influencing the level of transcription of several genes, so an indirect effect on a protein which is required for RA can not be excluded.

Regulation of other nucleotide-dependent systems

Data for the regulation of those restriction systems that cut modified DNA, and for type III restriction systems, are scarce. EcoP1I and EcoP15I can be transferred to *E.coli* cells by transfection, conjugation or transformation, become established without difficulty and the regulation occurs on the posttranscriptional level (Redaschi and Bickle, 1996). It was reported that type III R-M systems may undergo RA in *dam⁻* strains (Efimova *et al.*,1988a) but restriction alleviation was not observed after treatment with 2AP (Efimova *et al.*,1988b).

The response of four different systems of *E.coli* K-12 – *Eco*KI as a control, and three systems that restrict modified DNA (McrA, McrBC and Mrr) was monitored. All the systems showed some response to treatment with UV (Kelleher and Raleigh, 1994).

McrBC behaved in a similar way to *Eco*KI, showing the same kinetics of induced RA. However, the level of RA was smaller for the McrBC system, which might account for a previous report of the absence of RA for this system when measured by the frequency of transformation by an unmodified plasmid (Hiom *et al.*, 1991). The interpretation of the reduction of activity of the McrA system, encoded by e14, a cryptic prophage, was complicated by the induction and loss of the prophage after treatment with UV (Hiom *et al.*, 1991; Kelleher and Raleigh, 1994). A possible mechanism of regulation of the McrA system at the level of translation has been reported by (Shivapriya *et al.*, 1995).

The mechanism of RA of the McrBC and Mrr systems remains to be investigated.

1.3 Molecular mechanisms of specific recognition and degradation of proteins by the cytoplasmic proteases.

Proteolysis is one of the general mechanisms of regulation of important cellular processes such as the cell cycle, programmed cell death and pathogenesis. Proteases are widespread and have homologues in bacteria, archea, plants and animals (see for review (Gottesman 1996; Gottesman, 1999). Proteases play an important role in regulation of the cell cycle of certain bacteria such as *Caulobacter* and *Bacillus*, but the present review will concentrate on the action of the ATP-dependent proteases in *E.coli* and mostly on Clp proteases, as these have been shown to be involved in regulation of the activity of type I restriction enzymes (Makovets *et al.*, 1998; Makovets *et al.*, 1999).

Many of cytoplasmic proteases of bacteria require ATP for the degradation of the protein substrates and they belong to the AAA protein family of ATPases associated with a variety of cellular activities, defined by an AAA domain. This domain of 230
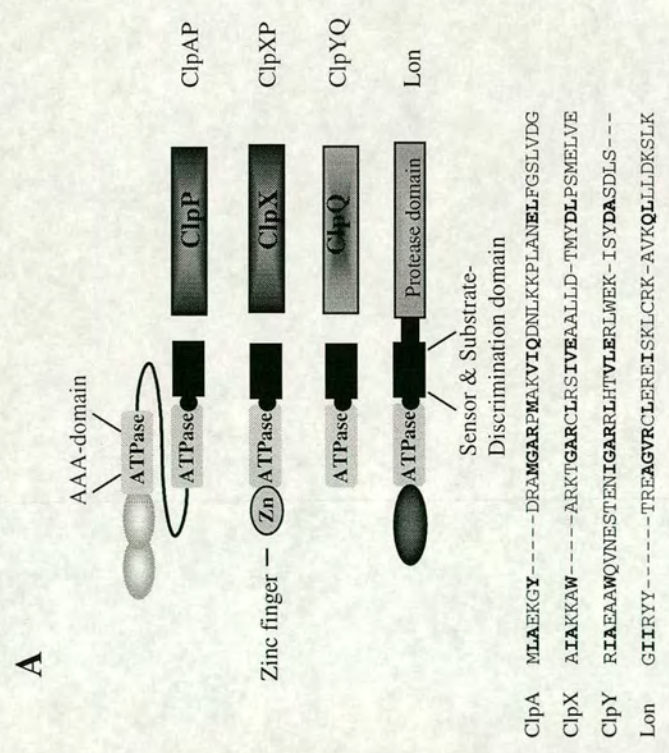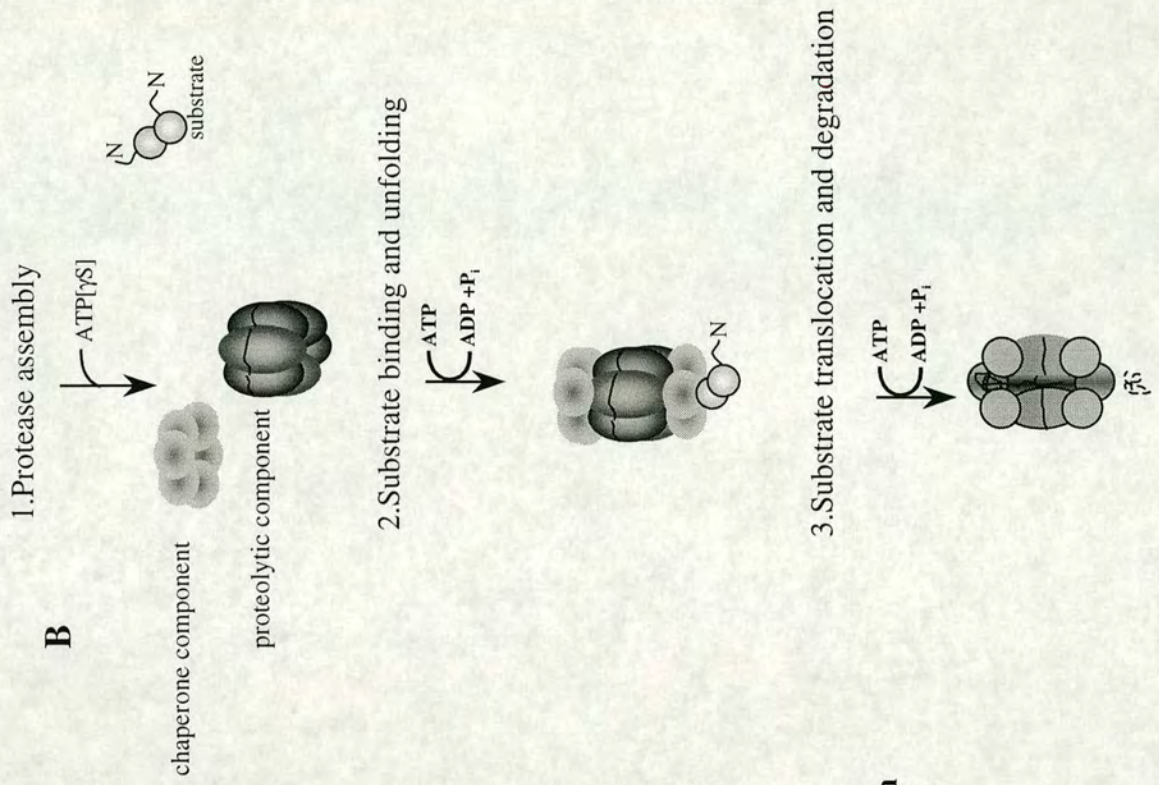
29

amino acid contains the Walker motifs and a conserved sequence, which is proposed to be associated with the conversion of the energy from ATP hydrolysis to the disassembly of protein complexes (Patel and Latterich, 1998; Neuwald *et al.*, 1999).

Intracellular proteases of *E.coli* include homooligomeric enzymes such as Lon (La) or HflB (FtsH) and heterooligomeric proteases ClpAP (Ti), ClpXP, ClpYQ (HslUV). For the heterooligomeric proteases ClpP and ClpQ are peptidase components, which alone can hydrolyse only short peptides. The chaperone-like subunits ClpA, X and Q, which can also act independently from the proteolytic components, provide substrate specificity to the complex (Suzuki *et al.*, 1997). The main difference between classical chaperones, like GroEL, and the specificity subunits of proteases like ClpA or ClpX is that classical chaperones recognise unfolded or misfolded and aggregated proteins and components of proteases disassemble complexes of "normal" proteins.

The term 'charonine' has been proposed to describe ATP-dependent proteases with intrinsic chaperone activity (Schumann, 1999). However, this is not a generally accepted terminology and this review will use the definitions given in (Gottesman, 1996).

The domain structures of the subunits of some proteases of *E.coli* are shown on Figure 1.6A (Smith *et al.*, 1999). Assembled heterooligomeric proteases are organised into ring of 6 or 7 subunits, stacked upon each other in such a way that the chaperone components serve as gates to the proteolytic chamber (Fig 1.6B) (Schmidt *et al.*, 1999).

Unlike the eukaryotic 26 proteasome, which mostly degrades ubiquinated proteins, the substrates of prokaryotic proteases do not possess a uniform signal that renders them susceptible to proteolysis. Prokaryotic proteases have substrate specificity; they degrade some proteins, but not others, even if similar in structure. So far it is not possible to predict that a protein will be recognised as a substrate for a particular protease. The least understood aspect of the proteolytic degradation is the recognition of the substrate by the chaperone. For example, the ClpXP protease was shown to

B

1. Protease assembly

ATP[γS]

chaperone component

proteolytic component

substrate
N
N

2. Substrate binding and unfolding

ATP
ADP + P$_i$

N

3. Substrate translocation and degradation

ATP
ADP + P$_i$

A

AAA-domain

ATPase

ATPase

ClpP — ClpAP

Zinc finger — (Zn) ATPase | ClpX — ClpXP

ATPase | ClpQ — ClpYQ

ATPase | Protease domain — Lon

Sensor & Substrate-Discrimination domain

ClpA MLAEKGY------DRAMGARPMAKVIQDNLKKPLANELFGSLVDG
ClpX AIAKKAW-----ARKTGARCLRSIVEAALLD-TMYDLPSMELVE
ClpY RIAEAAWQVNESTENIGARRLHTVLERLWEK-ISYDASDLS----
Lon   GIIRYY------TREAGVRCLEREISKLCRK-AVKQLLLDKSLK

Figure 1.6 Structure (A) and general mechanism of action

(B) of two-component ATP-dependent proteases

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recognise both the N-terminal part of a substrate – as in λO or UmuD', the C-terminal part as in MuA and the SsrA tag, or a sequence in the middle of a protein as in RpoS.

The current scheme of proteolysis is that, after recognition as a substrate, the chaperone binds and unfolds the substrate protein and threads it into proteolytic chamber using ATP as a source of energy.

Structure and general mechanism of action of Clp proteases.

In 1988 two groups independently discovered a protease in *E.coli,* which hydrolysed casein *in vitro,* in a reaction dependent on ATP and Mg^{2+} (Maurizi *et al.,* 1990; Katayama *et al.,* 1988).

It was found that the Clp protease (also called Ti) consists of two components - ClpA and ClpP – combined into multisubunit complex. ClpAP and ClpXP have mini-proteasome structures composed of four stacked rings, organised into chambers, where the unfolding and proteolysis of the substrate takes place (Schmidt *et al.,* 1999). ClpA is responsible for ATP hydrolysis but cannot degrade proteins in the absence of ClpP. The ClpP subunit has an active site characteristic of serine proteases, but can only degrade small peptides in the absence of ClpA. An alternative chaperone component of Clp proteases was identified later and named ClpX (Wojtkowiak *et al.,* 1993).

ClpA and ClpX belong to the Hsp100/Clp family of remodelling proteins and both are heat shock proteins (Schmidt *et al.,* 1999). ClpA was shown to exhibit a chaperone-like function reassembling and directing the RepA replication protein of the P1 prophage to degradation by ClpP (Wickner *et al.,* 1994). An elegant experiment in which the SsrA tag was fused to the green fluorescent protein (GFP) demonstrated that ClpA can unfold stable proteins (Weber-Ban *et al.,* 1999). When 70 amino acids from the N-terminus of RepA were fused to GFP, ClpAP degraded

the resulting fusion protein. It was shown that ClpA binds the Rep-GFP fusion without perturbing the native conformation of the protein, unfolds it in the presence of ATP and passes it to the proteolytic chamber, hydrolysing ATP (Hoskins *et al.*, 2000). Additionally, ClpA was able to bind unfolded GFP lacking a recognition signal but not native GFP.

ClpX, like ClpA, disassembles protein complexes, for example the MuA transposase tetramer or dimer of the replication protein of the R6K plasmid (Levchenko *et al.*, 1995), but it lacks the ability to bind unfolded proteins (Levchenko *et al.*, 1997a); ClpX interaction with proteins is facilitated by specific recognition motifs (Singh *et al.*, 2000).

a) SsrA tag

The SsrA tag is probably the closest approximation, in principle, to conjugation with ubiquitin in eukaryotes as a signal that targets proteins to degradation by the proteosome. It was shown that murine proteins produced in *E.coli* acquire an 11-residue C-terminal peptide tag -AANDENYALAA-COOH on their C-terminus and that this peptide is specified by the 10Sa RNA gene (*ssrA*) (Tu *et al.*, 1995). It was then shown that this mechanism of degradation works not only on foreign proteins but on *E.coli* proteins such as the LacI repressor (Abo *et al.*, 2000).

It was then shown that proteins translated from mRNA that lacks stop codons acquire an SsrA tag by switching the ribosomes from the damaged mRNA to *ssrA* tmRNA. Proteins tagged with the SsrA are sequentially degraded by cytoplasmic proteases (Keiler *et al.*, 1996).

The proteases responsible for the degradation of SsrA-tagged proteins are ClpAP and ClpXP (Gottesman *et al.*, 1998). *In vitro* both proteases act equally effectively, but *in vivo* ClpXP is more important. A minimal *in vitro* system of proteolytic degradation in this work consisted of λO protein tagged with SsrA, ATP, ClpP and either ClpA or ClpX. The degradation of λO-SsrA was shown to be mediated by Clp

proteases at 32°C but some other proteases were involved in degradation of the substrate at 39°C. One of these additional proteases, HflB, can compensate for the absence of Clp complexes, when overexpressed (Herman *et al.*, 1998).

More recently (Levchenko *et al.*, 2000) investigated additional factors that are involved in the SsrA tag-mediated degradation of abnormal proteins. A factor that enhances degradation of proteins fused to SsrA is a ribosome-associated protein, SspB (*stress starvation protein*), encoded by part of an operon important for the survival of bacterial cells in stationary phase (Williams *et al.*, 1994).

The recognition of SsrA-tagged proteins by ClpXP is enhanced 10 fold by SspB *in vitro*. The degradation of λO-N-SsrA was abolished *in vivo* in a ClpX mutant confirming that SspB protein is a specific factor enhancing degradation of SsrA-tagged proteins by ClpXP. Different parts of the -AANDENYALAA-COOH peptide were shown to be responsible for the binding of SspB protein and for recognition by Clp proteases. The degradation of some substrates, e.g. MuA and λO protein by ClpXP protease is not influenced by the presence of SspB.

b) Other substrates

These can be divided into broad groups of phage and plasmid replication proteins, cellular proteins and addiction modules.

Phage and plasmid replication proteins

ClpX as a chaperone and as a part of the ClpXP protease plays a dual role in the life cycle of phage Mu. ClpXP protease degrades Mu repressor, promoting the transposition of the phage. Virulent mutants of phage Mu, which successfully infect Mu lysogens and induce the resident prophage, have amino acid changes in the Mu repressor. These changes lead to an abnormally short half-life of the protein and their effect is *trans*-dominant (Geuskens *et al.*, 1991; Geuskens *et al.*, 1992).Other *vir* mutations, that result in the more rapid degradation of the repressor protein were

investigated. It was found that mutations that conferred the *vir* phenotype, and others that caused the reversion to the wild-type phenotype, affected the C-terminus of Mu repressor. A single amino acid change is sufficient to decrease the rate of proteolysis (Laachouch *et al.*, 1996).

MuA transposase binds to the ends of the Mu genome and assembles itself intö a tetramer that catalyses the transfer of each Mu end to the target DNA. ClpX disassembles the tetramer, releasing monomers of MuA into solution. The ten C-terminal amino acids of MuA, when transferred to the Arc - repressor of phage P22, were sufficient to convert the repressor protein into a substrate for ClpXP protease (Laachouch *et al.*, 1996).

It was also found that the region of transposase that interacts with MuB, the activator of transposition, was within the 30 amino acids at the C-terminus of MuA and this region overlaps the sequence recognised by ClpX . MuB inhibits disassembly of the MuA tetramer by ClpX, providing a mechanism for the regulation of the action of the chaperone (Levchenko *et al.*, 1997b).

Interestingly, the effect of a mutation, *Mucts62*, that leads to the thermosensitive derepression of Mu transposition, is dependent on ClpXP and Lon, and it requires carbon starvation in addition to the increase of temperature (Lamrani *et al.*, 1999). It would be of interest to investigate a possible involvement of SspB (see (Levchenko *et al.*, 2000) in the regulation of transposition of phage Mu.

λ O protein, which may be relevant to the switch of replication of the phage DNA to the rolling circle mode, has a very short half-life *in vivo* and its degradation was identified to be dependent on ClpXP protease (Wawrzynow *et al.*, 1995). The O protein is protected from degradation while bound to oriλ or as a part of oriλ:O:P:DnaB preprimosome complex but becomes susceptible to proteolysis under conditions permissive for transcription or as a result of increased negative supercoiling, e.g. after dissociation of λ O from the DNA (Zylicz *et al.*,1998).

Studies of recognition and targeting of λ O protein for binding to ClpXP and its hydrolysis have shown that deletion of first 18 amino acids from the N-terminus of the protein abolishes its degradation by ClpXP but not its binding to ClpX (Gonciarz-Swiatek *et al.*, 1999). The authors propose a hypothesis that ClpXP must first bind to an unidentified motif remote from the termini before the N-terminus sequence is recognised and used for unfolding the protein and its subsequent degradation.

Table 1.3 Examples of sequences recognised by ClpA or ClpX

| Protein	Source	Protease	Recognised sequence	Reference
RepA	P1 phage	ClpAP	N-MNQSTFISDILYADIE	(Hoskins *et al.*, 2000)
λ O	phage lambda	ClpXP	?	(Gonciarz-Swiatek *et al.*,1999)
Mu repres-sor	phage Mu	ClpXP	---QEVKKAV-C	(Laachouch *et al.*, 1996).
MuA	phage Mu	ClpXP	**ERDDEYETERDEYL**NHS LDI<u>LEQNRRKKAI</u>-C **Bold** – interaction with MuB, <u>underlined</u> – with ClpX	(Levchenko *et al.*, 1997b)

Cellular proteins

The sigma factor σ^S (Rpo^S), which replaces the major sigma factor, RpoD, in the stationary phase, is responsible for the transcription of a variety of genes expressed after cells enter the stationary phase. Rpo^S is rapidly degraded in the logarithmic phase (Hengge-Aronis, 1993) and its degradation is mediated by ClpXP protease (Schweder *et al.*, 1996). However, an additional protein, RssB, that has sequences

common to response regulator proteins, is necessary for the proteolysis(Muffler *et al.*,1996) as RpoS accumulates even in the logarithmic phase in an RssB mutant.

The action of RssB is modulated by phosphorylation (Bouche *et al.*, 1998) and a mutation affecting residue D58, the site of phosphorylation in MviA a *Salmonella thyphimurium* homologue of RssB, prevented degradation of RpoS (Moreno *et al*, 2000). RssB regulates the degradation of RpoS by direct interaction and sequences within the 180 N-terminal amino acids are necessary for the action of ClpXP (Zhou and Gottesman, 1998).

The turnover element, defined by the demonstration that the deletion of amino acids 177-183 completely abolished proteolysis of σ^S (Schweder *et al.*, 1996) was studied in detail by (Becker *et al.*, 1999). Site-directed mutagenesis was used to change the amino acids most dissimilar between RpoS and stable RpoD in the region identified by the deletion. This led to the identification of K173 as a critical residue. The change of this lysine to glutamic acid abolished proteolysis *in vivo* and led to the increased half-life of RpoS *in vitro*. E174T and V177K changes slightly increase the half-life of the corresponding mutant proteins (Figure 1.7).

$$\Delta(aa173-188)$$

RpoS   IHIV**K**ELN**VY**LRARELSHKLD
RpoD   VHMI**ET**IN**KL**NRISQMLQEMQ
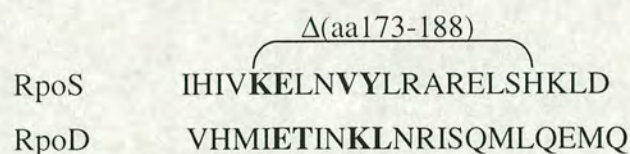
Figure 1.7 Alignment of the region of RpoS containing the turnover element with the corresponding region of RpoD. The in-frame deletion that interferes with RpoS proteolysis is shown. Residues changed in site-directed mutagenesis are shown in bold.

Unlike many other substrates of Clp proteases, the restriction subunit of type IA and IB restriction enzymes is a stable protein under normal conditions of growth of bacterial cells but it is degraded if cells acquire unmodified sequences on the

chromosome. The restriction subunits (HsdR) of type IA (EcoKI) and type IB (EcoAI) restriction enzymes were shown to be degraded if bacteria are grown in the presence of 2-aminopurine. A mutation that prevented translocation of DNA also abolished this ClpXP-dependent proteolysis and the HsdR subunit of only active complexes was degraded, even when a translocating complex from the other type I enzyme was present, i.e. the signal for degradation does not act *in trans* between translocating restriction complexes of different families (Makovets *et al.*, 1999).

Attempts to reproduce ClpXP proteolysis *in vitro* have been unsuccessful under conditions that permitted degradation of λ O protein (L.Powell, pers.com) suggesting that some additional factor(s) are required for degradation of HsdR subunit.

Unsolved problems of the proteolytic regulation of type I restriction systems.

The present introduction reviews the information on type I R-M systems, including the regulation of their restriction activity. Recent work by Makovets and colleagues provided evidence for the proteolytic regulation of type I systems (Makovets *et al.*, 1998; Makovets *et al.*, 1999). ClpXP protease was implicated in the degradation of the restriction subunit of both *Eco*KI (type IA) and *Eco*AI (type IB). The proposed hypothesis states that when a restriction complex recognises an unmodified target sequence in the bacterial chromosome and initiates DNA translocation, it is recognised by ClpXP and HsdR is degraded.

However, Makovets *et al.* (1999) base this hypothesis on the result of indirect evidence for unmodified chromosomal DNA. It was shown that the HsdR subunit is degraded in r^+m^+ cells after their treatment with 2AP. Incorporation of 2AP into the bacterial chromosome during DNA replication was presumed to lead to the appearance of unmodified targets in the chromosome, the consequent activation of the restriction enzyme and subsequent degradation of HsdR. Additionally, a mutation in *hsdR* that results in a restriction complex capable of binding to the target DNA, but not capable of restriction activity, prevented the proteolysis.

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Direct evidence that unmodified target sequences are the primary signal for the ClpXP-dependent degradation of HsdR will be shown in the present thesis. The data presented in Chapters 3-5 will also attempt to answer the following questions:

a) Is the presence of a restriction-proficient, modification-deficient type I restriction enzyme lethal for a bacterial cell?

b) What is the signal for RA?

c) At what stage of the restriction reaction is the restriction complex recognised by the ClpXP protease?

d) What is a possible explanation of the residual restriction activity during RA?

e) Might the localisation of *Eco*KI explain the different cellular response to foreign and resident DNA?

Chapter 2 Materials and methods

Bacteria

E.coli K-12 strains	Relevant genotype or phenotype	Reference
AB1157	$hsd_k rac^-$	De Witt & Adelberg, 1962
C600	hsd_k	Appleyard,1954
DH5α	endA1hsdR17recA1gyrA	Grant et al., 1990
ED8654	hsdR514supE44supF58	Murray et al., 1977
JC9935	AB1157 $recA13sup^0$	J.Clark
KL32	recA Δ (hsdRM)	S. Makovets
NK224[b]	Δ(hsdMS) gyrA	S.Makovets
NK301	rac^- gyrA lacY thr leu thi	Makovets et al., 1999
NK304*	clpX::kan	Makovets et al., 1999
NK308*	recA::cat	Makovets et al., 1999
NK311*	Δ(mcr hsd mrr)	Makovets et al., 1999
NK312*	Δ(mcr hsd mrr)recA::cat	Makovets et al., 1999
NK352*	Δ(hsdRM)	Makovets et al., 1999
NK379*	hsdRΔ4	NK301 x P1(NM802)
NK380*	hsdRΔ4 clpX::kan	NK379 x P1(NK304)
NK382*	hsdRΔ4 hsdM(F269G)	NK380 x λNM1394
NK383*	hsdRΔ4 hsdM(F269G) recA::cat	NK352 x P1 (NK312)
NK384 *	hsdRΔ4 hsdM(F269G) clpX::kan	NK352 x P1 (NK304)
NK386*	hsdM(F269G)	NK301 x λNM1394
NM679[a]	W3110Δ(mcr hsd mrr)	King and Murray, 1995
NM181	hsd_k^+ $r^+_{RI} m^+_{RI}$	Kelleher et al.,1991
NM146	hsdR $r^+_{RI} m^+_{RI}$	Murray and Murray,1974

NM522	*Δ(hsdMS)*	Gough and Murray, 1983
NM890[¶]	*hsdR*(F629Y)	N.Murray
NM904[¶]	*hsdR*(D298E)	N.Murray
NM908[¶]	*hsdR*(E312H)	N.Murray
VC301*	*lac⁺*	NK301 x P1(AB1157)
VC3017*	*lac⁺ hsdM*(F269G)	NK386 x P1(AB1157)
VC4[¶]	*ΔhsdMS recA::cat lamB::tet*	This study
VC2[¶]	*ΔhsdR recA::cat lamB::tet*	This study
VC48*	*clpX::kan tsx::tet*	NK301x P1
VC802[¶]	*ΔhsdR gyrA*	This study
VC797[¶]	*hsdR*(H577D) *hsdM*(F269G)	NM797 x λNM1394
VC799[¶]	*hsdR*(A619V) *hsdM*(F269G)	NM799 [x] λNM1394
VC801[¶]	*hsdR*(K477R) *hsdM*(F269G)	NM801 [x] λNM1394
VC802[¶]	*hsdRΔ4 gyrA*	NM802 x P1(NK301)
VC803[¶]	*hsdRΔ4 recA::cat*	VC803 x P1(VC802)
VC892[¶]	*hsdR*(G177C) *hsdM*(F269G)	NM 892 [x] λNM1394
VC893[¶]	*hsdR*(D502Y) *hsdM*(F269G)	NM893 [x] λNM1394
VC898[¶]	*hsdR*(R826H) *hsdM*(F269G)	NM898 [x] λNM1394
VC904[¶]	*hsdR*(D298E) *hsdM*(F269G)	NM904 [x] λNM1394
VC908[¶]	*hsdR*(E312H) *hsdM*(F269G)	NM908 [x] λNM1394
VC890[¶]	*hsdR*(F629Y) *hsdM*(F269G)	NM890 [x] λNM1394
VC914[¶]	*hsdRΔ4 hsdM177gyrA*	VC802 x λNM1332
VC8026[¶]	*hsdRΔ4 hsdM269 gyrA*	VC802 x λNM1394
VC3003[¶]	*hsdRΔ4 hsdM269 clpP::cat gyrA*	VC802 x P1 (NK303)

[a] ⁻ different isolates have different properties, see Section 3.2

[b] – all NK strains are derivatives of C600, but some are rac⁻

* - derivatives of NK301

[¶] ⁻ derivatives of AB1157

Bacteriophages

Bacteriophage	Relevant genotype or phenotype	Reference
T7ocr⁻	D104 Δ1.3-7.2	Garcia & Molineux,1999
M13 mpAT38	M13mp18 with targets for *Eco*KI	A.J.B. Titheradge
λ*vir*	5 targets for *Eco*KI	Laboratory collection
λNM1326	*hsdM*(F269G) *hsdS⁺ imm²¹*	Willcock *et al.*,1994
λNM1327	*hsdM*(F269C) *hsdS⁺ imm²¹*	Willcock *et al.*,1994
λ NM1329	*hsdM*(F269Y) *hsdS⁺ imm²¹*	Willcock *et al.*,1994
λNM1330	*hsdM*(N266D) *hsdS⁺ imm²¹*	Willcock *et al.*,1994
λNM1331	*hsdM*(N266D, F269G) *hsdS⁺ imm²¹*	Willcock *et al.*,1994
λNM1332	*hsdM*(G177D) *hsdS⁺ imm²¹*	Willcock *et al.*,1994
λNM1384	λ*hsdR*(A619V) *cI*857	N.E.Murray
λNM1394	*hsdM*(F269G) *hsdS⁺ cI*857	Makovets *et al.,* 1999
NM150	h^{82} *imm*$^\lambda$ with 4 targets	Laboratory collection
NM106	phage 82 with two targets for *Eco*KI	Laboratory collection
NM105	h^{80} *imm*$^\lambda$ with two targets for *Eco*KI	Laboratory collection

Plasmids

Plasmid	Relevant genotype or phenotype	Reference
pACYC184	*cat tet*	Chang and Cohen, 1978
pNK3	pACYC184/*hsdR*	Makovets *et al.,* 1999
pBRsKI	pBR322, one *Eco*KI target	Davies, 2000
pJK2	pBR322/*hsdR bla*	Kelleher *et al.,* 1991
pBRK	pBR322 *tet neo*	Laboratory collection
F'101-102	*hsdR⁻M⁺S⁺ zjj::*Tn*10*	Makovets *et al*, 1998
F'101-103	*hsdR⁺ zjj::*Tn*10*	This study

Standard solutions and reagents

All solution were made using sterile, deionised water

Agarose gel load dye

10% (w/v) Ficoll 400, 50mM EDTA (pH 8.0), 0.5% SDS, 0.25% bromphenol blue or Orange G.

ATP

100mM ATP, 10mM Tris, pH adjusted to 7.0 with NaOH. Concentration determined from the A_{260nm} of a 10^{-3} dilution using $\varepsilon_{ATP(pH\ 7.0)} = 15.4$. Aliquots were stored at -70°C.

Boehringer Mannheim Buffer A

33 mM Tris-acetate (pH 7.9), 10 mM Mg-acetate, 66 mM K-acetate, 0.5 mM Dithiothreitol.

50 x TAE Buffer

242g Tris, 571 ml glacial acetic acid and 100 ml 0.5M EDTA (pH.8.0)

20 x TBE Buffer

1.78M Tris, 1.78M Boric Acid, 50 mM EDTA (pH.8.3)

TE Buffer

10 mM Tris (pH 7.5), 1 mM EDTA (pH.8.0)

10 x TBS Buffer

60g Tris, 87.6 g NaCl, adjust to pH 7.5 with HCl, H_2O up to 1 l.

Transfer Buffer

9 g Tris, 43.2 g Glycine, H_2O up to 3 L.

Nuclease Buffer

20 mM Tris-HCl (pH7.5), 10 mM $MgCl_2$ 7 mM 2-mercapthoethanol, 10% glycerol, 10 mM PMSF, 10 MM benzamidine.

Phage buffer

22 mM monopotassium phosphate (KH_2PO_4), 5mM disodium phosphate (Na_2HPO_4), 8.5 mM sodium cloride(NaCl), 0.1 MM magnesium sulphate ($MgSO_4$), 1 MM calcium chloride ($CaCl_2$), 1 x 10^{-3} (w/v) gelatine.

Ethidium Bromide Stock

10 mg/ml in dH_2O, stored in dark at 4°C.

Antibiotics:

- Ampicillin (Amp): stock solution 100 mg/ml in dH_2O, 100µg/ml final concentration;
- Chloramphenicol (Cat): stock solution 10 mg/ml in 50% ethanol, 10µg/ml final concentration;
- Kanamycin (Kan): stock solution 10 mg/ml in dH_2O, 100µg/ml final concentration;
- Rifampicin (Rif): stock solution 10 mg/ml in methanol, 50µg/ml final concentration;
- Streptomicin (Str): stock solution 20 mg/ml in dH_2O, 50µg/ml final oncentration;
- Tetracyclin (Tet): stock solution 10 mg/ml in dH_2O, 10µg/ml final concentration;

Media

All media were sterilised by autoclaving at 15 lb/in^2 for 30 minutes

Luria-Bertany Broth (LB)

10 g Difco Bacto Tryptone, 10 g NaCl, 10 g Difco Bacto yeast extract, pH adjusted to 7.2 with NaOH, dH_2O up to 1 L.

LB agar

1.5g of Difco agar in 1 L of Luria-Bertany Broth

Baltimore Biological Laboratory (BBL) agar

5 g NaCl, 10 g BBL trypticase, 10 g Difco agar (6.5 g for BBL top agar).

Microbiological and genetic techniques

Long-term storage of bacterial cells

A single freshly grown colony was picked up with a loop and stabbed into a thick layer of LB-agar in a small glass bottle. The stabs were incubated overnight at 30°C to allow bacterial growth, leads were wrapped by Parafilm and kept at room temperature.

Preparation of plating cells

An overnight culture was diluted 5-fold in L-broth, grown to mid-log phase, and the cells were harvested by centrifugation (4,000 x g, 5 min) and resuspended in the same volume of 10mM $MgSO_4$.

Preparation of phage lysates

A single plaque was picked using a toothpick and resuspended in 1 ml phage buffer containing a few drops of chloroform (@10^7 p.f.u. /ml). 0.1 ml of the appropriate dilution of the phage, to give confluent lysis (@10^6 p.f.u. /ml), was mixed with an equal volume of plating cells, left for 15 minutes to allow phage adsorption, and 2.5 ml of molten BBL top agar was added before the mixture was poured on fresh BBL-

agar in a plate. The plate was incubated overnight at 37°C. 1 ml of L-broth was added, the top layer of BBL was harvested and after adding a few drops of chloroform and vortexing, resulting mixture was clarified by centrifugation (4,000 x g, 15 min). The phage lysate was collected and transferred to a fresh bottle and stored at 4°C.

Preparation of M13 lysates

F^+ bacteria were used as plating cells and lysate was clarified by centrifugation (12,000 x g, 5 min)

Restriction assays

Bacteria to be tested for restriction and a control restriction-deficient and restriction-proficient strains were grown until mid-log phase, and 1 ml of the cultures were mixed with 0.1 ml aliquots of the appropriate dilution of unmodified phage lysate (.0). After 15 minutes incubation at room temperature to allow phage adsorption, 2.5 ml BBL top agar was added and used to overlay BBL-bottom agar plate. Plaques were counted after overnight incubation and the ratio of p.f.u. on the tested strain to one on the control was taken as an. e.o.p. The reverse ratio displayed restriction. The experiments were accompanied by a control of a modified phage (. K).

For a quick non-quantitative test, 10 µl of appropriate dilutions of .0 and .K phages were spotted on lawns of test-bacteria and two control strains. After overnight incubation .0 phage produced a spot of confluent lysis on the restriction-deficient bacteria and a few plaques on the restriction-proficient control strain. Test bacteria were compared with controls.

Modification assay

A λ plaque grown on a strain to assay for modification was picked with a toothpick, resuspended in 1 ml phage buffer, treated with a drop of chloroform and the phage titre was determined on restriction-deficient and restriction-proficient bacteria.

If the phages are modified they form plaques with the same efficiency on both strains.

Lysogenisation of *E.coli* with λ and λ/ *E.coli* chromosome exchange

In this work we used different lambda phages with *cI857* and an *att⁻* mutations. *cI857* is a thermosensitive mutation in the lambda repressor which prevents maintenance of lysogenic state above 37°C. A mutation in the *att* site prevents phage integration into the attachement site and forces integration of the phage into the bacterial chromosome via homologous recombination between the chromosome and a fragment cloned in the phage.

To make an *E.coli* lysogen a lysate was diluted 100-fold in phage buffer and spotted (10 μl) onto a lawn of bacteria. The spots were dried and the plate was incubated overnight at 37°C to allow lysogenisation. Bacteria from the spots were streaked with a toothpick on fresh L-agar seeded with λ*imm*²¹*cI⁻* and h⁸²*imm*²¹*cI⁻*, homoimmune phages which lyse cells that do not contain a prophage and therefore select for lysogens. Alternatively, when the phage used for lysogenisation had a drug resistance gene, bacteria from the spots were streaked on agar with appropriate antibiotic. The plates were incubated overnight at 32°C and single colonies were purified at the same temperature and tested for the presence of a prophage as lysogens survive the infection by homoimmune phages but not λ*vir*.

The prophage can be induced at 42°C. On excision via homologous recombination, allelic exchange can occur between the sequences common to the λ and bacterial chromosome. The phage progeny can be analysed for allele exchange and phage with a new genotype can be obtained *in vivo*.

Conjugation

Cultures of donor and recipient bacteria strains were grown until mid-log phase (A$_{600}$ 0.3-0.4) and mixed in a flask, taking no more than 1/10 of volume. The ratio of donor:recipient was 10:1. Conjugation mixtures were incubated with gentle aeration on a rotating wheel for 2-3 hours, and appropriate dilutions were plated on selective media.

The frequency of transfer (f.o.t.) of a plasmid was calculated as the ratio of the titre of recipient cells that acquired the plasmid to the titre of the recipient cells in the conjugation mixture.

Preparation of P1 lysates

Overnight cultures were diluted 50-fold in L-broth and incubated until late log-phase and harvested by centrifugation. After decanting the supernatant, bacteria were resuspended in MC buffer 1/10 of the original volume. An 0.1 sample of cells was mixed with $10^5 - 10^6$ P1 phages in small test tubes and incubated in a 37°C water bath for 15-20 min. 2.5 ml of BBL-top agar was added to each tube, mixed and poured in a freshly prepared BBL bottom plate. Plates were incubated overnight and those with confluent lysis were used for obtaining the lysate.

P1 transduction

Cells were prepared as for P1 lysate but after incubation in a water bath sodium citrate was added to final concentration of 100 mM and the cells were plated on selective agar media. After overnight incubation the colonies were purified on the appropriate selective media and used for further experiments.

Preparation of competent cells for transformation

Overnight cultures were diluted 50-fold in flasks with L-broth and incubated at 37°C with vigorous aeration. When A_{600} reached 0.3-0.5 the cultures were chilled on ice-water bath for 15 minutes. The bacteria were transferred to pre-chilled McCartney bottles, harvested by centrifugation at 4°C, resuspended in ice-cold 0.1M $CaCl_2$ (1/10 of the initial volume) and incubated on ice for 1 hour. The cells were centrifuged and resuspended in ice-cold 0.1M $CaCl_2$ containing 20% glycerol (1/20 of the initial volume).

0.1 ml aliquots were used for transformation or transferred to pre-chilled eppendorfs and stored at -70°C.

Transformation

An aliquot of competent cells was thawed on ice, DNA was added (0.5-10 μl) and was incubated on ice for 1 hour. The cells were incubated at 42°C for 2 minutes and returned to ice for a minute. 1 ml of L-broth was added to each tube and the tube was incubated at 37°C for 1 hour. Bacteria were plated on selective media and incubated overnight to obtain transformants.

Restriction alleviation

Stock solution of 2AP in L-broth at the final concentration 400 μg/ml was added to mid-log cultures grown at 37°C in L-broth. Intensive aeration was provided before and during the treatment. After 1h, the cells were centrifuged at room temperature (4.000 rpm, 10 min) resuspended in 1/10 of initial volume of LB and tested for restriction or used for preparation of subcellular fractions.

Isolation of subcellular fractions

Preparation of spheroplasts.

All steps were carried at 4°C. Cells were grown until A_{600} ~0.6, harvested by centrifugation and washed 0.9%NaCl. Supernatant was completely removed. Cells were converted to spheroplasts by incubation for 30 min in plasmolysis buffer [50mM Tris-HCl pH8.0, 20% w/v sucrose, 1mM DTT]. Then 2 ml of a fresh solution of lysozyme 10mg/ml in 10 mM Tris-HCl pH8.0 was added. 100μl of 1M $MgSO_4$ and DNase, RNase (10 mg/ml) were added and the cells were incubated 0.5h. 0.5M EDTA pH 8.0 was slowly added and cells were incubated for 1.5 h on ice with gentle mixing every 10 minutes. The convertion to spheroplasts was monitored by phase contrast microscopy.

Spheroplasts were frozen at -70°C and were kept until required.

Spehoroplasts then were lysed by three cycles of freezing at -70°C for 30 min followed by 15 min at 37°C. After the first lysis an inhibitor of proteases methylphenylflouoride was added to the final concentration 1μM.

Manipulation of nucleic acids

Small-scale preparation of plasmid DNA

Samples of bacterial cells obtained from 5ml o/n cultures of *E.coli* were used and the procedures were carried out using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer instructions.

Small-scale preparation of λ DNA

0.1 ml of a culture of an appropriate host (~10^8 cells) was infected with the required phage (~10^8 p.f.u), left for 15 minutes to allow phage adsorption, diluted by 4ml of L-Broth supplemented with 10mM $MgSO_4$ and grown at 37°C with aeration until full lysis of the cells (3-5 hours). Remaining cells were lysed by the addition of the chloroform (0.1 ml), cells debris was removed by centrifugation (10,000 x g, 10 min), and nucleases (5 µl, 10 mM RNase and DNase) added to supernatant in a fresh tube. The lysate was incubated at 37°C for 30 minutes, and the phage precipitated by the addition of polyethylene glycol (PEG) and salt solution (4ml 20g 6K PEG, 11.7 g NaCl, 78 ml phage buffer). The mixture was left overnight at 4° C and the phage particles sedimented by centrifugation (13,000-x g, 20 min, 4°C). The supernatant was completely removed and the phages were resuspended in 0.5 ml of phage buffer. Chloroform was added (0.5 ml) to remove any remaining PEG, the mixture clarified by centrifugation (11,000 x g, 3 min) and the aqueous phase containing the phage particles was transferred to a tube containing phenol (0.5 ml) and TE buffer pH8.0(0.1 ml) and gently mixed.

The two phases were separated by centrifugation (11,000 x g, 3 min), and the aqueous phase transferred to a tube containing 0.5 ml of phenol: chloroform (1:1) mixture in Tris buffer pH 8.0, gently mixed and was separated into aqueous phase containing naked phage DNA and organic fraction. The aqueous phase was treated with chloroform to remove traces of phenol and ethanol precipitated. 1/10 volume of

3M sodium acetate (pH5.3) and two volumes of chilled ethanol were added and the solution was left at -70°C for 20 minutes. The DNA was sedimented by centrifugation (11,000 x g, 15 min), rinsed with 70% ethanol, dried, resuspended in TE (0.1 ml) and stored at 4°C.

Measuring DNA concentration

DNA concentrations were calculated by measuring UV-adsorption on a Perkin Elmer lambda 5 spectrophotometer. An A_{260} of 1 = 50 µg/ml for double-stranded DNA.

Cutting DNA with type II restriction enzymes

Endonuclease reaction was performed in a volume 10-40 µl containing 1-2 µg of DNA. The reaction contained the appropriate Boehringher Manheim (Rosche) or New England Biolabs restriction buffer 1 x concentration. BSA was added (to 1mg/ml) if recommended by the manufacturer of the enzyme. The digests were made up to final volume using distilled sterile water. The complete reaction mixtures were incubated at the temperature recommended for a particular enzyme for 1-4h. The products of the reaction were analysed using agarose gel electrophoresis. If digested DNA was used for ligation the restriction enzymes were inactivated (20 min at 65°C or 80°C according to the supplier recommendations). When an enzyme can not be heat inactivated an AG cartridge (Advanced Genetic Technologies Corp.) that allows purification DNA from proteins was used. If the DNA was to be subjected to purification from an agarose gel, it was not necessary to inactivate the enzyme.

The polymerase chain reaction

PCR was used to screen phages or plasmids for certain sequences. Reactions were performed in an OmniGene thermal cycler (Hybaid Ltd). Primers were designed about 20 base pairs long with GC pairs at both ends and GC content 60-70%. The melting temperature (T_m) was calculated $T_m \sim 4(G+C) + 2(A+T)$, the annealing temperature used in each reaction was 5 degrees lower than T_m.

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Typically the reaction was performed in 50 µl, with 1 x reaction buffer containing 2mM MgSO$_4$, 1 unit of polymerase, 200 µM dNTP mix, 0.4 µM primers and 10ng of DNA template and 1 unit of Vent® DNA polymerase (New England Biolabs). A layer of 20 µl mineral oil was added to cover each reaction mix.

A typical cycle was: 96°C for 5 minutes, then 20 cycles of (96°C for 1 minute, 52°C for 40 seconds, 72°C for 40 seconds), then 72°C for 5 minutes.

DNA sequencing

The nucleotide sequences of plasmid or PCR DNA templates were identified by automated sequencing using ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 377 DNA Sequencer. The procedure consists of several stages: template preparation, cycle sequencing, extension product purification, sample electrophoresis and data analysis. The last two stages were carried out by Nicola Preston in the department sequencing service.

Plasmid DNA templates were prepared by Qiagen mini-prep method and no further purification was required.

100 ng of dsDNA template were added to 4 µl terminator ready reaction mix and 1.6 pmol of an appropriate ssDNA primer in a total volume of 10 µl. The mixed reactions were then overlayed with 20 µl light mineral oil and cycled through 25 cycles of 96°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min. Samples were held at 4°C on completion of the programme before brief centrifugation of the contents in a microfuge.

After complete removal of mineral oil, 1 µl 3M sodium acetate (pH 4.6) and 25 µl 100% ethanol was added to the samples. Tubes were vortexed, placed at -70°C for 20 min, and centrifuged for 15 min on maximum speed. Supernatant was discarded and the pellet washed with 70% ethanol. Residual ethanol was removed by evaporation.

The samples were stored at 4°C and submitted to the department sequencing service. Data were analysed using Factura and Sequence Navigator Software.

Agarose gel electrophoresis

Agarose gels for electrophoretic analysis of DNA were prepared with TBE or TAE buffer. Agarose concentration varied from 0.3 to 1% according to the sizes of expected DNA fragments. Samples containing 1 x loading dye (6 x stock is 0.25% bromphenol blue, 0.25% xylene cyanol and 40% (w/v) sucrose in H_2O) were always loaded after immersion of the gels in the TBE or TAE buffer with 0.5 µg/ml of ethidium bromide. The gels were run at 50-70 mA at room temperature. These conditions usually led to separation of the fragments in less than an hour. For better separation, especially with larger fragments and higher agarose concentration, the gels were run 20-40 mA overnight. For better quality pictures gels were destained in distilled water for an hour. The DNA was visualised by using UV transilluminator.

Recovery of DNA from agarose gels

DNA was purified from agarose gel slices using a DNA Purification Kit II (Hybaid).

Ligation of DNA

DNA ligation was usually performed in a final volume of 10-20 µl. The reaction contained between 0.5-2 µg of total DNA with insert DNA in a 2 to 5-fold molar excess over the vector DNA, 1 x New England Biolabs ligation buffer and T4 DNA ligase from the same manufacturer. The reactions were incubated overnight at 16°C. 5-10 µl of the reaction mixture was directly used to transform competent cells of an appropriate strain of *E.coli*. When λ vectors were used the ligase was heat inactivated and the ligated DNA was packaged using λ packaging extracts.

Phage λ DNA packaging

A 50 µl aliquot of λ packaging extract (Promega) stored at -70°C was defrosted on ice. 15 µl of the extract was mixed with 10 µl of ligation reaction with inactivated ligase and containing at least 200 ng DNA. The mix was left at room temperature for

2h. 0.5 ml of phage buffer was added and appropriate dilutions were plated on *E.coli* ED8654. After overnight incubation the plaques were analysed.

Manipulation of proteins

Tris-glycine SDS polyacrylamide gel

Solutions:

- 4 x stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

15.25 g of Tris base was dissolved in 200 ml dH_2O, adjusted to pH 6.8 with concentrated HCl, made up to 250 ml, filtered and kept at 4°C.

- 4 x resolving gel buffer (1.5 M Tris-HCl, pH 8.8)

45.5 g of Tris base was dissolved in 200 ml dH_2O, adjusted to pH 8.8 with concentrated HCl, made up to 250 ml, filtered and kept at 4°C.

- 10% SDS (w/v)

20 g of SDS, made up to 200 ml, filtered and kept at room temperature.

- 10% ammonium persulphate (w/v) – freshly made

0.2g of ammonium persulphate was dissolved in 2-ml dH_2O

- running buffer

3 g of Tris base and 144 g of glycine were dissolved in 990 ml dH_2O. SDS was added to the final concentration 0.1% (10 ml of 10% stock solution)

- 2 x loading buffer

4 x stacking gel buffer (2.5 ml), 10% SDS (2.0 ml), glycerol (2.0 ml), dH_2O (2.5 ml), β-mercaptoethanol (1 ml), and bromphenol blue (some crystals to give blue colour).

E.coli proteins were routinely separated using SDS-polyacrilamide gel electrophoresis with a discontinuous buffer system (Laemmli, 1970). Usually a 7.5% resolving gel and 5% stacking gel were employed and a mini-gel apparatus (SE 250) manufactured by Hoeffer Scientific Instruments was used.

Staining of proteins with Coomassie Blue

Solutions:
- staining solution

    10% (v/v) acetic acid, 50% (v/v) methanol and 0.1% (w/v) Coomassie Brilliant Blue R250.

- destaining solution

    7% (v/v) acetic acid, 5% (v/v) methanol.

Gels were separated from glass plates, transferred to a plastic box with staining solution and incubated on a shaker for 30 min at room temperature. The staining solution was replaced by destaining solution and two pieces of polypropylene sponge were placed into the box to absorb Coomassie Blue. Gels were incubated on a shaker for 2-3 hours, until protein bands become clearly seen.

Preparation of *E.coli* whole cell lysates

Protein samples to be loaded onto an SDS PAGE gel were prepared from cultures grown to late logarithmic phase (O.D.$_{600}$ ~ 0.7-0.9) at 37°C with shaking. 1.5 ml of culture was centrifuged for 1 minute, the pellet was resuspended in 150 µl of distilled water and mixed with an equal volume of 2 x SDS load dye. The samples were boiled for 5 minutes and used immediately or frozen at -20°C to be used for western blotting in aliquot 20-30 µl. Samples require 3 minutes spin at 13 000g before use for removal of insoluble debris.

Protein samples from cell fractions.

Lysed spheroplasts were subjected to 15,000-rpm/1.5h centrifugation at 4°C to remove cell debris. The supernatant was centrifuged 60,000/1h/4°C to sediment the membrane fraction. Supernatant was completely removed and used as cytoplasmic fraction (CF). CF was mixed with equal volume of mixed with an equal volume of 2 x SDS dye, frozen at -20°C.

Membrane was washed in 50mM Tris-HCl pH8.0, resuspended 50mM Tris-HCl pH8.0, 1mM DTT, methylphenylflouoride, mixed with an equal volume of 2 x SDS load dye. Samples were stored -70°C.

Western blotting

After protein gel electrophoresis was completed apparatus was disassembled; one of the two glass plates removed and the gel was covered with PVDF membrane briefly wetted with methanol. No air bubbles should be left between the gel and the membrane. The proteins were transferred to the membrane by electrophoresis at 4°C for 1.5h at a constant voltage of 42V.

After electrophoresis membrane was used for protein detection using the chemoluminescence detection system (POD) of Rosche. Through all the procedure membrane was incubated on a shaker at room temperature. The membrane was blocked to prevent non-specific adsorption of the antibodies during immunodetection, in block solution (1:10) dilution of the stock in TBS buffer for 2h. The block solution was removed and replaced by solution with primary antibodies (block diluted in TBS 1:20 plus 1: 2500) for 2h.Then the membrane was washed 2 x 10 minutes in TBST (0.1% Tween in TBS) and 2 x 10 min in block solution. The secondary antibody solution was added (block diluted in TBS 1:20 plus 1:1000 anti-rabbit IgG peroxidase conjugate (Sigma)) for 1 hour and then the membrane was washed 4 x 15 minutes in TBST. After last wash the membrane was incubated with substrate solution for 1 min covered with Saranwrap film and exposed to X-ray film (Kodak) for 3-60 seconds.

Assay of β-galactosidase

The assays were performed following the procedure described by Miller (1972).

Measuring protein concentration

Protein concentrations were estimated by a modification of the Lowry method (Peterson, 1979).

Assay of NADH oxidase

The oxidation of DPNH (Sigma) was monitored (de Maagd and Lugtenberg, 1986) at 37°C in 100 μL volume of 50 mM Tris-HCl pH 7.5 supplemented with 0.12 mM DTT and 0.12 mM DPNH. The decrease in substrate absorbance at A_{340} was monitored over 5 minutes period using a Perkin Lambda 15 UV/VIS spectrophotometer. The rates were calculated from change in A_{340} with time using A_{340} of 1 mM DPNH = 6.22 nM. All assays were performed at least in two duplicates.

In vitro assembly of $R_2M_2S_1$ complex

The method of Dryden *et al.* (1997) was used. The assembly of $R_2M_2S_1$ complex from M_2S_1 complex and R subunit kindly provided by L.Cooper was carried out for 30 minutes on ice in 10 mM Tris HCl pH 7.5. The proteins were mixed in molar ratio 1:4 in nuclease buffer and then used directly for nuclease assays

Endonuclease assays

Endonuclease assays monitored the degradation of a covalently closed circular plasmid DNA (Vipond *et al.*, 1995). pBRsK1, a derivative of pBR322 with one EcoKI recognition site (Davies, 2000) .0 or .K was used as a substrate. Reactions were carried out at 37°C in Boehringer Manheim buffer A with 5 nM plasmid DNA, 100 μM SAM, 50 μg/ml BSA and varying concentrations of wild type or mutant $R_2M_2S_1$ complex. The enzyme was preincubated with SAM for 10 minutes and the reaction started by addition of ATP to a final concentration of 2 μM. Samples (10 μl) were removed at various times and mixed with 0.2 volumes stop solution (10%

Ficoll 400, 50mM EDTA, 50 mM Tris (pH8.0), 0.5% SDS, 0.1% Bromphenol Blue) to terminate the reaction. DNA fragments were separated by electrophoresis.

Chapter 3. Mutations that block methyltransferase activity but permit the binding of AdoMet lead to a modification-deficient, restriction-proficient (r^+m^-) *Eco*KI complex

3.1 Introduction

Bacterial cells in which *hsdR* is expressed from a multicopy plasmid die after the acquisition of the *hsdM* and *hsdS* genes, as the consequence of the formation of a functional type I restriction complex. This was shown to be the case when type IB *hsd* genes are transferred via either λ (Fuller-Pace *et al.*, 1985) or an F'plasmid (Suri and Bickle, 1985).A similar phenomenon was used in the analysis of the type IA restriction enzyme, *Eco*KI (Kelleher *et al.*, 1991).

The phenomenon of cell death resulting from the sudden formation of a functional type I enzyme in a cell that cannot modify its DNA or has not had the opportunity to modify its DNA was called "the kill-effect" because it was shown that the e.o.p of modified λ carrying *hsd* genes is reduced and it was interpreted as due to the degradation of chromosomal DNA.

Thus the kill-effect can be used to assay the restriction proficiency of restriction complexes that cannot be maintained *in vivo*, for example, a restriction-proficient, modification-deficient (r^+m^-) type I restriction enzyme. It has generally been anticipated that a mutation that confers an r^+m^- phenotype will be lethal.

Good candidates for the formation of r^+m^- restriction enzymes are mutations affecting motifs common to N6-adeninemethyltransferases. *Eco*KI mutants with single amino acid changes in either motif I or IV, motifs that are essential for catalysing the transfer of methyl groups from the cofactor AdoMet to the target adenine residues, were shown to form M_2S_1 complexes. The *hsdM*(F177G) mutant (motif I) was unable to bind the cofactor, whereas the mutants with substitutions in the NPPF motif (motif IV) were able to bind AdoMet but most of them failed to

modify DNA *in vivo* and were inactive in the methylase assay (Willcock *et al.*, 1994). However, the properties of these methylases as a part of the restriction complex have not been characterised.

3.2 Mutations in motif IV but not in motif I result in *EcoK*I that is restriction-proficient but modification-deficient *in vivo* and *in vitro*.

In vivo assays

One of the logical expectations concerning type I systems is that there is no possibility of isolating r^+m^- mutants or, at least, of maintaining such a mutant *in vivo*. Therefore we decided to use the kill-effect to check the ability of restriction complexes containing mutant HsdM subunits to restrict DNA.

Strain NM679, which lacks the three *hsd*-genes, was transformed by pACYC/*hsdR*$^+$. λ*hsdMS* phages that include the *hsdM* mutations mentioned above were assayed on NM679 and on its *hsdR*$^+$ derivatives transformed by pNK3 (Table 3.1).

Only mutants with single amino acid changes in motif IV have the kill-effect on the NM679 pNK3 strain (column 6), which supports the idea that restriction-proficient complexes are formed. Neither a mutant with a substitution in motif I (λ1330) and unable to bind AdoMet, nor a mutant with a substitution that did not affect the modification ability of the corresponding enzyme (λ1326) were restricted. However, none of the phages were restricted if *hsdR*$^+$ was expressed from the chromosome (column 7).

The drop in the efficiency of plating (e.o.p.) of a λ*hsdM*$^-$*S*$^+$ phage on the strain with *hsdR* on the plasmid could be explained by the restriction of phage DNA by *EcoK*I

or by the death of the cells as a result of chromosome cutting. Since experiments with these phages grown on m_k^+ strains showed no drop of e.o.p., EcoKI derived from the defective methylase still retains the restriction specificity of the original enzyme and cuts the phage DNA, and thus the chromosomal DNA may be unaffected.

The experiments shown in Table 3.1 are the summary of 3 independent assays that were done on the same strains. However, the attempts to reproduce this experiment on another isolate of NM679 were unsuccessful. No kill-effect was observed, although both isolates of NM679 behaved in the same way in all other tests, e.g. routine tests with phages and the deletion of *hsd* genes was confirmed by a PCR reaction. It is possible that the plasmid is better maintained in the isolate used in the experiment (Table 3.1), than in the initial NM679 strain, due to an additional mutation, but the difference that enables the detection of kill-effect in some NM679 isolates remains unclear.

In vitro assays

For further analysis, *hsdM*(F269G) and *hsdM*(G177D) were chosen as representatives of two classes of modification-deficient mutants: the first mutation causes the kill-effect *in vivo* and the second one does not.

The expectation that a r^+m^- restriction enzyme would kill the cells that produce it, because of restriction of the unprotected chromosomal DNA, led to the alternative approach of assembling EcoKI *in vitro*. The *in vitro* assembly from the corresponding mutant methyltransferases and HsdR subunits was done as described by (Dryden *et al.*, 1997) and the resulting complexes were used in standard endonuclease assays (Davies *et al.*, 1999a) (Fig. 3.1).

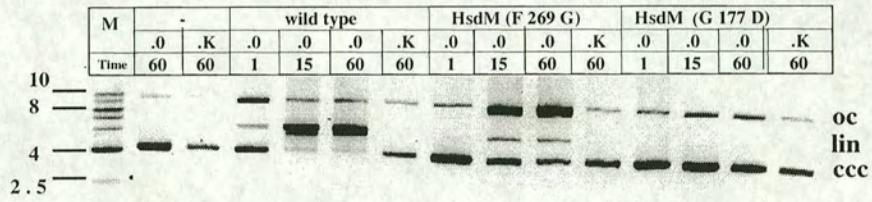**Table 3.1 The effect of mutations within *hsdM* on restriction and modification activities.**

λ*hsdMS*[+] phages	Substitution specified by *hsdM*	Motif	AdoMet binding	The efficiency of plating of the λ*hsdM S*[+] phage[a]		
				C600	NM679* pNK3[b]	NM522 *hsdR*[+]*M*[-]*S*[-]
λ1332.0	D177G	I	-	4×10^{-4} (1)[c]	0.8 (1)	0.8 (1)
λ1326.0	F269G	IV	+	3×10^{-4} (1)	4×10^{-4} (1)	0.8 (1)
λ1327.0	F269C	IV	+	2×10^{-4} (1)	9×10^{-5} (1)	0.9 (1)
λ1330.0	N266D	IV	+	4×10^{-4}(1)	5×10^{-4} (1)	0.8 (1)
λ1331.0	N266D F269G	IV	+	4×10^{-4}(1)	4×10^{-4} (1)	0.7 (1)
λ1329.0[d]	F269Y	IV	+	0.8 (1)	0.6 (1)	1 (1)

[a] – Defined relative to the (*hsdRMS*)∇ strain NM679

[b] – pNK3, a derivative of pACYC184

[c] – Figures in parentheses are for .K phages

[d] – The substitution F269Y results in a wild-type phenotype and serves as a positive control

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M	-		wild type				HsdM (F 269 G)				HsdM (G 177 D)			
	.0	.K	.0	.0	.0	.K	.0	.0	.0	.K	.0	.0	.0	.K
Time	60	60	1	15	60	60	1	15	60	60	1	15	60	60

Figure 3.1 Endonuclease activity of modification-deficient enzymes.

The modification enzyme used in the *in vitro* assembly of *Eco*KI is
identified at the head of the figure. The substrate DNA was plasmid
pBRsKI, a plasmid with one unmodified (.0) or modified target (.K).
The incubation times are indicated in minutes. M identifies marker
DNAs from 10 to 2.5 kb in length, **oc** = open circular, **lin** = linear , **ccc**
= covalently closed circular plasmid DNA.

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*Eco*KI specifically cleaves DNA in the presence of AdoMet, ATP, and Mg^{2+}, where AdoMet acts as an allosteric effector (Hadi *et al.*, 1975). Unmodified pBR322 with one *Eco*KI site was used as a substrate. Fig. 3.2 shows the conversion of covalently closed circular DNA via open-circles to linear molecules by both the wild-type *Eco*KI and the F269G mutant complexes, but not by the G177D mutant complex. This is in agreement with the results obtained *in vivo*.

The M_2S_1 complexes used in the endonuclease assay were shown to be unable to methylate the target DNA (Willcock *et al.*, 1994). These data were confirmed recently (O'Neill M., pers.com) using a more sensitive assay (Roth and Jeltsch, 2000): the M_2S_1 complexes containing either HsdM(G177D) or HsdM(F269G) have shown no methyltransferase activity.

In 4 out of 5 experiments that were carried out using the same sample of purified methylase and freshly assembled complex, the F269G mutant enzyme cut the DNA more slowly than the wild-type enzyme. For example, in the experiment shown in Fig 3.2 after 15 minutes of incubation of the restriction complexes with unmodified DNA, 80% of the plasmid DNA was linearised in the case of incubation with wild-type enzyme but only 10% in the case of the HsdM (F269G) mutant. This fact can be explained by a change in the local structure of the mutant enzyme that might have led the enzyme to have a higher binding constant for AdoMet.

A similar mutant in motif IV of the *Bcg*I system (see section 1.1) blocked methyltransferase activity but the mutant enzyme was still able to cleave DNA *in vitro,* showing approximately 40% of restriction activity of the wild-type enzyme. The expression of this mutant protein from a multicopy plasmid was lethal to the host (Kong, 1998).

A similar change (Y269G) in the motif IV of *Eco*R124I (IC family) has recently been shown to result in a restriction-proficient, modification-deficient phenotype (L. Powell and N. Murray, pers.com).

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Therefore, the finding that substitutions in motif IV of the methyltransferase block methylation but not the restriction reaction can be extended from type IA restriction enzymes to other groups of restriction enzymes where AdoMet is required for endonuclease activity.

Chapter 4 HsdR subunits of *Eco*KI complexes are degraded if the complexes are able to translocate DNA.

4.1 Conjugation experiments show that survival of $hsdR^+hsdM269(F269G)hsdS^+$ cells depends on ClpXP protease, but does not depend on RecA for the repair of double-strand breaks.

It is expected that bacterial cells, which have the *hsdM*(F269G) mutation (abbreviated to *hsdM269* in further sections) in the presence of $hsdS^+$, should die if a functional *hsdR* gene is present, because the *Eco*KI complex formed will fragment the unmodified bacterial chromosome. For example, if there is a resident methylase able to interact with HsdR to form a restriction complex but unable to methylate the targets present in the chromosome, recipient cells should die if they acquire *hsdR* on a plasmid. However, for the correct interpretation of the results of such an experiment it is important to maintain the ratio of Hsd subunits and their concentration in the cell to that determined by one copy of the *hsd* genes per bacterial chromosome. An F'plasmid was chosen because it is a low copy number plasmid – 1-2 copy per chromosome. The F'101-102 $hsd_KR^-M^+S^+$ (Prakash-Cheng and Ryu, 1993) was available and used for the construction of an F'$hsdR^+$ necessary for the designed conjugation experiments.

F'101-102 is a derivative of F'101 (Makovets *et al.*, 1998) with Tn*10* inserted into the *zjj* gene. *zjj::*Tn*10* is closely linked to the *hsd* region and this allows the use of an F' *hsd zjj:*Tn*10* for the construction of F'derivatives with different *hsd* genes via chromosome-plasmid allele exchange (Figure 4.1):

1. A strain with a functional *hsdR* gene on the bacterial chromosome, but in which a deletion removed *hsdM* and *hsdS* (NK224) was chosen. A *zjj202::* Tn*10* insertion was transferred to this strain by P1 transduction to provide a marker closely linked to the $hsdR^+$ gene.

2. An F' $hsdM^+S^+$ carrying the hsd region but deleted for $hsdR^+$ was transferred to the $hsdR^+$ $zjj202::$ Tn10 recipient and clones were checked for Nal^r Tet^r and the r^+m^+ phenotype anticipated for a strain with all of the three hsd genes.

3. Derivatives of the partial diploid were sought in which exchange of markers between the chromosome and F' generated an F' in which $hsdM^+hsdS^+$ were replaced by $hsdR^+\Delta hsdMS$ $zjj202::$ Tn10. To recognise recombinant F' factors, selection was made for the transfer of Tn10 to a $recA::cat\Delta hsdRM$ recipient and transconjugants were checked for the predicted r^+m^+ phenotype.
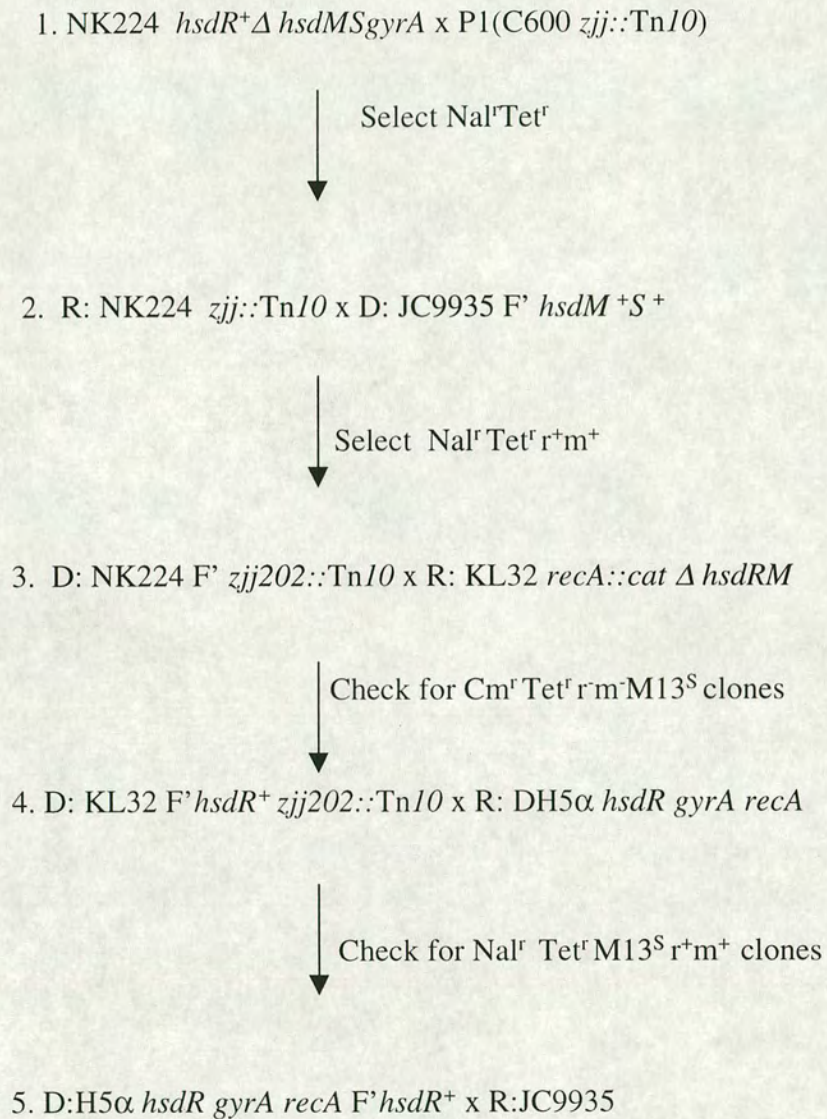
4. The presence of a functional $hsdR$ gene in the F' was confirmed by transferring the F' to an $hsdM^+S^+$ recipient lacking $hsdR$. The Nal^r Tet^r derivative was checked for the predicted r^+m^+ phenotype.

5. The new F' designated 101-103 $hsdR^+$ was transferred to JC9935.

The resultant JC9935 F'101-103 hsd_KR^+ was used in the conjugation experiments where strains $\Delta hsdR$ $hsdM^+S^+$, $\Delta hsdR$ $hsdM269S^+$ and $\Delta hsdR$ $hsdM177S^+$ were used as recipients and JC9935 F'101-102 or JC9935 F'101-103 as donor strains (Table 4.1).

Unexpectedly, the transfer of F'$hsdM^+S^+$ and F'$hsdR^+$ was equally efficient to both $hsdM269$ and $hsdM177$ recipients (Table 4.1). Samples of transconjugants that acquired resistance to tetracycline, presumably because of transfer of the F', were purified and tested for restriction. All of them were sensitive to the male-specific phage M13 which confirms F' transfer from donor to the recipient cells.

Although cells of NM802 with F' $hsdR^+$ have a r^+ phenotype (λ v.0 has an e.o.p. of 10^{-4}), cells of VC8026 $hsdM269$ F' $hsdR^+$ restrict λ v.0 poorly (e.o.p. 10^{-1}) while λ v.K has an e.o.p. close to 1. The e.o.p. of λ v.0 and λ v.K was close to 1 when assayed on $hsdM$(G177D) F' $hsdR^+$ transconjugant clones, e.g. no restriction was detected in these transconjugants.

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1. NK224 *hsdR+Δ hsdMSgyrA* x P1(C600 *zjj::*Tn*10*)

Select Nal^{r}Tet^r

2. R: NK224 *zjj::*Tn*10* x D: JC9935 F' *hsdM $^+$S $^+$*

Select Nal^r Tet^r r$^+$m$^+$

3. D: NK224 F' *zjj202::*Tn*10* x R: KL32 *recA::cat Δ hsdRM*

Check for Cm^r Tet^r r$^-$m$^-$M13^S clones

4. D: KL32 F'*hsdR+ zjj202::*Tn*10* x R: DH5α *hsdR gyrA recA*

Check for Nal^r Tet^{r}M13^S r$^+$m$^+$ clones

5. D:H5α *hsdR gyrA recA* F'*hsdR+* x R:JC9935

Figure 4.1 The construction of F'*hsdR+*

See comments in the text

Table 4.1 The effect of *clpX*, *clpXP* and *recA* mutations on the acquisition of F'plasmids.

Recipient strains (all Δ*hsdR*)	JC9935 F' *hsdM⁺S⁺*			JC9935 F' *hsdR⁺*		
	Titre of recipients after conjugation	Titre of Tc^r clones	Relative frequency of survival of recipients	Titre of recipients after conjugation	Titre of Tc^r clones	Relative frequency of survival of recipients
VC802	5.0×10^7	6.9×10^7	0.72	3.1×10^7	3.8×10^7	0.62
VC914 *hsdM177*	1.6×10^7	1.5×10^7	0.93	2.0×10^7	1.8×10^7	1.13
VC914 *clpX::kan*	4.1×10^7	3.5×10^7	0.85	3.5×10^7	3.7×10^7	0.85
VC8026 *hsdM269*	1.0×10^8	1.2×10^7	1.2	7.5×10^7	6.8×10^7	0.75
VC802 *clpX::kan*	3.2×10^7	2.7×10^7	0.84	5.0×10^7	4.4×10^7	1.26
VC8026 *clpX::kan*	2.8×10^7	1.9×10^7	0.69	4.5×10^3	2.1×10^{3}[a]	1.6×10^{-4}
VC3003 *clpP::cat*	3.3×10^7	3.9×10^7	0.85	9.0×10^3	1.3×10^{3}[a]	2.7×10^{-4}
VC803 *recA::cat*	4.8×10^6	2.5×10^6	0.52	5.4×10^6	3.9×10^6	0.81
NK8026 *recA::cat*	9.3×10^6	4.9×10^6	0.53	3.7×10^6	2.1×10^6	0.40

a – all conjugants tested were restriction deficient

Why should restriction be virtually absent in *hsdM269hsdS*$^+$ F' *hsdR*$^+$ transconjugants while the experiment in section 3.1 supported the hypothesis that this mutation in *hsdM* leads to an r$^+$m$^-$ complex? One of the possible explanations is that mutations in F' *hsdR*$^+$, which allow the survival of *hsdM269 hsdR*$^+$ cells, were selected during the experiment.

Twelve *hsdM269* F' *hsdR*$^+$ transconjugant clones were used as donors and the *hsdR* gene was functional in all cases when transferred to a restriction-deficient, modification-proficient strain. The e.o.p. of λ v.0 on these transconjugants was close to 10^{-4}, e.g. restriction activity was restored in these cells. Therefore there was no evidence for mutations in the F'.

A second possible explanation of the phenotype of the *hsdM269hsdR*$^+$ cells is that restriction has been alleviated. In the experiments that were done in our laboratory by S.Makovets the wild-type cells treated with 2AP showed some similarity in their r$^{+/-}$ phenotype to the low level of restriction shown by the *hsdM269hsdR*$^+$ strain (r$^{+/-}$m$^-$ phenotype). Survival of the cells treated with 2AP is dependent on ClpXP protease so it was logical to check whether survival of *hsdM269* F' *hsdR*$^+$ transconjugants is dependent on the *clpXP* genes .

However, if the cell death expected after the formation of restriction-proficient, modification-deficient *Eco*KI is prevented by the recombinational repair of bacterial chromosomes, residual restriction activity would be lethal in a *recA* strain in which repair of DSBs is impaired.

Mutations in *clpX, clpP* and *recA* were transferred to the recipient strains by P1-transduction. Each mutation was tagged by a transposon and could be selected by the antibiotic resistance marker. The strains obtained were used as recipients in conjugation experiments (Table 4.1).

The frequency of transfer of the control F'*hsdM*$^+$*S*$^+$ was independent of the genotype of the recipient cells. The frequency of acquisition of the selective marker of the F'

was as low as 10^{-4} if an *hsdM269clpX::kan* or *hsdM269 clpP::cat* cells were used as recipient cells. The frequency was close to one in the case of *hsdM269recA::cat* recipient. These results imply that ClpX and ClpP but not RecA are critical for the survival of the clones that encode a r^+m^- enzyme and have $r^{+/-}m^-$ phenotype.

All F'plasmids that were acquired by *clpX::kan* recipient cells (VC8026) were shown to be deficient in restriction activity after their transfer to an r^-m^+ strain, and they may represent deletion variants of the plasmid which were selected by the survival of the cells that received them.

The mutation in the *clpP* gene, which inactivated the proteolytic component of the ClpXP protease, also reduced the survival rate of recipient cells of VC3003 slightly less prominently than the *clpX* mutant, probably because disassembling of restriction complexes by ClpX chaperone might reduce the number of DSBs on the chromosome.

The dynamics of killing of *hsdM* (F269G) transconjugant cells during conjugation was also monitored, but in a different background – in a C600 Rac $^-$ strain to check that the results obtained are not an artefact of the strains used and because the recent work on restriction alleviation was done in a C600 Rac $^-$ background (Makovets *et al.*, 1999) (Fig 4.2).

The graphs shown in Figure 4.2 indicate the death of the recipient cells in the absence of either ClpX or ClpP but not in the absence of RecA. Control experiments with F'101-102 revealed no drop in titre of any of the recipient bacteria tested.

Clearly, both *clp^-* genes show a similar effect on the survival of the *ΔhsdR hsdM(F269G)* recipient strain, which confirms that a functional ClpXP protease, rather than the ClpX chaperone alone is required for the survival of the recipient cells.
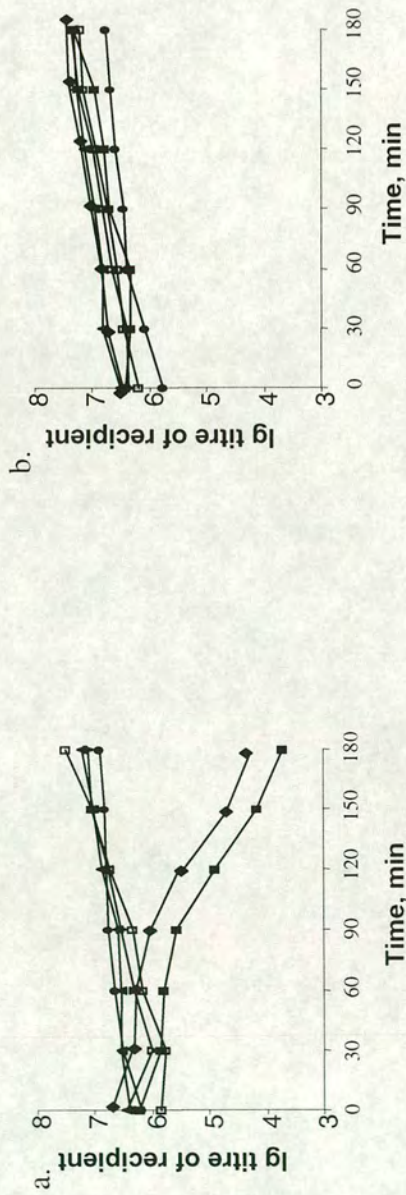
Figure 4.2   The survival of hsdR+hsdM(F269G) hsdS+ cells was assessed after the conjugative transfer of hsdR+ to ΔhsdRhsdS+ recipients.
(a) The experiment using F'101-103 (hsdR+). (b) The control experiment with F'101-102 (hsdM+S+).

Data are plotted for the following recipients: NK379, hsdR⁻ (▲); NK380, hsdR⁻ clpX (□): NK382,

hsdR⁻M269 (▲); NK384, hsdR⁻ hsdM269 clpX (■); VC3003, hsdR⁻ hsdM269 clpP (◆); and NK383,

hsdR⁻ hsdM269 recA (●). The data show that hsdR+M(F269G)S+ cells require ClpXP, but not RecA

to survive.

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4.2 Survival of r⁺m⁻ cells depends on the copy number of the *hsdR* gene – a high copy number of *hsdR* is not tolerated even in Clp⁺ cells.

The establishment of type I systems is dependent on *clpXP* when *hsd* genes enter the bacterial cell by conjugation, transduction or transformation (Makovets *et al.*, 1998). Thus transformation provides the possibility to assess the effect of the dosage of *hsdR⁺* on the cells that are able to make a r⁻m⁺ restriction complex.

The *hsdR⁻M269S⁺* strain was transformed with a mixture of two plasmids, an *hsdR⁺* test plasmid and a control plasmid tagged with a different antibiotic resistance. The ratio of the clones transformed by a test plasmid to those transformed by a control plasmid was compared (Table 4.2). Two *hsdR⁺* plasmids were tested. These were pNK3, a derivative of the low copy number plasmid pACYC184 which has 5-10 copies per bacterial chromosome (Chang and Cohen, 1978), and pJK2, a derivative of pBR322 (20-25 copies, (Sutcliffe, 1978).

The ratio of clones transformed by the test plasmid to those transformed by the control plasmid depended only on the ratio of plasmid DNA in the *ΔhsdR* strain (NM802) (Table 4.2). In a *clp⁺* strain it was possible to maintain pNK3 in the cells in the presence of the *hsdM269* mutation (NK384); the ratio of transformants by pNK3 and pACYC184 is close to that obtained for NM802. However, the transformants containing pNK3 grew slowly on the solid medium containing chloramphenicol and it was not possible to grow these transformants in liquid broth.

No transformants were observed if a mutation in *recA* was introduced into the *hsdM269* strain (VC36), which is in contrast to the result of the experiment with F'transfer by conjugation (see Fig 4.2).

The presence of the *clpX* mutation in the *hsdM269* mutant prevented transformation by any *hsdR⁺* plasmid. In the case of the higher copy number plasmid, even Clp⁺ cells failed to give *hsdR⁺* transformants (Table 4.2).

A possible explanation of these findings is that the restriction-proficient, modification-deficient enzyme obtained as a result of the interaction HsdR with the defective methylase leads to a situation in which the enzyme binds to the unmodified target sequences in the chromosomal DNA and initiates the restriction pathway. The action of the basal level of ClpXP is enough to degrade the HsdR subunits produced from one copy of *hsdR*, hence the survival of Clp$^+$ cells in case of the transfer of the F'plasmid by conjugation, but the level of ClpXP in the cell is insufficient to prevent *Eco*KI from cutting the chromosome if the level of HsdR is increased. *rec*$^+$ cells are able to grow slowly in the presence of a low copy number plasmid, pNK3, but repair mechanisms cannot prevent cell death if the number of functional *Eco*KI complexes is increased, as shown by the presence of the pBR322/*hsdR*$^+$ plasmid (pJK2).

When an *hsdR*$^+$ *hsdM269hsdS*$^+$ strain was lysogenized by λ *att*$^+$ *hsdM*$^+$*S*$^+$ the resulting strain was able to modify phage DNA in a routine test of modification activity but restriction was still 10^{-1}, which suggests that the *hsdM269* mutation is dominant over the wild-type with respect to the induction of RA. If there is no preference in assembly between wild-type and mutant HsdM there should be 25% of wild-type $R_2M_2S_1$ complexes, 50% complexes which have one wild-type and one defect M-subunit and 25% of mutant restriction enzymes. The chromosome will still be undermethylated and HsdR subunits of *Eco*KI are probably degraded.

Table 4.2 The effect of *hsdM* (F269G) mutation on acquisition of *hsdR⁺* by transformation

Recipient strains	The ratio and the phenotype of transformants with *hsdR* plasmid to a control plasmid	
	pACYC184/*hsdR⁺*	pBR322/*hsdR⁺*
NM802 (Δ*hsdR*)	4.2 ($r^+ m^+$)	3.4 ($r^+ m^+$)
NK384(Δ*hsdR* *hsdM269*)	4.1 *	$< 5 \times 10^{-3}$ ᵃ
VC36((Δ*hsdR* *hsdM269recA*)	$< 10^{-3}$ ᵃ	$< 10^{-3}$ ᵃ
NM802 *clpX::kan*	3.1 ($r^+ m^+$)	2.1 ($r^+ m^+$)
NK384 *clpX::kan*	$< 2 \times 10^{-3}$ ᵃ	$< 8 \times 10^{-2}$ ᵃ

a)   approximately 1000 transformants were isolated after transformation by a control plasmid, but none was observed after transformation by a plasmid with *hsdR⁺* .

* - cells were sick.

The restriction phenotype of the transformants is shown in brackets.

4.3 HsdR is degraded in *hsdM*(F269G) mutant

The survival of transconjugant cells after the acquisition of F'*hsdR⁺* suggested that it might be possible to maintain *hsdM269* in the presence of a functional *hsdR* allele on the chromosome. The *hsdM*(F269G) mutation was transferred to the chromosome of NK301, an *hsdR⁺M⁺S⁺* strain, by phage-chromosome allele exchange using λ*hsdM269hsdS⁺*. The *hsdM269* recombinants were recognised by their modification-deficient phenotype.

Sections 4.1 and 4.2 have shown that the survival of *hsdM269S*$^+$ cells is ClpXP-dependent in those cases where either conjugation or transformation provided *hsdR*$^+$ *in trans* on a plasmid. If the survival of *hsdR*$^+$ *M269S*$^+$ cells is still Clp-dependent when all *hsd* genes are present *in cis* on the chromosome, it should be impossible to transfer a *clp*$^-$ allele to *hsdR*$^+$*M269S*$^+$ cells, for example, by P1 transduction.

To check the possibility of constructing an *hsdR*$^+$*M269 clp*$^-$ strain, a P1 lysate was prepared on a *clpX::kan tsx::*Tn*10* strain (VC48) where Tn10 has 50% linkage with the *clpX::kan* allele. Transductants of a wild-type strain, the *hsdR*$^+$*hsdM269* strain, and the *hsdR*(A619V)*hsdM269S*$^+$ strain, in which the restriction activity of the *Eco*KI complex is inactivated as the result of a single amino acid change in one of the DEAD-box motifs, were selected by their resistance to tetracycline and screened for the transfer of the mutation in *clpX* (Table 4.3). The linkage between *tsx::*Tn*10* and the *clpX::kan* allele is close to 50% for the wild-type strain and for the *hsdR*(A619V)*hsdM269* strain. No *clpX::kan* derivative of *hsdM(F269G)* was isolated, which strongly supports the hypothesis that ClpXP is essential to maintain the viability of cells that contain the r$^+$m$^-$ enzyme.

Table 4.3 The effect of *hsdM*(F269G) on the acquisition of the *clpX* gene by P1 transduction.

Recipient strains	CAG12148 *tsx::*Tn*10 clpX::kan*	
	Sample size	*tsx::*Tn*10 clpX::kan* linkage
NK301 w.t.	100	0.51
NK382 *hsdM*(F269G)	100	0.00
VC3017-1 *hsdM*(F269G) *hsdR*(A619R)	100	0.47

A possible mechanism of control of *Eco*KI by ClpXP protease is proteolysis of the HsdR subunit of the restriction complex. The proteolysis would prevent the restriction reaction and therefore prevent cell death. To check this hypothesis a Western-blot analysis was done on the cell extracts of the strains used in the previous experiment (Fig 4.3A) . The antibodies identify HsdR and HsdM in the wild-type strain (lane 1 of Fig 4.3). Lane 2 shows that HsdR was virtually absent in cells with the *hsdM*(F269G) mutation. However, HsdR could be seen on a film after a long exposure(Fig. 4.3B), which can explain the residual restriction activity of *hsdM*(F269G) strain. Substitution of *hsdM*$^+$ for the *hsdM269* using a $\lambda\Delta hsdRhsdM^+S^+$ donor prevented the degradation of HsdR, which allows us to attribute the cause of degradation of HsdR in *hsdR*$^+$*hsdM269* strain to the mutation in *hsdM* (Fig 4.3A, lane 2). The presence of the *hsdR* (A619V) mutation transferred from a $\lambda hsdR$(A619V) (Fig. 4.3A, lane 4) prevents the degradation of HsdR in *hsdM269* mutant. The mutation in one of the DEAD-box motifs results in an enzyme that is incapable of restriction (Davies et al.,1999) and therefore is not lethal in the presence of the *hsdM269* mutation (Table 4.1), despite the retention of the restriction subunit.

The analysis of cell extracts shown in Fig 4.3 indicates that *hsdM*(F269G) is a mutation that leads to the constitutive degradation of the HsdR subunit and an extremely low level of restriction, despite the restriction-proficient genotype. This is an example of a mutation that leads to constitutive restriction alleviation. Constitutive restriction alleviation in *hsdM*(F269G) mutants supports the hypothesis that unmodified targets on the chromosome are the primary condition for the degradation of HsdR.

The proteolytic control is very effective because it even prevents cell death in *recA* strains, where a single DSB should be lethal (Murialdo, 1988) and there are some 600 *Eco*KI targets on the chromosome of *E.coli* K-12 (Winter M., unpublished observations).
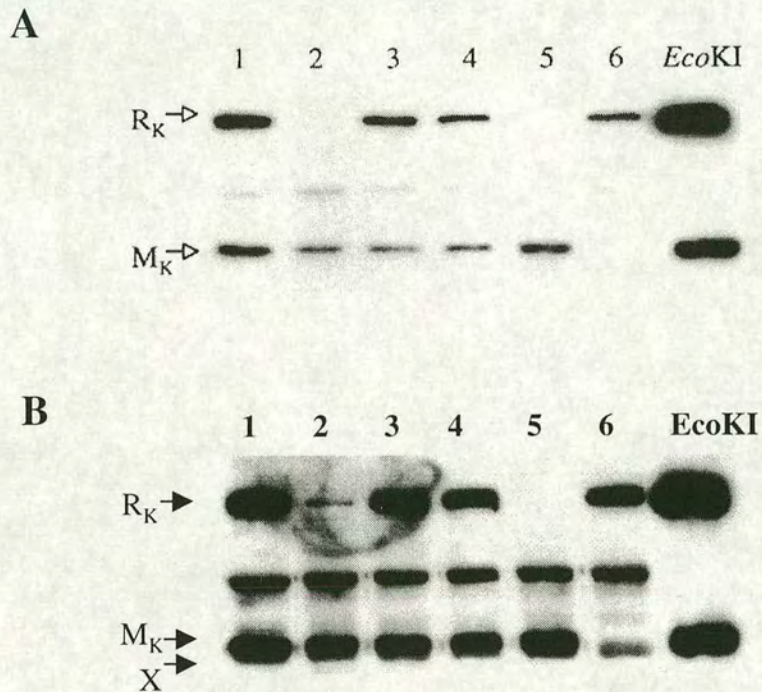
Figure 4.3 The level of HsdR was monitored by Western blots using antibody against *Eco*KI.
Lanes 1-6 include extracts of strains: 1, NK301 (*hsd+*); 2, NK386 [an *hsdM*(F269G) derivative of NK301]; 3, an *hsd+* derivative of NK386; 4, an *hsdR*(A619V) derivative of NK386 (NK388) in which alleles of *hsd* genes was replaced by using *hsd* phages that included only *hsdM+S+* or *hsdR*(A619V), respectively, 5, NM802 (an Δ *hsdR* strain); and 6, NK352 (an Δ *hsdMS* strain).
A. Short exposure
B. Long exposure. Some HsdR subunit can be seen in NK386 (lane 2), but not in the strain with deletion *hsdR* (lane 5). An unknown protein X with a mobility close to the mobility of HsdM is detected in the strain deleted for *hsdM* (lane 6).

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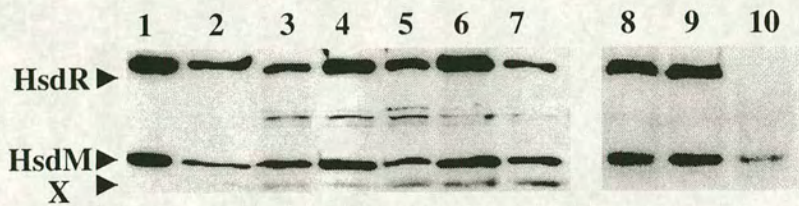
Figure 4.6 The effect of changes in DEAD-box motifs on the degradation of HsdR in *hsdM269* mutant.

The positions of amino acid changes are given in brackets followed by the motif. An unidentified protein (X in the figure) is detected by our polyclonal antibodies and it is present in strains deleted for *hsdM* (Fig 4.5B).

Lane 1 - EcoKI, Lanes 2-8, extracts of *hsdM*(F269G) strains with mutations in *hsdR* that affect DEAD box motifs:2 - NM797, *hsdR*(H577D), motif II; 3 - NM799, *hsdR*(A619V), III ; 4 - NM801, *hsdR* (K477R), I ; 5 - NM892 *hsdR*(G799C), V; 6 - NM893, *hsdR*(D502Y), Ia ; 7 - NM898, *hsdR*(R826H), VI; 8 - NM890, *hsdR*(F629Y), IV; lane 9 - wild type (AB1157), and lane 10 - VC1, *hsdM*(F269G).

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In addition to the DEAD-box motifs, HsdR subunits of type I restriction enzymes were reported to contain another conserved motif localised in the N-terminal part of the polypeptide (Titheradge *et al.*, 1996). This motif formerly known as "region X" is reminiscent of the AD (E/D) X_n (E/D)ZK sequence of type II restriction enzymes which is responsible for the endonuclease activity of the enzyme (Aggarwal, 1995; Selent *et al.*, 1992). Type I R-M enzymes with substitutions in this motif retain ATPase activity *in vitro* and translocation activity *in vivo* (Davies *et al.*, 1999b; Janscak *et al.*, 1999) but lack the DNA nicking activity characteristic of DEAD-box mutants (Davies *et al.*, 1998). Double mutants with changes in the endonuclease motif of HsdR and in addition to *hsdM269*(F269G) substitution were constructed using λ*hsdM269S*⁺ as a donor of the *hsdM* mutation. The Hsd subunits were assayed by Western-blot analysis (Figure 4.7). The HsdR subunits of *Eco*KI with conserved changes in the endonuclease motif (either D298E or E312D) in the presence of *hsdM269* were degraded (lanes 3 and 12). The HsdR of the mutant (E312H) that retains only 10% of the wild-type ATPase activity was partially degraded (lane 5).

Mutants in the endonuclease motif do not introduce DSBs into DNA so their degradation by ClpXP protease supports the hypothesis that DSBs are not the signal that triggers degradation of HsdR by the ClpXP protease.

The experiments presented above suggest that the signal for restriction alleviation depends upon the translocation of unmodified DNA by the type I restriction enzyme. But while translocation of chromosomal DNA leads to RA (Makovets *et al.*, 1999). the phenomenon of restriction of unmodified phage or plasmid DNA by *E.coli* K12 raises the question of the difference between unmodified DNA that causes RA and unmodified DNA that does not.
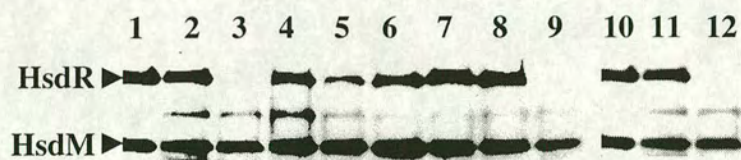
Figure 4.7 The effect of changes in the nuclease motif on the degradation of HsdR in *hsdM269* mutant.

Lane 1 - *Eco*KI, 2 - NM904, *hsdR*(D298E); 3 - VC904, *hsdR*(D298E)*hsdM*(F269G); 4 - NM908, *hsdR*(E312H), 5 - VC908,*hsdR*(E312H)*hsdM(F269G)*; 6 - NM890, *hsdR*(F629Y) - a mutation in DEAD-box motif IV ; 7-VC890, *hsdR* (F629Y) *hsdM(F269G)*; lane 9 -VC1, *hsdM*(F269G), lane 10 - *Eco*KI, lane 11 - NM907, *hsdR*(E312D), lane 12 - VC907 *hsdR*(D298E)*hsdM*(F269G). The substitution E312H (lane 5) in contrast to D298E (lane 3) and E312D (lane 12) greatly impairs ATPase activity

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Chapter 5 HsdR is depleted from the cytoplasm during restriction alleviation.

5.1 Introduction

The Western blot analysis of cell extracts has shown that the amount of HsdR is significantly depleted in the *hsdM269* mutant (Fig 4.3). Nevertheless, these cells retain the ability to restrict up to 90% of incoming unmodified lambda phage. If *Eco*KI had been acting with the same efficiency on unmodified chromosomal targets, cells should die as a result of the chromosome breakage. Their survival raises the question about the differences between DNA substrates and the recognition of "self", rather than "non-self" DNA: how does a cell distinguish between unmodified targets on chromosomal DNA and those on foreign DNA.

First, these two substrates differ in their localisation. DNA that enters the cell – phage or plasmid DNA - has to cross an outer membrane of a bacterial cell, its periplasmic space, and the inner membrane in order to be replicated within a bacterial cell. Early work by Schell and Glover (Schell and Glover, 1966a; Schell and Glover, 1966b) suggested that phage lambda DNA that crossed the membranes of *E.coli* K-12, is not degraded but becomes modified. In recent work by (Holubova *et al.*, 2000) *Eco*KI was found in the inner membrane fraction of bacterial cells after overproduction of the restriction enzyme. On the other hand, there are some reports that type II restriction enzymes are cytoplasmic (Lacks and Neuberger, 1975). But the definitive *Eco*KI evidence for the localisation of *Eco*KI in wild-type cells is still lacking.

5.2 Phages that contain double-stranded but not those that contain single-stranded DNA are subject to residual restriction activity during RA.

DNA of lambda phage and plasmid DNA during transformation are substrates for residual restriction activity under conditions of restriction alleviation, although the level of RA is much lower in the case of transformation (Hiom *et al.,* 1991). These substrates for restriction by *Eco*KI are double-stranded while crossing the membranes of the bacterial cell.

The analysis of residual restriction activity during restriction alleviation was extended to include T7 – a double-stranded DNA phage with a different mode of entry from λ) (Garcia and Molineux ,1999) and   a derivative of M13 – a single-stranded DNA phage, which has to be replicated in the cytoplasm of a bacterial cell in order to become a substrate for restriction enzymes.

The residual restriction activity remaining when RA is induced by treatment with 2AP is more noticeable than that in the *hsdM269* mutant (Table 5.1). The residual restriction activity (RRA) was observed for T7 *ocr⁻* phage and for derivatives of lambda with different numbers of targets. However, RRA was not detected using a derivative of M13 with 2 targets for *Eco*KI. This might be explained if the residual *Eco*KI is associated with the membrane. If DNA enters the bacterial cell as an unmodified double-stranded molecule, the restriction enzyme recognises and cuts it. However, single-stranded DNA becomes double-stranded only after the synthesis of the complementary strand; this will occur in the cytoplasm where, under conditions of restriction alleviation, HsdR is depleted.

TABLE 5.1 Residual restriction under conditions of RA is detected for T7 and λ but not M13.

STRAIN	PHAGE							
	λ with 4 targets		T7 0.3 (4 targets)		M13 with 2 targets		λ with 2 targets[a,b]	
	.0	.K	.0	.K	.0	.K	.0	.K
$hsdR^-M^+S^+$ NK379	$1.8 \pm 0.1 \times 10^9$	$7.0 \pm 0.1 \times 10^9$	$3.5 \pm 0.1 \times 10^9$	$2.0 \pm 0.1 \times 10^9$	$1.6 \pm 0.4 \times 10^{10}$	$3.0 \pm 0.4 \times 10^9$	$2.2 \pm 0.1 \times 10^{10}$	$1.8 \pm 0.5 \times 10^{10}$
$hsdR^+M^+S^+$ NK301	$1.4 \pm 2 \times 10^5$	$7.5 \pm 0.3 \times 10^9$	$1.5 \pm 0.2 \times 10^6$	$5.0 \pm 0.1 \times 10^9$	$2.5 \pm 0.2 \times 10^6$	$2.2 \pm 0.5 \times 10^9$	$5.5 \pm 0.4 \times 10^6$	$1.9 \pm 0.5 \times 10^{10}$
$hsdR^+M^+S^+$ NK301 + 2-AP	$1.3 \pm 0.1 \times 10^8$	$7.5 \pm 0.3 \times 10^9$	$1.5 \pm 0.1 \times 10^8$	$3.9 \pm 0.1 \times 10^9$	$1.6 \pm 0.3 \times 10^{10}$	$2.1 \pm 0.1 \times 10^9$	$1.8 \pm 0.2 \times 10^9$	$1.9 \pm 0.7 \times 10^{10}$
$hsdR^+M$ F269G)S^+ NK380	$1.2 \pm 0.1 \times 10^8$	$7.4 \pm 0.3 \times 10^9$	$1.1 \pm 0.5 \times 10^8$	$3.5 \pm 1 \times 10^9$	$1.5 \pm 0.3 \times 10^{10}$	$3.0 \pm 0.3 \times 10^9$	$3.4 \pm 2.0 \times 10^9$	$2.1 \pm 0.7 \times 10^{10}$

[a] The data are for phage 82, a lambdoid phage with two targets for *EcoKI*.

[b] Similar data were obtained for a hybrid phage (h^{80} $imm\lambda$) with two targets, but these tests require different hosts since NK301 and its derivatives are resistant to *phi*80.

5.3 HsdR is depleted from the cytoplasmic fraction under conditions of RA.

The hypothesis that the localisation of *Eco*KI influences the residual restriction activity under conditions of RA was checked after bacterial spheroplasts had been separated into cytoplasmic and membrane fractions. The purity of the fractions was controlled measuring the activity of β-galactosidase and NADH oxidase as a marker of cytoplasmic and membrane fractions, correspondingly (Table 5.2).

**Table 5.2 Activity of marker enzymes in different cellular fractions.**

Fraction		β-galactosidase, Miller units	NADH oxidase, u/mg protein	Protein concentration, mg/ml
Cytoplasmic fraction	NK301	645	2.0	0.50
	VC3017	586	1.5	0.24
Membrane fraction	NK301	ND	50	1.0
	VC3017	ND	45	0.6

ND – not detected

While some NADH oxidase activity was observed in the cytoplasmic fraction, which might be explained by incomplete sedimentation of the membrane, β-galactosidase activity was detected only in the cytoplasmic fraction and therefore contamination of the membrane fraction by cytoplasmic proteins is unlikely.

HsdR and HsdM subunits of *Eco*KI were detected in both cytoplasmic and the inner membrane fraction of the wild-type strain. HsdR was depleted in the cytoplasmic fraction of *hsdM269* mutant (Fig 5.1A) and to the lesser extent when 2AP was added to the wild-type cells in the early logarithmic phase (Fig 5.1B).

The wild-type cells, treated with 2AP, showed some depletion of HsdR from the cytoplasmic fraction (Fig. 5.1B). However, the degradation was incomplete.

A

HsdR ▶

HsdM ▶
X ▶

B
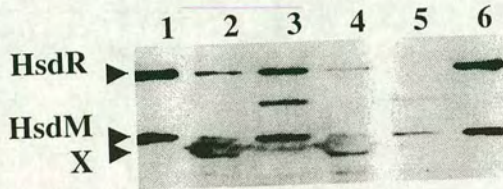
HsdR ▶

HsdM ▶
X ▶

Figure 5.1 The effect of RA on the localisation of HsdR.

Subcellular fractions of bacteria were analysed by Western blot under conditions of restriction alleviation. X is not a derivative of either HsdR or HsdM and shows an anomalous mobility in the lipid-rich membrane fraction.

A Lane1 and 6 - *Eco*KI, 2 - membrane fraction of NK301 r⁺m⁺, 3- cytoplasmic fraction of NK301 r⁺m⁺, 4 - membrane fraction of VC3017 *hsdM269*,5 - cytoplasmic fraction of VC3017 *hsdM269*.

B Lane1 - *Eco*KI, 2- cytoplasmic fraction of NK301 grown in the presence of 2AP(+2AP); 3 - membrane fraction of NK301+2AP; 4- cytoplasmic fraction of NK301; 5 - membrane fraction of NK301

Obviously, RA in the *hsdM269* mutant is more severe because the chromosome is not protected by modification and this results in an enhanced level of degradation of HsdR.

The ratio of HsdR to HsdM of the purified protein estimated from the intensity of bands on a film afteer a short exposure was taken as 1:1 in each of the three independent experiments using the ImageQuant^{TM} program. The ratio of HsdR and HsdM in the cellular fractions was normalised relatively to the value of that of the *Eco*KI protein on the same film. Protein X on the films used for the analysis was represented as an individual peack and was not taken into calculations.

The ratio of the subunits detected in the membrane fractions of the wild-type and the mutant was close to that of the $R_2M_2S_1$ complex (Table 5.3). For the wild-type cells the ratio was 0.4:1 for the cytoplasmic fraction, which probably reflects the abundance of the of M_2S_1 complex in it. These data are in accord with those obtained by (Weiserova *et al.*, 1993) for *Eco*KI, expressed from a multicopy plasmid in minicells.

Table 5.3 Ratio of HsdR:HsdM subunits in cellular fractions as determined by Western-blot analysis

Strain	Fraction	
	Cytoplasmic	Membrane
NK301 wild type	0.45	1.23
VC3017 *hsdM269*	**ND***	1.22

* ND, not detected. At least 50 times less than in the cytoplasmic fraction of the wild type

5.4 RA during conjugation is dependent on *clpX*.

RA in a recipient following the transfer of unmodified bacterial chromosomal DNA into r^+m^+ cells has been reported by (Glover and Colson, 1966). The level of restriction was reduced 60-90 minutes after the start of conjugation and it was restored later to the initial level. The authors used different Hfr donors and F'plasmids for the transfer of DNA into recipient cells, although the data for F' were not shown. This type of experiment was reproduced using a donor of F'101-103 and the results are shown on Fig 5.2.

Donor and recipient cells were grown until mid-logarithmic phase and mixed in the ratio 10:1. Donor cells were r^-m^- and resistant to phage λ adsorption. Recipient cells were $r_K^+m_K^+$ and contained plasmid with the *mod* gene of a type II R-M system EcoRI ($r_{RI}^+m_{RI}^+$ phenotype) so that only phages that were replicated in the recipient cells will be detected. The conjugation mixture was incubated with gentle aeration and samples were taken at time points indicated in Fig. 5.2B. Each sample was divided into two aliquots. λ.0 was added to one aliquote and λ.K was added to the other. The infected cells were mixed with an $r_{RI}^-m_{RI}^+$ indicator culture so that unadsorbed phage will be restricted by the *Eco*RI system of these cells. Infective centres were estimated from plaque counts.

A transient drop in the level of restriction activity was detected with λ.0 in clp^+ cells but not in *clpX* cells. Therefore, RA caused by conjugation is dependent on ClpXP protease as in the case of the establishment of *hsd* genes (Makovets *et al.*, 1998).

A

R_1: r+ m + pEcoRI$_{mod}$

R_2: r+ m + *clpX* pEcoRI$_{mod}$

X

D: *Δhsd recA*λ^r

F'*101-103*

adsorb λ, plate mixture on r$_k$+ m$_k$+ r$_{RI}$+ m$_{RI}$+ strain

↓

Restriction of lambda v.0 by conjugation mixture

B

Figure 5.2 RA during conjugation is dependent on *clpX*.

A. Scheme of experiment.

B. Restriction of λ.0 bythe recipient in the conjugational mixture.

The result from one of three independent experiments is shown.

Chapter 6. Discussion

6.1 Regulation of type I systems

This thesis investigates the details of the regulation of the restriction activity of *Eco*KI, a type IA restriction enzyme. Three different situations relevant to the control of type I restriction systems have been shown to be dependent on the ClpXP protease:- the acquisition of genes specifying these restriction systems, inducible RA and constitutive RA (Makovets *et al.*, 1998; Makovets *et al.*, 1999).

The experiments in Chapter 3 show that some mutations in the *hsdM* gene of a type I restriction enzyme (*Eco*KI) can result in a restriction-proficient, modification-deficient restriction complex (r⁺m⁻). This complex was produced *in vivo* when cells in which $hsdR^+$ was expressed from pNK3, a derivative of pACYC184 (~ 8-10 copies per cell), were infected with $\lambda hsdM^- hsdS^+$ phages. The low e.o.p. of unmodified, but not modified, $\lambda hsdM^- hsdS^+$ phages can be explained by the restriction of phage DNA. In earlier experiments (Kelleher *et al.*,1991) the e.o.p. of $\lambda hsdM^+ hsdS^+$ phages was found to be low, irrespective of the modification state of the phage DNA, when the phage infected a strain containing pJK2, a pBR322 derivative including $hsdR^+$ (~ 20-25 copies per cell). The authors therefore concluded that the bacterial DNA was degraded. The apparent discrepancy between these experiments, all of which used Clp⁺ bacteria, can be explained by the difference in the copy number of pNK3 and pJK2 and consequent difference in the amount of *Eco*KI.

However, when even a low copy number plasmid (pNK3) is used, $\lambda hsdM^- hsdS^+$.K phages have a low e.o.p. on modification-deficient strains in the absence of ClpXP, consistent with the degradation of the unmodified bacterial chromosome (Doronina and Murray, 2001). This suggests that ClpXP can protect the bacterial chromosome from degradation when $hsdR^+$ is expressed from a low copy number plasmid. Preliminary experiments (V.Doronina) have checked for the degradation of the bacterial chromosome following the infection of modification-deficient bacteria with $\lambda hsdM^- hsdS^+$ phages. Using a method described by G. Cromie and D. Leach (2001),

degradation of host DNA was detected in ClpX⁻ but not in Clp⁺ cells, and this occurred after infection with λ$hsdM269hsdS^+$.K but not λ$hsdM177hsdS^+$.K. This is consistent with the *in vivo* experiments in which a low e.o.p. of the phage was observed when *clp⁻ΔhsdRMS* cells were infected by λ$hsdR^+M^+S^+$. The low e.o.p. is likely to reflect the degradation of the chromosome of infected cells (Doronina and Murray, 2001).

Mutations conferring a r^+m^- phenotype have not been described previously, because bacteria effectively regulate the restriction component of the R-M system so these mutations, if characterised *in vivo*, would have been classified as conferring a r^-m^- phenotype. This phenotype is characteristic of the transconjugant *hsdRΔ4hsdM269hsdS⁺* cells that have an F'$hsdR^+$. Survival of these bacteria, that contain a r^+m^- type I restriction enzyme, depends on the ClpXP protease. Mutations that result in a very low level of residual restriction activity can be classified as leading to constitutive RA.

This discrepancy between the behaviour of a mutant restriction complex *in vitro* and the phenotype of bacteria that contain the mutant type I restriction enzyme was also observed when a number of single amino acid changes in the N-terminal TRD of HsdS of *Eco*KI were shown to result in a poor ability of mutant complexes to bind DNA and the $r^-m^{-/+}$ or r^-m^- phenotype of the corresponding strains. The survival of these mutants is dependent on the ClpXP protease (M.O'Neill *et al.*, 2001). For example, the *hsdS*(K92R) mutant has 70% of methyltransferase activity of the wild-type enzyme on an unmodified oligonucleotide DNA substrate and 90% of activity on the hemimethylated oligonucleotide DNA. Nevertheless, this mutation leads to the ClpXP-sensitive phenotype, which suggests that even a modest decrease in the modification activity of a restriction complex would lead to the DBSs in the bacterial chromosome.

A mutant that is apparently inactive *in vitro* but retains the need for ClpXP *in vivo*, *hsdS*(S103E), has a remarkable difference between its action on three substrates: double-stranded oligonucleotides and phage DNA as opposed to the chromosomal DNA. *hsdS*(S103E) mutant complex completely lacks the ability to bind an

oligonucleotide *in vitro* or restrict phage λ DNA *in vivo*. At the same time this amino acid change led to the ClpXP-sensitive phenotype of the mutant strain and to the low e.o.p. of λ*hsdM⁺hsdS*(S103E).K on a ClpX⁻ strain (V.Doronina). Both these pieces of evidence support the idea that, despite its complete inactivity *in vitro,* S103E can introduce DSBs into bacterial chromosome *in vivo*. Therefore, ClpXP sensitivity is probably the best test of *in vivo* restriction activity.

One of the critical factors in the regulation of the restriction activity of *Eco*KI in the case of an undermethylated chromosome is the copy number of the *hsdR* gene. Overproduction of the HsdR polypeptide can lead to cell death as shown by experiments in which the *hsdM* (F269G) mutant is transformed by *hsdR⁺* plasmids with different copy number (section 4.2.).

The process of inducible RA, also controlled by ClpXP protease mechanism, was similarly dependent on the copy number of *hsdR*. The level of residual restriction activity was 10 times higher in cells with pNK3, approximately 250 times higher in cells with pJK2 and was independent of the copy number of either *hsdM* or *hsdS* (Makovets, 1999).

The HsdR subunit was shown to be degraded in an *hsdM* (F269G) mutant if the *Eco*KI complexes can translocate DNA. The recognition of translocating complexes must occur before DSBs are introduced (section 4.4). We hypothesise that a part of the HsdR is exposed during the process of translocation and is recognised by the ClpXP protease. A mutation in HsdR that abolishes the recognition of translocating complexes by ClpXP protease would lead to the cell death in responce to induction that naturally evoke RA as a result of DSBs introduced into the bacterial chromosome.

Some mutations in HsdR isolated in our laboratory led to the bacteria with a 2AP-sensitive phenotype, the phenotype predicted if the process of restriction alleviation is blocked. Using a Tn7-based system for random mutagenesis *in vitro* (Biery *et al.,* 2000) insertion mutations were obtained in the *hsdR* gene of *Eco*KI (W.Pigaga and A. Titheradge, pers.com). One mutation resulting in the insertion of five amino acids

close to the C-terminus of HsdR, in a linker between the helicase domain and the C-terminal domain (Davies et al., 2000, see Fig 1.3A) that is involved in the interactions with the methylase complex. The mutation results in the 2AP sensitivity of an $r_k^+m_k^+$ strain while present on pNK3 suggesting a defect in the RA. The amino acid sequence affected by the insertion is conserved in *Eco*KI and *Eco*AI, both of which are substrates for Clp-dependent regulation, and the insertion is not predicted to change the local secondary structure. The sequence affected has some similarity with the sequences of some other substrates of ClpXP, i.e. those within RpoS and the Phd protein of phage P1, which have been shown to be important for the degradation of these proteins (V. Doronina).

Chemical mutagenesis of wild-type *E.coli* and a subsequent screen for 2AP-sensitive mutants led to the isolation of four mutations mapped to the *clpXP* genes and one in *hsdR* with a single amino acid change (E590K) between DEAD-box motifs II and III of the (S.Makovets, A. Titheradge and N.Murray, pers.com). The level of restriction activity is enhanced in this mutant. Motif II (Walker motif B) and motif III are implicated in coupling of ATP-hydrolysis and DNA translocation (Davies *et al.*, 1999b; Hall and Matson, 1999) and the change of the local conformation may lead to the less effective translocation and an introduction of an early DSBs before the translocating complex is recognised by ClpXP and HsdR is degraded. This explanation is supported by the finding that HsdR is still degraded in the presence of 2AP, although possibly less effectively than the wild-type HsdR (S.Makovets, pers.com). However, the direct involvement of this sequence in the recognition of HsdR by ClpXP cannot be excluded.

Despite the effective regulation of *Eco*KI by ClpXP protease, RA is always incomplete and some residual *Eco*KI remains. The residual restriction activity is efficient against phages that cross bacterial membranes as double-stranded DNA but not those that enter as a single-stranded DNA (Chapter 5, Table 5.1). This difference in sensitivity is consistent with the detection of HsdR in the inner membrane fraction but not in the cytoplasmic fraction, under conditions of restriction alleviation (Fig. 5.1). HsdR is depleted from the cytoplasmic fraction under conditions of restriction

alleviation and therefore single-stranded DNA would replicate in the environment lacking EcoKI.

In wild-type cells EcoKI was detected both in the cytoplasmic fraction and in the inner membrane fraction of cells (Fig. 5.1, lane 2 and 3). This is consistent with results of (Holubova *et al.*, 2000). However, the results presented by Holubova *et al.* (2000) appear to contradict each other. In one experiment EcoKI was no longer associated with the inner membrane if cells were treated by an enzyme that hydrolyses nucleic acids and the authors concluded that EcoKI was associated with the inner membrane via DNA. But the other experiment treating the spheroplasts with a proteolytic enzyme resulted in fragmentation of HsdR and this was taken as evidence that parts of HsdR are exposed to the periplasmic space.

Obviously, the question of the localisation of type I restriction enzymes requires further investigation.

Finally, what is the signal for the RA when unmodified DNA enters a restriction-proficient cell? The data in Chapter 5 (section 5.4) suggest that postconjugational RA, like in the case of establishment of a type I R-M system, is ClpXP - dependent even if cells contain an established type I system.

Our preliminary evidence supports the hypothesis that incoming DNA has to be recombined with the resident chromosome in order to provide the trigger for the restriction alleviation. RA was not observed using pOX38Km, a derivative of F plasmid that lacks homology with the bacterial chromosome and therefore cannot undergo homologous recombination with the chromosome of recipient cell. It also does not include any transposable element that could lead to the integration of the an F plasmid to the bacterial chromosome and mobilisation of chromosomal DNA was used and this plasmid was effectively restricted (Doronina and Murray, 2001).

Additionally, the restriction alleviation is blocked in a *recA* mutant. However, *recA* mutation leads to a pleiotropic phenotype and a functional RecA is necessary for some types of RA (section 1.2, Inducible restriction alleviation).

To test this hypothesis it is critical to restore the recombination proficiency of the recipient cells by providing an alternative recombination system and accessing the restriction alleviation.

Summarising, this thesis describes certain aspects of regulation of type I R-M systems. It has been a generally accepted opinion that the modification itself is sufficient and necessary for a restriction system to distinguish between the resident DNA and foreign DNA. Fully modified resident DNA becomes hemimethylated after the replication and hemimethylated DNA is a substrate for a modification enzyme but not the restriction enzyme. However, chromosome might lose the property, distinguishing it from the foreign DNA, i.e. modification as in the case of mutations that impair modification activity or lead to a restriction enzyme with a new specificity. This situation requires the additional mechanisms of regulation of a resident restriction system.

6.2 Biological aspects of restriction systems.

Restriction-modification systems are widespread in nature. At least one candidate is found in 80% of the completely sequenced genomes of Bacteria and Archaea, 75% of these genomes appear to contain multiple restriction systems (Roberts, 1998; (Kong *et al.*, 2000). Type II R-M systems are predicted to be the most abundant (in terms of number predicted for one strain) and widespread (number of species predicted to have at least one) group and type I is the second most abundant and widespread. Some bacteria for which a complete genome sequence is published, notably intracellular parasites *Ricketsia provazekii* and three *Chlamidia* species,

have no candidates for any type of restriction systems. The number of restriction systems predicted for a species does not correlate with genome size. The correlation between the presence of putative restriction systems and their functionality, detected by biochemical methods, has been analysed using *Helicobacter pylori* (*H.pylori*) as a model organism for detection of type II systems. *E.coli* was used for the study of biology of type I systems.

The genome of *H.pylori* is of a medium size (~1.65 Mb) but as much as 4% of the DNA is predicted to encode putative restriction systems and more than ten of these are type II systems (Kong *et al.*, 2000). Three type I systems and two type III systems were also predicted in each strain.

The biochemical analysis of two strains, *H.pylori* J99 and *H.pylori* 26695, for which complete sequences of genomes are available revealed that 30% (4 in each strain) of putative type II systems are functional. Those restriction enzymes for which endonuclease activity is detected belong to the strain-specific R-M systems (Kong *et al.*, 2001). There are also 6 systems where only the modification component is active and the authors propose that these systems are in the process of inactivation due to the accumulation of mutations.

Ando et al. (2000) analysed 19 strains of *H.pylori* and found that all of them have at least one functional R-M system but no two strains have an identical functional type II restriction system. Therefore, most of the strains of *H.pylori* screened so far have a functional type II R-M system and most of these restriction systems have a different specificity.

In contrast to *H.pylori*, the sequences of the genomes of two strains of *E.coli* - K12 and enteropathogenic O157:H7 – contain no candidates for type II R-M systems (Blattner *et al.*, 1997; Perna et al., 2001). Each has one type I system - *Eco*KI and an unidentified R-M system that shows a high level of identity with *Eco*AI of IB family (G. Davies. pers.com), correspondingly.

The biochemical activities of type I restriction enzymes are more difficult to detect because a type I restriction enzyme requires cofactors for the restriction activity and does not produce a distinct pattern of restriction fragments. A search for type I R-M systems in the collection of *E.coli* strains relied on the hybridisation of probes derived from the representatives of four families (IA, IB, IC and ID) with chromosomal DNA. Seventeen of thirty-seven strains tested were probe-positive, but the authors do not exclude the possibility that probe-negative strains have a type I system non-homologous to the probes used (Barcus *et al.*, 1995).

Restriction systems have been compared with the immune system of higher organisms. Using an absence or presence of a specific modification as a "foreign antigen" they distinguish between "self" and "non-self" DNA and eliminate the latter. The most obvious role for the restriction systems is to protect the host DNA against an invasion by foreign DNA: bacteriophages, conjugative plasmids and naked DNA. The presence of antirestriction systems in the majority of phages (Kruger and Bickle, 1983) and transmissible plasmids (Chilley and Wilkins,1995), absence of the recognition sites for the restriction systems (Kruger *et al.*, 1995) and modification of DNA determined by phage modification systems (Bickle and Kruger, 1993) are the result of the arms race in host-parasite interaction.

In the case of lateral transfer (for review see Ochman *et al.*, 2000) of homologous DNA R-M systems can play an additional role in facilitating recombination. Restriction enzyme fragment DNA and fragmented DNA is degraded until sequences that facilitate recombination are encountered and the recombination can start.

However, a potential damage to the resident DNA in the process of the establishment of an RS or as a result of loss of the RS raises the question of regulation of restriction activity of restriction systems. Nucleoside-independent restriction systems (NIRS) and nucleoside-dependent restriction systems (NDRS) have a fundamental difference in the mechanisms of their regulation. There is accumulating evidence that the first group is controlled at the level of transcription and the second group at the

posttranslational level (Murray, 2000). The differences between NIRS and NDRS which may be the cause of the distinct regulatory mechanisms will be discussed using type II R-M systems as representatives of NIRS and type I restriction enzymes as representatives of NDRS.

There is a bewildering variance in the nucleotide sequences of the genes, gene arrangements and sequences recognised among type II systems. The division into families like the "natural" classification of type I systems is non-applicable to type II systems. The restriction and modification enzymes that are part of one type II R-M system apparently do not have a common ancestor (Wilson and Murray, 1991).

In contrast, the organisation of type I R-M systems into families, within which the subunits of the enzymes are interchangeable and the finding that enzymes that recognise similar sequences have similar TRDs is consistent with divergent evolution from a common ancestor (Barcus and Murray, 1995).

The hypothesis of type II R-M systems as "selfish genes" is fully explored by Kobayashi and co-workers. According to them, R-M systems behave like "molecular parasites". A resident R-M system eliminates the host cells that have lost the resident restriction system by killing the host when the residual modification enzyme is no longer able to modify all the target sequences. In the experiments performed to check this hypothesis R-M systems serve as addiction modules for plasmid maintenance (Kulakauskas *et al.*, 1995; Naito *et al.*, 1995). Loss of a plasmid encoding a type II system leads to cell death as a result of an attack on the bacterial chromosome by the restriction enzyme and introduction of DSBs (Handa *et al.*, 2000).

However, a type II system which is established and situated on the bacterial chromosome will be harmful if a mutation inactivates inactivates the modification gene or if the specificity of one of either components is changed.

The finding of a group of type II R-M systems, in two *H.pylori* strains (Kong *et al.*, 2000; Kong *et al.*, 2001), which have inactive restriction but active modification

components supports a hypothesis that a restriction gene accumulates mutations more rapidly than the cognate modification gene.

Moreover, the DNA-binding specificity of restriction endonucleases are difficult to change (for recent review see (Lukacs and Aggarwal, 2001. Despite numerous attempts to engineer an enzyme with the new specificity it has been possible only to change *Bam*HI so that it prefers a methylated substrate (Dorner *et al.*, 1999) or the preferred target sequence of *Eco*RV from six base pairs to eight base pairs (Lanio *et al.*, 2000).

Unlike type II R-M systems, type I R-M systems are prone to change of the specificity not by change within a TRD but as the result of rearrangements affecting *hsdS* (for review see Bickle and Kruger, 1993; Murray, 2000). However, the hypothesis of "selfishness" as defined by Kobayashi and co-workers is non-applicable to type I R-M systems.

Type I systems can be readily acquired and established (Ryu et al., 1993; Makovets, 1998) and lost (O'Neill *et al.*, 1997). Type I R-M genes in the chromosome are readily replaced by the mutant alleles and by alleles encoding a type I R-M system of different specificity ((O'Neill *et al.*, 1997).

This thesis investigates the consequences of a mutation that led to the inactivation of modification but not restriction activity of a type I restriction enzyme. The results indicate that this mutation is not lethal due to the proteolytic control of the restriction enzyme and they support the idea that type I R-M systems are not "selfish". The system of control of the restriction activity of *Eco*KI that distinguishes between the resident DNA and foreign DNA is consistent with the idea that some R-M systems are involved in "cellular defense" and therefore are advantageous to the cell.

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Regulation of endonuclease activity by proteolysis prevents breakage of unmodified bacterial chromosomes by type I restriction enzymes

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ABSTRACT   ClpXP-dependent proteolysis has been implicated in the delayed detection of restriction activity after the acquisition of the genes (*hsdR, hsdM,* and *hsdS*) that specify *Eco*KI and *Eco*AI, representatives of two families of type I restriction and modification (R-M) systems. Modification, once established, has been assumed to provide adequate protection against a resident restriction system. However, unmodified targets may be generated in the DNA of an *hsd*+ bacterium as the result of replication errors or recombination-dependent repair. We show that ClpXP-dependent regulation of the endonuclease activity enables bacteria that acquire unmodified chromosomal target sequences to survive. In such bacteria, HsdR, the polypeptide of the R-M complex essential for restriction but not modification, is degraded in the presence of ClpXP. A mutation that blocks only the modification activity of *Eco*KI, leaving the cell with ≈ 600 unmodified targets, is not lethal provided that ClpXP is present. Our data support a model in which the HsdR component of a type I restriction endonuclease becomes a substrate for proteolysis after the endonuclease has bound to unmodified target sequences, but before completion of the pathway that would result in DNA breakage.

Within a bacterium that has a classical restriction and modification (R-M) system, the nucleotide sequences that define the targets for attack by the resident restriction endonuclease are concealed by the modification of appropriate bases within them. For some systems this modification is achieved by the methylation of specific adenine residues, and for others it is achieved by methylation of cytosine residues. The restriction endonuclease has the potential to attack DNA from different strains of the same species because foreign DNA generally lacks the protective imprint of the relevant methyltransferase (for reviews see refs. 1 and 2). Restriction of the host cell's newly synthesized DNA normally is avoided, because the unmethylated strand of each target sequence produced by DNA replication is methylated before the next round of replication. If, however, resident DNA were to acquire unmodified target sequences, would it, like foreign DNA, become a substrate for restriction? In this paper we show that in situations where the modification of the host DNA by a type I R-M system fails, an alternative level of protection impairs the endonuclease activity of the restriction system and the bacteria survive.

A type I R-M system is encoded by three genes: *hsdR, hsdM,* and *hsdS*. The three polypeptides, HsdR, HsdM, and HsdS, often designated R, M, and S, assemble to give an enzyme ($R_2M_2S_1$) that modifies hemimethylated DNA and restricts unmethylated DNA. A smaller complex (M_2S_1) has only the methyltransferase activity. The S subunit confers target spec-

ificity; hence, both complexes and both activities respond to the same nucleotide sequence.

Type I systems of enteric bacteria have been divided into discrete families by tests for cross-hybridization between genes and cross-reactivity with antibodies raised against the archetypal member of each family (3–5). Four families of distantly related systems have been identified (types IA, IB, IC, and ID), and where complementation tests have been done they indicate that enzymes in the same family can interchange subunits, but those from different families cannot (6, 7).

No transcriptional regulation of type I R-M genes has been detected; yet these genes are transferred readily to recipient bacteria devoid of the relevant modification activity (8–10). It is presumed that the cells survive the acquisition of the new R-M system because they become restriction proficient only after the modification activity is established. Experiments in support of this identify a lag of ≈ 15 generations before the cells become restriction-proficient after the acquisition of *hsd* genes by conjugation (11). The ClpXP protease was shown to be essential for the effective acquisition of genes specifying type IA and IB systems, and for this reason proteolysis has been implicated in the delayed expression of restriction activity (10).

The acquisition of a new specificity system is not the only situation in which a temporary loss of restriction proficiency has been detected. A well documented example, referred to as restriction alleviation (RA), occurs in response to treatments that damage DNA (12–14). UV light, nalidixic acid, and 2-aminopurine (2-AP) have been shown to induce restriction alleviation. It is possible that the temporary loss of restriction proficiency associated with the establishment of a new specificity is an example of RA. If this is so, ClpXP would be required for the alleviation of restriction in response to DNA damage. We have tested this hypothesis and show ClpXP to be a common requirement for RA in response to the various agents that damage DNA. This led us to identify steps in the molecular pathway that protect bacteria against the potentially lethal effects of restriction after DNA damage in a cell with a resident type I system or after the acquisition of a type I system capable of attacking the resident DNA.

MATERIALS AND METHODS

Bacterial Strains, Phages, Plasmids, and General Microbial Methods. Bacterial strains are listed in Table 1. Integration-deficient, $\lambda hsdc$I857 phages were used to transfer *hsd* alleles to bacterial chromosomes: λNM1367 includes $hsd\Delta RM(F269G)S^+$; λNM1376, $hsdM^+S^+$; λNM1394, $hsdM(F269G)S^+$; and λNM1384, $hsdR(A619V)$ (17). JC9935 was used as the donor of the following derivatives of F′101: F′101–102, $hsd_KR^-M^+S^+$ (11); F′101–301, $hsd_K^- hsd_A^+$ (10);

Table 1.   *E. coli* K-12 strains

Strain	Relevant genotype	Source or origin
C600	hsd_K^+	See ref. 10
5K	hsdR514	See ref. 10
CB51	dam-3	C. Boyd
JC9935	recA13	See ref. 10
LE451	rac-0 recA srl::Tn10	Ref. 15
NM477	Δ(hsdMS)5	See ref. 10
NM659	ΔrecA::cat	This laboratory
NM679	Δ(hsdRMS)	Ref. 16
NM799	hsdR(A619V)	Refs. 17 and 18
NM802	ΔhsdR4	This laboratory
SG22007	ΔclpP::cat	Ref. 19
SG22080	ΔclpX::kan	Ref. 20
SG22129	ΔclpP::cat Δ clpX::kan	S. Gottesman
RH6972	dnaQ::miniTn10 (mutD)	D. R. F. Leach
RS2	topA10	Ref. 21
TPC48	zjj::Tn10 dnaC^{ts}	See ref. 10
NK31	gyrA96	Ref. 10
NK167	$hsd_K^- hsd_A^+$	Ref. 10
NK300	rac-0 recA$^+$srl$^+$	LB451 × P1(C600)
NK301	rac-0 gyrA96	NK300 × P1(NK31)
NK302	dam	NK301 × P1(CB51)
NK303	ΔclpP	NK301 × P1(SG22007)
NK304	ΔclpX	NK301 × P1(SG22080)
NK308	ΔrecA	NK301 × P1(NM659)
NK309	zjj::Tn10 dnaC^{ts}	NK301 × P1(TPC48)
NK310	hsdR	NK301 × P1(5K)
NK311	Δ(hsdRMS)	NK309 × P1(NM679)
NK312	Δ(hsdRMS) ΔclpX	NK311 × P1(SG22080)
NK315	dam ΔclpX	NK302 × P1(SG22080)
NK320	ΔclpX	NK300 × P1(SG22080)
NK323	ΔclpX ΔrecA	NK304 × P1(NM659)
NK324	Δ(hsdRMS) ΔclpX ΔrecA	NK312 × P1(NM659)
NK325	hsdR ΔclpX	NK310 × P1(SG22080)
NK326	mutD	NK301 × P1(RH6972)
NK327	mutD ΔclpX	NK326 × P1(SG22080)
NK329	topA10 ΔclpP ΔclpX	RS2 × P1(SG22129)
NK351	hsdR(A619V)	NK309 × P1(NM799)
NK352	Δ(hsdMS)5	NK309 × P1(NM477)
NK354	$hsd_K^- hsd_A^+$	NK309 × P1(NK167)
NK355	$hsd_K^- hsd_A^+$ ΔclpX	NK354 × P1(SG22080)
NK378	ΔhsdR hsdM(F269G)	NM802 × λNM1367
NK379	ΔhsdR	NK309 × P1(NM802)
NK380	ΔhsdR ΔclpX	NK379 × P1(SG22080)
NK382	ΔhsdR hsdM(F269G)	NK309 × P1(NK378)
NK383	ΔhsdR hsdM(F269G) ΔrecA	NK382 × P1(NM659)
NK384	ΔhsdR hsdM(F269G) ΔclpX	NK382 × P1(SG22080)
NK386	hsdM(F269G)	NK301 × λNM1394
NK388	hsdR(A619V)hsdM(F269G)	NK386 × λNM1384

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and F'101–103, zjj::Tn10 hsd$_K$R$^+$Δ(MS)5. F'101–103 was selected after plasmid–chromosome allele exchange, as described for F'101–301 (10). pNK3 was made by transferring the HindIII-SmaI fragment containing hsdR from pBg3 (22) to pACYC184 (23) digested with HindIII and NruI. Media and general methods were as described previously (10).

Restriction Alleviation. 2-AP (400 µg/ml) was added to midlogarithmic cultures grown at 37°C in LB medium. Intensive aeration was provided before and during the treatment. After 1 h, the cells were washed, resuspended in fresh broth, and tested for restriction. UV-induced RA was measured as described in ref. 24, and RA in response to nalidixic acid was measured as described by (13).

Analysis of Proteins. Polypeptides were separated by electrophoresis through SDS/polyacrylamide gels (25). Western

blots used rabbit antisera against EcoKI or EcoAI and the chemiluminescence detection system (POD) of Boehringer Mannheim.

The stability of proteins was monitored after pulse-labeling with [^{35}S]methionine. Bacteria were grown at 37°C with intensive aeration to an OD$_{600}$ of 0.2–0.3 in minimal medium supplemented with thiamin and all amino acids except methionine and cysteine. Chloramphenicol (20 µg/ml) maintained the presence of pNK3. Each culture was divided, and 2-AP (400 µg/ml) was added to one aliquot. After 1.5 h, a 1-min pulse of [^{35}S]methionine (25 µCi/ml) was given. Labeling was stopped by diluting each culture with an equal volume of prewarmed LB supplemented with L-methionine (15 µM) or with L-methionine and 2-AP (400 µg/ml). Intensive aeration was maintained, and samples were taken at appropriate intervals. Bacteria were collected by centrifugation, resuspended in SDS sample buffer, and boiled for 5 min, and samples were applied to SDS/polyacrylamide gels for the separation of polypeptides by electrophoresis.

RESULTS

ClpXP Is Necessary for RA. A simple quantitative test for restriction relies on the fact that most unmodified λ phages are killed when they infect *Escherichia coli* K-12; the phage genome is a substrate for EcoKI, the resident restriction system. The titer of an unmodified phage lysate (λ.0) on a restricting host relative to that on a nonrestricting derivative is referred to as the efficiency of plating (EOP). Therefore, the inverse of EOP quantifies restriction. RA is detected as a temporary reduction in restriction (hence, an increased EOP) after treatment of genetically restriction-proficient cells with agents that damage DNA.

We examined RA for Clp$^+$ and Clp$^-$ strains in response to each of three treatments; UV light, nalidixic acid, and 2-AP. For each treatment, ClpX was essential for efficient RA (Fig. 1). A clpP strain was tested for RA in response to 2-AP, and it also was deficient in RA (data not shown). The results support our hypothesis that RA, in response to agents that damage DNA, and the delayed expression of restriction activity after the acquisition of hsd$^+$ genes by an hsd$^-$ recipient are both the outcome of a common ClpXP-dependent process. RA for the EcoAI system in response to 2-AP also was shown to be dependent on ClpX (data not shown).

"Constitutive" RA. Restriction is alleviated in dam strains (26). It is known that the Dam-methylase identifies the parental DNA strand during mismatch repair, and in dam mutants mismatch repair leads to double-strand breaks (DSBs) (27). This alleviation of restriction in dam strains led us to question whether other mutations that impair the efficiency or fidelity of DNA replication might induce RA. If such a phenotype occurred, would it be dependent on ClpXP? We tested topA, mutD, and dam strains.

Mutants deficient in topoisomerase I, like wild-type cells treated with nalidixic acid, have problems in DNA replication; DSBs may occur when the replication forks stall (28). In
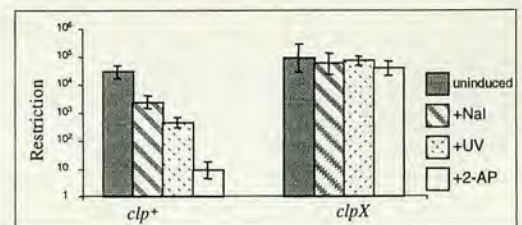

FIG. 1.   Restriction of unmodified phage λ by clp$^+$ (NK301, NK300 for nalidixic acid) and clpX (NK304, NK320 for nalidixic acid) bacteria. Only clp$^+$ cells show restriction alleviation.

contrast, a *mutD* mutation enhances the error rate of DNA polymerase III (29) and the increased frequency of mismatches may mimic the effect of 2-AP, an analogue of adenine that causes base pair transitions.

Restriction by *dam, topA*, or *mutD* strains was at least 100-fold less efficient than restriction by wild-type *E. coli* K-12 (Fig. 2). If this poor restriction is the result of constitutive expression of RA activated in response to either DNA damage or mismatches, then a mutation in *clpX* or *clpP* should restore restriction. Consistent with this prediction, the efficiency of restriction was enhanced by approximately 100-fold in the absence of ClpXP protease (Fig. 2).

ClpXP-Deficient, Restriction-Proficient Bacteria Die During Prolonged Exposure to 2-AP. After prolonged treatment with 2-AP (3–4 h at 400 µg/ml), *clp⁻* (NK303 and NK304) but not *clp⁺* (NK301) bacteria become filamentous, a phenotype characteristic of the SOS response. 2-AP does not normally activate the SOS response but, in the absence of ClpXP, it could induce a chain of events that leads to DNA damage. The relevance of a RecA-dependent repair pathway is supported by the observation that *recA clpX* double mutants (NK323) are supersensitive to 2-AP and do not survive low concentrations (40 µg/ml) of 2-AP in the medium. In contrast, a *recA clp⁺ hsd⁺* strain (NK308) is no more sensitive to 2-AP than its *rec⁺* counterpart (NK301); *recA* strains resemble *rec⁺* in their RA response to 2-AP.

Is ClpXP needed in the presence of 2-AP to prevent DNA damage by the resident restriction endonuclease? We made the *clpX* bacteria deficient in restriction both by deleting the *hsd* genes (NK312) and by including a mutation in *hsdR* (NK325), the gene essential for restriction. The restriction-deficient bacteria were not sensitive to 2-AP. Similarly, the hypersensitivity of the *recA clpX* strain was relieved by inactivation of the endonuclease activity. We suggest that during prolonged treatment with 2-AP, the ClpXP-dependent pathway is essential to prevent *Eco*KI from causing DNA damage and consequent cell death.

RA Induced by 2-AP Is Associated with a Deficiency of HsdR. RA is not correlated with a loss of modification activity (14, 30). It could, therefore, be the result of a deficiency in HsdR and the consequent depletion of *Eco*KI (R₂M₂S₁), but not the modification enzyme (M₂S₁).

The HsdR and HsdM subunits were monitored by Western blots after the addition of 2-AP to both *clp⁺* and *clpX* bacteria (Fig. 3). After a lag of 20 min, a reduction in the concentration of HsdR, but not HsdM, was detected. This deficiency of HsdR was found only in *clp⁺* cells in response to 2-AP. RA, therefore, correlated with a ClpX-dependent reduction in the concentration of HsdR, the polypeptide essential for restriction, but not modification.

HsdR Is Degraded in *clp⁺* Cells Treated with 2-AP. The very low concentration of HsdR detected in Clp⁺ cells after a period of growth in the presence of 2-AP (Fig. 3) is consistent
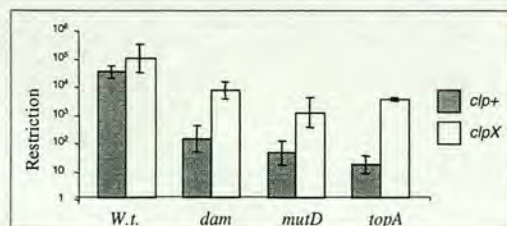

FIG. 3. Assays for HsdR and HsdM polypeptides after treatment with 2-AP. (*a*) *clp⁺* bacteria (NK301). (*b*) *clpX* bacteria (NK304). In the absence of 2-AP (data not shown), the assays for *clp⁺* and *clpX* bacteria were indistinguishable from those seen in *b*. *Eco*KI polyclonal antibody, used in these Western blots, fails to detect HsdS, but detects HsdR and HsdM and some other *E. coli* proteins.

with the degradation of HsdR in the presence of ClpXP, but it could be argued that ClpXP in some way affects the synthesis rather than the degradation of HsdR.

We therefore assayed the stability of HsdR in *clp⁺* and *clpX* cells in response to treatment with 2-AP. The preferred experiment was to rely on the chromosomal *hsdR* gene, but the signal generated from a single copy of *hsdR* was weak compared with those generated by other proteins. Gene dosage was increased by cloning *hsdR* in pACYC184, a low-copy-number vector. *clp⁺ hsd⁺* (NK301) and *clpX hsd⁺* (NK304) bacteria transformed with the *hsdR⁺* plasmid (pNK3) were treated with 2-AP for 90 min to allow the establishment of RA before they were pulse-labeled with [³⁵S]methionine. HsdR was unstable in Clp⁺ but not ClpX⁻ cells after 2-AP treatment (Fig. 4). In the absence of 2-AP (data not shown) the HsdR polypeptide was stable in *clp⁺* and *clpX* cells for at least 180 min.

These results are consistent with 2-AP as the activator of a RA pathway in which HsdR is susceptible to ClpXP-dependent proteolysis.

Functional *Eco*KI Is Obligatory for the Loss of HsdR That Is Characteristic of RA. Is active *Eco*KI necessary to generate the signal that leads to ClpXP-dependent degradation of HsdR? To answer this question we tested whether 2-AP-induced depletion of HsdR occurs in restriction-deficient mutants. One of the mutants tested has a missense mutation in *hsdR* (NK351), and the other (NK352) has a wild-type *hsdR* gene, but *hsdM* and *hsdS* are deleted so that HsdR cannot form an *Eco*KI complex.

HsdR was not depleted in either mutant in response to 2-AP (Fig. 5*a*). This finding implies that a functional endonuclease is required for induction of the pathway that leads to degradation of HsdR. If the products of restriction by a type I enzyme are the stimulus for RA, the endonuclease activity of one R-M system should induce RA for a different system. We tested whether a functional type IB system (*Eco*AI), for which RA is regulated in a ClpXP-dependent manner, induced degradation of the HsdR polypeptide of the inactive type IA system, *Eco*KI.

We transferred F'*hsd*ₐR⁺M⁺S⁺ (F'101–301) to the three strains used in the previous experiment (Fig. 5*a*): *hsd*ₖR⁺M⁺S⁺, *hsd*ₖR⁻M⁺S⁺, and *hsd*ₖR⁺Δ(MS). The transconjugants, both untreated and treated with 2-AP, were

FIG. 2. Restriction of unmodified phage λ by *dam* (NK302), *mutD* (NK326), and *topA* (RS2) strains and their *clpX* derivatives (NK315, NK327, and NK329). It is known that *topA* strains accumulate compensatory mutations in *gyrA* or *gyrB* (21), but the *topA10* strain (RS2) is not known to have a compensatory mutation (21), and the *topA* mutation itself correlates with impaired restriction (G. P. Davies, personal communication).
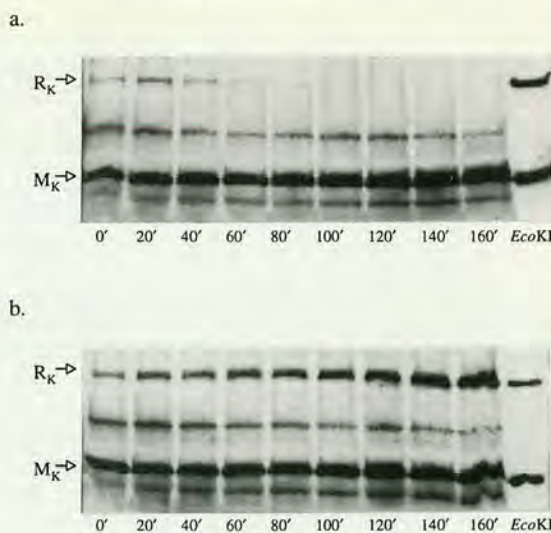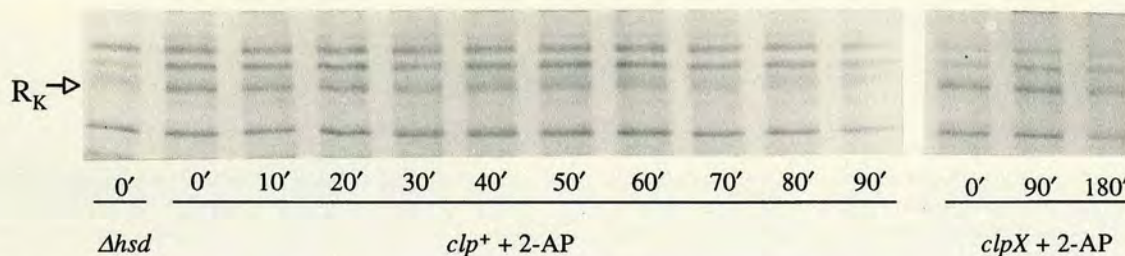
FIG. 4. The stability of HsdR *in vivo* after treatment with 2-AP. Labeled polypeptides separated by electrophoresis through SDS-polyacrylamide gels (6%) were detected by autoradiography. An extract from a strain lacking HsdR (NK311/pACYC184) was analyzed in the first track. Samples from *clp*[+] and *clpX* bacteria containing pNK3 were taken at the time intervals indicated after pulse labeling.

assayed for *Eco*AI- and *Eco*KI-dependent restriction *in vivo* and for the presence of HsdR polypeptides. 2-AP caused RA of functional R-M systems, and HsdR from any restriction-proficient complex was lost (Fig. 5*b*). However, for the non-functional *Eco*KI complex, R_K remains even in the presence of functional *Eco*AI. These data require that the stimulus for RA is family-specific and therefore is not simply the product of restriction.
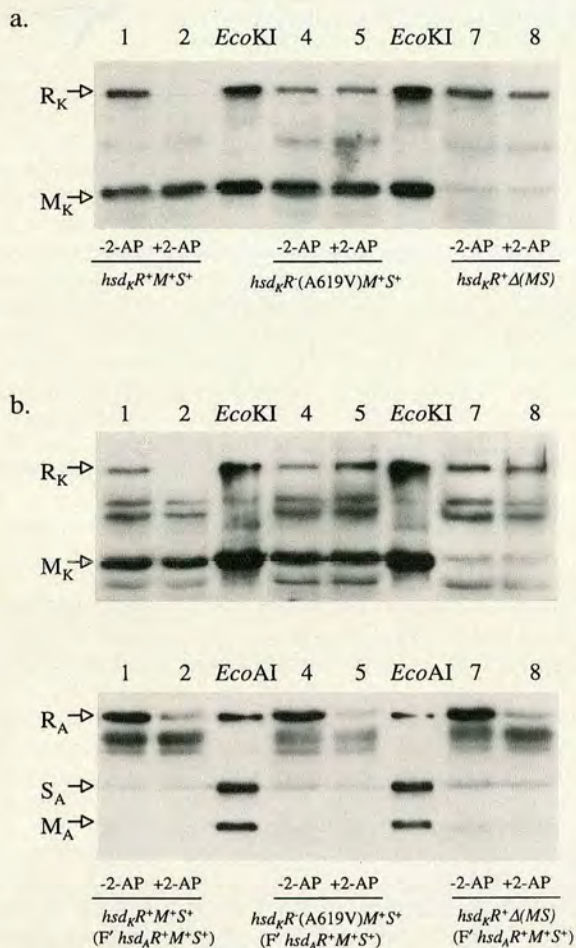
FIG. 5. Hsd subunits were monitored, after treatment with 2-AP, using antibodies raised against the relevant R-M complex. HsdR is degraded only when it is a part of a functional complex. (*a*) Degradation of HsdR is prevented by a missense mutation in *hsdR* (track 5) or by the absence of HsdM and S (track 8). (*b*) The presence of functional *Eco*AI has no effect on the degradation of the HsdR subunit of *Eco*KI (*Upper*), even though the HsdR subunit of *Eco*AI itself is degraded (*Lower*, lanes 2, 5, and 8). The control tracks for *Eco*AI contain a mixture of polypeptides in which HsdM and HsdS are present in molar excess to give strong signals with antibody.

Mutations Predicted to Confer a Restriction-Proficient, Modification-Deficient (r[+]m[−]) Phenotype Cause Restriction Alleviation. It is logical to expect that a mutation conferring an r[+]m[−] phenotype would be lethal. We chose to investigate a mutation in *hsdM* (F269G) that abolishes methyltransferase activity but has no effect on the binding of the cofactor *S*-adenosylmethionine and therefore is predicted to leave a functional endonuclease (31). This *hsdM* mutation was transferred from a λ*hsd* phage (λNM1367) to the chromosome of an *hsdR* strain. The presence of *hsdM*(F269G) (NK378) was associated with the anticipated m[−] phenotype. We tested the naive prediction that the acquisition of an F′ with a functional *hsdR* gene would generate r[+]m[−] transconjugants and these would die. However, we found no difference between the survival of the recipients upon acquisition of F′*hsdR*[+] and the survival of recipients receiving the control F′ lacking an *hsdR*[+]
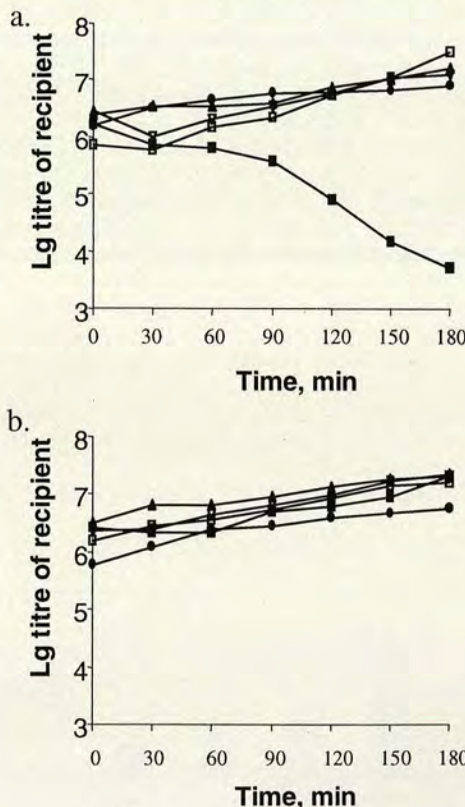

FIG. 6. The survival of *hsdR*[+]*M*[−](F269G)*S*^{+} cells was assessed after the conjugative transfer of *hsdR*[+] to *hsdR*[−]*M*[−]*S*[+] recipients. (*a*) The experiment using F′101–103 (*hsdR*[+]). (*b*) The control experiment with F′101–102 (*hsdM*[+]*S*[+]). Data are plotted for the following recipients: NK379, *hsdR* (▲); NK380, *hsdR clpX* (▽); NK382, *hsdRM* (□); NK384, *hsdRM clpX* (■); and NK383, *hsdRM recA* (●). The data show that *hsdR*[+]*M*[−](F269G)*S*^{+} cells survive only if the recipient is ClpX[+].

allele (Fig. 6). We extended our experiment to include a *recA* recipient in which DSBs would not be repaired; transfer of the $hsdR^+$ allele still occurred efficiently (Fig. 6).

The EOP of λ.0 on the $hsdR^-M^-S^+$ (F'$hsdR^+$) transconjugants was 10^{-1} in contrast to 5×10^{-4} when the F'$hsdR^+$ was transferred to an $hsdR^-M^+S^+$ recipient (the EOP of λ.K was 1 in both cases). The low level of restriction by the m^- transconjugants is consistent with induction of the RA response. Therefore, the conjugation experiments were extended to include *clpX* recipients. In the absence of ClpX, transfer of the F'$hsdR^+$ to the $hsdR^-M^-S^+$ recipient was lethal, consistent with the presence of functional restriction endonuclease (Fig. 6). Our hypothesis predicts that the transconjugant bacteria can survive in the presence of ClpXP because of the activation of the RA pathway. If this suggestion is correct, $hsdR^+M^-S^+$ bacteria would be deficient in HsdR.

We chose to use chromosomal genes in preference to a plasmid-borne *hsdR* to test this prediction. We transferred the *hsdM*(F269G) mutation to the chromosome of NK301, an hsd^+ strain. The *hsdM* recombinants were recognized by their m^- phenotype and could not be transduced to give *clpX* derivatives (data not shown). These derivatives restricted λ.0 with an efficiency indicative of RA (EOP = 10^{-1}). Consistent with the induction of RA, HsdR was missing in the *hsdM*(F269G) strain that encodes a functional restriction enzyme and present in a derivative with a missense mutation in *hsdR* (Fig. 7). Importantly, when *hsdM*(F269G) was replaced with the wild-type allele (see legend to Fig. 7), HsdR was restored. Therefore, the loss of HsdR is a consequence of the *hsdM* mutation. Our experiments with the modification-deficient mutant show that *E. coli* has an extraordinary capacity to protect itself against potential DNA damage elicited by a resident type I R-M system.

DISCUSSION

The diagnostic feature of RA is an r^- phenotype despite a restriction-proficient genotype (hsd^+). The r^- phenotype that persists for many generations in a transconjugant after the acquisition of functional *hsd* genes by an hsd^- recipient (11, 32) may be viewed as an example of RA. In this case, the establishment of hsd^+ genes in a naive bacterium depends on the ClpXP protease (10). We now have shown that RA in response to a variety of stimuli, including external agents and mutations that affect the fidelity of DNA replication, also requires ClpXP. In two quite different situations the presence of subunits of *Eco*KI was monitored after the induction of RA. In the first, the bacteria were treated with 2-AP, and in the second, a mutation in *hsdM* (F269G) was introduced that blocks only the methyltransferase activity of *Eco*KI (31). In both these examples of ClpXP-dependent RA, a negligible level of HsdR remained. We propose a general pathway for RA in which ClpXP is necessary for the degradation of HsdR and the consequent r^- phenotype. According to this scheme, unmodified chromosomal DNA targets would be a signal for the cell to protect its own DNA from restriction. We believe that all the stimuli for RA examined by us rely on the presence of unmodified target sequences.

A particularly severe stimulus is provided by the mutation in *hsdM* (F269G) that results in a modification-deficient, restriction-proficient *Eco*KI complex (Fig. 6*a*). For this mutant to survive, despite an unmodified chromosome, restriction alleviation must be extraordinarily effective. A more common stimulus is DNA damage that elicits RecA-dependent repair. UV irradiation and mutations in *dam* can cause DSBs (26, 33); nalidixic acid and mutations in *topA* are likely to generate DSBs by stalling replication. Damage by UV light also leads to lesions in one strand that are repaired postreplicatively (34). RecA-dependent repair relies on homologous recombination. If homologous recombination involves two segments of hemimethylated DNA, the annealing of unmethylated strands or DNA synthesis may generate a localized region of unmethylated DNA. In contrast, both 2-AP and *mutD* increase the frequency of base pair transitions (29, 35). Some mutations will generate new target sequences, all of which will be unmodified.

Our experiments have shown a ClpXP-dependent loss of HsdR in response to 2-AP. It seems likely that the ClpXP protease itself degrades HsdR, rather than being necessary to maintain or activate another protease. The only protease-deficient mutants found to affect the transmission of the genes encoding *Eco*KI were *clpX* and *clpP* (10). Our experiments also show that HsdR is lost only in cells in which HsdR could produce functional *Eco*KI. Thus, in the absence of HsdM and HsdS, wild-type HsdR is not degraded; likewise, in the presence of HsdM and HsdS, a missense mutation in *hsdR* prevents degradation of the nonfunctional polypeptide. The requirement for unmodified targets and functional *Eco*KI might suggest that DNA breakage initiates the RA response. We argue that DSBs are not involved in the initiation of RA. One reason for doubting this idea is our observation that a $recAclp^+hsd^+$ bacterium is no more sensitive to 2-AP than its rec^+ counterpart. This finding is not consistent with the creation of DSBs in response to 2-AP. Second, we tested whether active *Eco*AI, a member of the type IB family of enzymes is sufficient to induce loss of the HsdR subunit of *Eco*KI in response to treatment with 2-AP. It is not, although it is susceptible to ClpXP-dependent RA. If DSBs are the signal for RA, those made by *Eco*AI do not provide a signal for degradation of the HsdR subunit of *Eco*KI. Finally, even in the absence of RecA we readily made strains in which *Eco*KI is defective in methyltransferase activity (Fig. 6). Because DSBs cannot be repaired in a $recA^-$ strain (36), it would appear that in this $hsdR^+M$(F269G) S^+ bacterium DSBs are avoided, despite the presence of ≈600 unmodified targets and the coding potential for restriction-proficient, modification-deficient *Eco*KI. We conclude that ClpXP-dependent degradation of HsdR is able to prevent cutting of the bacterial chromosome. In the absence of ClpXP, however, even rec^+ cells fail to survive because *Eco*KI cuts their chromosomes.

If DSBs are not the stimulus for RA, why does a missense mutation in *hsdR* prevent degradation of HsdR? The amino acid substitution (A619V) is associated with a defect in the hydrolysis of ATP and probably, therefore, with the ATP-dependent translocation of DNA that precedes the generation of DSBs (18). The missense mutation does not prevent either the binding of *Eco*KI to its target sequence or the associated ATP-dependent conformational change that is a prerequisite for the restriction pathway (18, 37). Other missense mutations in HsdR also prevent degradation of HsdR (V.A.D. and N.E.M., unpublished observations); therefore, it seems probable that the functional defect, rather than the amino acid substitution *per se*, determines whether the enzyme is a substrate for ClpXP. We conclude that HsdR is recognized only after the *Eco*KI complex has embarked on its restriction pathway. It remains to be determined what renders the HsdR
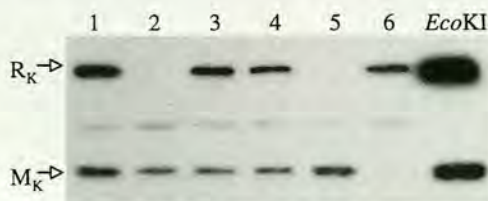

FIG. 7. The effect of *hsdM* (F269G) on the level of HsdR. The mutation *hsdM* (F269G) destroys only the modification activity of *Eco*KI. The level of HsdR was monitored by Western blots by using antibody against *Eco*KI. Lanes 1–6 include extracts of strains. Lanes: 1, NK301 (hsd^+); 2, NK386 [an *hsdM* (F269G) derivative of NK301]; 3, an hsd^+ derivative of NK386; 4, an *hsdR*(A619V) derivative of NK386 (NK388) in which alleles of *hsd* genes were replaced by using λ*hsd* phages that included only *hsdMS* or *hsdR*, respectively; 5, NM802 (an *hsdR* deletion strain); and 6, NK352 (an *hsdMS* deletion strain).

subunits susceptible to proteolysis. Nevertheless, the present experiments promote the concept of a remarkably specific control mechanism, effective only once the relevant restriction pathway has been initiated, but able to act before any damage is inflicted on unmodified chromosomal DNA.

The RA response can protect the bacterial chromosome from restriction in the complete absence of modification, but the alleviation is not entirely complete when analyzed by infection with λ.0 (EOP = 10^{-1}). These facts raise two new, but probably related, problems. First, why does phage DNA entering the cell show some susceptibility to restriction whereas the resident bacterial chromosome does not? Second, why do unmodified targets on the chromosome, but not those on incoming phage DNA, stimulate the RA response? At present, it should be borne in mind that the two substrates differ in their location and their association with other proteins.

Our current experiments document the disappearance of HsdR under conditions of RA, and we interpret this as ClpXP-dependent degradation of HsdR. Initial but unsuccessful attempts to detect degradation *in vitro* used purified HsdR, or *Eco*KI, as substrate. The *in vivo* experiments indicate that the substrate is unlikely to be protein alone but, rather, a functional protein–DNA complex.

The role of ClpXP in the disassembly and degradation of the Mu transposase already is known to be complex. MuB apparently protects the MuA–DNA complex from recognition by ClpX and, hence, from disassembly and potential degradation by the protease activity of ClpP (38). These authors suggested "that a protein–complex architecture that uses overlapping sequences for subunit interactions and for targeting a protein for remodeling or destruction provides a useful design for this type of regulation." By analogy we would suggest that some step in the ATP-dependent DNA translocation by *Eco*KI leads to the exposure of the target sequence for ClpX.

Our investigation of the relevance of ClpXP to RA has been confined to the type IA and IB families of R-M systems. There is evidence for Dam-mediated RA of a type III system (26). Members of the type IC and ID families are susceptible to RA in response to 2-AP (unpublished results), but transmission of the plasmid-borne type IC *hsd* genes by conjugation is not dependent on ClpXP (10, 32). Although the assembly pathway of the $R_2M_2S_1$ complex may provide a lag in the production of the endonuclease after plasmid transfer (39), it would not prevent the cutting of unmodified targets created in cells in which functional endonuclease is already assembled. It is not known whether RA can involve other proteases or other mechanisms, but RA is found for some methylation-dependent restriction systems (24), where DNA damage would not generate target sequences. RA has not been detected for any type II system; rather, RA appears to be characteristic of complex R-M systems.

Our experiments demonstrate that control of the restriction activity of *Eco*KI is extraordinarily sensitive. It not only copes with the acquisition of *hsd* genes conferring new specificities and the production of unmodified targets created by repair and mutation, but *clp*$^+$ cells also survive a mutation that destroys the modification activity of the R-M complex. A similar control system could permit the efficient phase variation of type I R-M systems, a phenomenon recently documented for *Mycoplasma pulmonis* (40). Molecular mechanisms of the sophisticated interactions that mediate the proteolytic control remain to be determined.

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The proteolytic control of restriction activity in *Escherichia coli* K-12

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Summary

The endonuclease activity of *Eco*KI is regulated by the ClpXP-dependent degradation of the subunit that is essential for restriction, but not modification. We monitored proteolysis in mutants blocked at different steps in the restriction pathway. Mutations that prevent DNA translocation render *Eco*KI refractory to proteolysis, whereas those that permit DNA translocation, but block endonuclease activity, do not. Although proteolysis alleviates restriction in a mutant that lacks modification activity, some restriction activity remains; our evidence indicates residual *Eco*KI associated with the membrane fraction. ClpXP protects the bacterial chromosome, but little effect was detected on unmodified foreign DNA within the cytoplasm of a restriction-proficient cell. The molecular basis for the distinction between unmodified resident and foreign DNA remains to be determined.

Introduction

Recent experiments have shown that the endonuclease activity of some restriction and modification (R–M) systems is regulated by proteolysis (Makovets *et al.*, 1998; 1999). This regulation of restriction activity becomes relevant to the survival of a restriction-proficient bacterium should its chromosome acquire unmodified target sequences. Previously, it was assumed that, in a restriction-proficient bacterium, the imprint provided by modification was essential to distinguish DNA in the bacterial chromosome from unmodified DNA entering the cell by phage infection or conjugation. Contrary to the classical view, modification of chromosomal DNA is not essential for its identification as 'self' rather than 'foreign'. For some, if not all, type I R–M systems, *Escherichia coli* has an additional, 'fail-safe' mechanism of protection against its own restriction system. This protection is relevant under a variety of conditions including those in which DNA damage, either as the result of

external agents or as the consequence of defects in DNA metabolism, generates unmodified target sequences in the bacterial chromosome (Makovets *et al.*, 1999).

The published data support a model in which one of the three subunits of a type I R–M system is the substrate for proteolysis by a cytoplasmic protease, ClpXP, before the completion of the complex reaction that leads to DNA breakage (Makovets *et al.*, 1999). Degradation of the subunit of an R–M complex that is essential for restriction, but not for modification, can result in a restriction-deficient, modification-proficient phenotype (r⁻m⁺). This phenotype was reported in response to treatment with UV light in the very first paper that identified a classical R–M system (Bertani and Weigle, 1953). More recently, the phenomenon has been referred to as restriction alleviation (RA) (Day, 1977; Kelleher and Raleigh, 1994). Usually, the alleviation is incomplete, and some residual restriction activity remains (Bertani and Weigle, 1953; Makovets *et al.*, 1999).

Type I R–M systems are encoded by three genes: *hsdR*, *hsdM* and *hsdS*. The three polypeptides, HsdR, HsdM and HsdS, often designated R, M and S, assemble to give an enzyme (R₂M₂S₁) that modifies hemi-methylated DNA and restricts foreign DNA if it includes unmethylated target sequences (for a recent review, see Murray, 2000). HsdS confers sequence specificity to the R–M complex. The HsdM subunit contains the active site for the methyltransferase activity, including the binding site for the cofactor AdoMet (Willcock *et al.*, 1994; for a review, see Dryden, 1999). HsdS and HsdM are sufficient for modification activity; restriction requires the additional subunit, HsdR, which includes an ATP binding site and other motifs essential for endonuclease activity (Murray *et al.*, 1993). The pathway that eventually leads to DNA breakage begins when an R–M complex binds to an unmodified target sequence in the presence of AdoMet and ATP (see Fig. 1). This pathway includes the translocation of DNA in an ATP-dependent process; the enzyme remains bound to its target sequence and moves the flanking DNA towards itself. DNA cutting is triggered when DNA translocation is impeded (Studier and Bandyopadhyay, 1988; Janscak *et al.*, 1999a; for reviews, see Murray, 2000; Szczelkun 2000). Mutations in *hsdR* are available that block the restriction pathway (Webb *et al.*, 1996; Davies *et al.*, 1998; 1999a,b). These mutations define the relevance of seven motifs, the so-called DEAD-box motifs characteristic of many DNA and RNA

1 Recognition of the target sequence

ATP + AdoMet
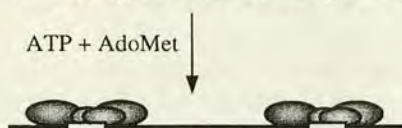
2 ATP-dependent DNA translocation

ATP

ADP +P$_i$
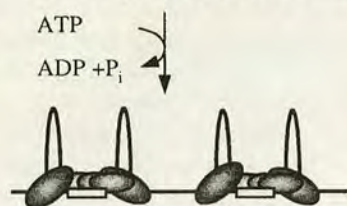
3 Double-strand break
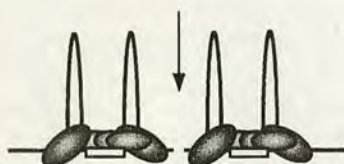
Fig. 1. The restriction pathway.
Step 1. The binding of AdoMet is blocked by the substitution in motif I of HsdM (G177D) but not those in motif IV.
Step 2. ATP-dependent translocation is prevented by substitutions in any of the seven DEAD-box motifs.
Step 3. DNA breakage but not DNA translocation is prevented by conservative mutations in the endonuclease motifs.

helicases, to the ATP-dependent translocation process (Davies *et al.*, 1999b). Conservative mutations that impair restriction but do not impair DNA translocation define an endonuclease motif (Davies *et al.*, 1999a,b; Janscak *et al.*, 1999b). A missense mutation that blocks DNA translocation was shown to prevent the proteolytic degradation of HsdR, and a model was proposed in which HsdR became susceptible to proteolysis as the result of a conformational change associated with DNA translocation (Makovets *et al.*, 1999). In support of this model, we show that substitutions in any of the motifs essential for DNA translocation prevent degradation of HsdR, whereas those that permit DNA translocation, but block endonuclease activity, do not prevent proteolysis of HsdR.

RA was shown to protect the bacterial chromosome from breakage in the absence of DNA modification, but RA was found to be incomplete when assayed by infection with unmodified phage λ. Why are most of these unmodified phage (90%) restricted, whereas the bacterium with approximately 600 unmodified targets in its chromosome survives? It was noted that these two DNA substrates differ both in their location and in their association with other proteins (Makovets *et al.*, 1999). In this paper, we provide biological and biochemical

evidence in support of the suggestion that the cellular location of the substrate DNA can influence its susceptibility to restriction. We find that, under conditions of RA, the residual restriction activity for *Eco*KI attacks phage λ but not M13 and that the residual R–M complex is recovered preferentially in the membrane fraction. We show, however, that foreign DNA within the cytoplasm remains susceptible to *Eco*KI even in the presence of ClpXP.

Results

Mutations that block methyltransferase activity

A mutation in *hsdM* that impaired the methyltransferase activity of a type I R–M enzyme was presumed to be lethal unless the mutation concomitantly abolished the endonuclease activity of the R–M complex. Mutations in the *hsdM* gene of *E. coli* K-12 have permitted a test of this prediction. Site-directed mutagenesis demonstrated the relevance of two conserved sequences within the active site for the methyltransferase activity of *Eco*KI (Willcock *et al.*, 1994). A change in the sequence predicted to be within motif I (G177D) abolishes the binding of AdoMet, whereas changes (F269G, F269C and N266D) within a motif previously called motif II, but now known as motif IV (Dryden, 1999), prevent catalysis but do not inhibit the binding of AdoMet. It is known that AdoMet is an essential cofactor for endonuclease activity (Meselson and Yuan, 1968); therefore, the amino acid change G177D should result in an inactive complex and an $r_K^- m_K^-$ phenotype (see Fig. 1). In contrast, changes in motif IV are anticipated to result in a restriction-proficient, modification-deficient ($r_K^+ m_K^-$) complex and, if this restriction-proficient complex were to break the unmodified bacterial chromosome, it would lead to cell death. We have previously provided indirect evidence that a mutation in motif IV (F269G) of the active site for methyltransferase activity does not block the endonuclease activity of the modification-deficient complex and that the mutant cell survives because the HsdR polypeptide of the functional complex is degraded by the ClpXP protease (Makovets *et al.*, 1999). Direct evidence from the following *in vivo* and *in vitro* experiments confirms that a modification-deficient *Eco*KI complex able to bind AdoMet retains endonuclease activity, whereas the complex that fails to bind AdoMet does not.

The *in vivo* tests for restriction used λ*hsdMS* phages in which the *hsdM* gene included a mutation that alters the sequence of either motif IV (e.g. F269G) or motif I (G177D). Restriction was monitored after the infection of bacteria that encode a functional *hsdR* gene in the absence of resident *hsdM* and *hsdS* genes. Infection leads to the expression of the phage-encoded *hsd* genes

and the consequent assembly of *Eco*KI complexes with defective M subunits ($R_2M_2^-S_1$). Lysates of the *hsdM*⁻ test phages were prepared on both modification-deficient and modification-proficient hosts, so that the effect of K-specific modification of the test phages could be assessed.

The data (Table 1) indicate that, when unmodified phages (.0) specify a methyltransferase-deficient enzyme able to bind AdoMet (e.g. λ1326 with a mutation in motif IV), they have a low efficiency of plating (e.o.p.) on an $r_K^- m_K^-$ strain in which a plasmid-borne *hsdR* gene is maintained; the e.o.p. is indistinguishable from that on C600, a standard restriction-proficient strain. We conclude that the resident HsdR subunit is assembled into an active $r_K^+ m_K^-$ complex after the expression of the *hsdM* and *hsdS* genes of the λ transducing phage and that the unmodified phage genomes are restricted. In contrast, the phage encoding a methyltransferase defective in its ability to bind AdoMet (λ1332) forms plaques with high efficiency in the presence of HsdR subunits. The survival of these unmodified phages is consistent with an *Eco*KI complex deficient in both restriction and modification. A control phage encoding a methyltransferase-proficient enzyme (λ1329) also forms plaques with high efficiency as its DNA will remain modified.

The experiments in Table 1 relied on the expression of *hsdR* from a multicopy plasmid in an *hsdM*⁻ *hsdS*⁻ strain. Earlier experiments had shown the need to enhance the level of HsdR to observe efficient restriction when the *hsdM* and *hsdS* genes were expressed from the p_L promoter of an incoming λ phage (Fuller-Pace *et al.*, 1985). More recently, it has been shown that the unmodified targets in the bacterial chromosome will induce restriction alleviation, i.e. the degradation of HsdR (Makovets *et al.*, 1999). The necessity for high levels of HsdR may reflect the large number of unmodified target sequences ($\approx$600) in the bacterial chromosome. When *Eco*KI binds to unmodified target sequences in the bacterial chromosome, ClpXP will degrade the HsdR subunits and deplete the restriction activity of the cell. In addition, the sensitivity of the present tests may be decreased by the replication of phage genomes. This would increase the chance that one or more phage genomes will escape restriction.

When the experiments used modified, rather than unmodified, λ*hsdM*⁻ S^+ phages (.K), an e.o.p. of $\approx$1 was obtained irrespective of which mutation was present in *hsdM* (data not shown). K-specific modification therefore protects phage genomes from the endonuclease activity produced within the infected cells. These experiments clearly show that *Eco*KI with a substitution in motif IV remains proficient in K-specific restriction, whereas that with a substitution in motif I is restriction deficient.

The modification complex (M_2S_1) purified from *hsdM* mutants conferring any of the following changes (F269G, F269C, N266D or G177D) was shown to be defective in methyltransferase activity (Willcock *et al.*, 1994). Protein purified from one mutant defective in motif I (G177D) and from one affecting motif IV (F269G) have been reinvestigated recently using the sensitive assay system for methyltransferase described by Roth and Jeltsch (2000); no methyltransferase activity was detected for either protein (M. O'Neill, personal communication). It is not possible to purify the R–M complex from the mutant with the F269G change in motif IV, as survival of the mutant bacteria depends upon the degradation of HsdR by ClpXP. It was necessary therefore to assemble *Eco*KI ($R_2M_2^-S_1$) *in vitro* from the modification complex and purified HsdR (Dryden *et al.*, 1997). Endonuclease activity of the assembled complexes was assayed using a plasmid substrate with a single target for *Eco*KI (Fig. 2). Enzyme with the F269G substitution in motif IV retained K-specific endonuclease activity, whereas that with the G177D substitution in motif I did not. In four out of five experiments, the wild-type enzyme was more proficient in the linearization of unmodified plasmid DNA than enzyme with the substitution in motif IV, but each of the experiments provides evidence that *Eco*KI with the F269G substitution retains K-specific endonuclease activity.

The *in vitro* assays endorse the conclusions deduced from biological tests of the *hsdM* mutations. In particular,

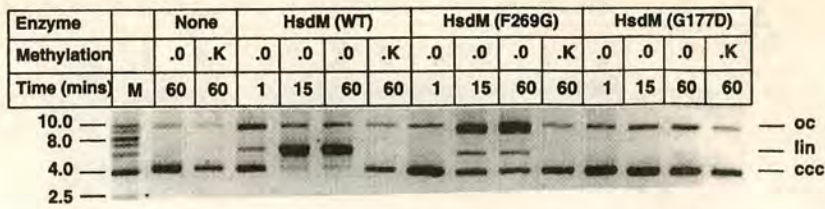**Table 1.** *In vivo* tests for restriction by *Eco*KI deficient in modification activity.

λ*hsdM*⁻ S^+ phage	Substitution specified by *hsdM*	Motif	AdoMet binding	E.o.p. on test strains[a,b] Δ*hsdRMS* + p*hsdR*[c]	hsd⁺ (*C600*)
λ1332.0	G177D	I	−	0.8	4×10^{-4}
λ1326.0	F269G	IV	+	4×10^{-4}	3×10^{-4}
λ1327.0	F269C	IV	+	9×10^{-5}	2×10^{-4}
λ1330.0	N266D	IV	+	5×10^{-4}	4×10^{-4}
λ1329.0	F269Y	IV	+	0.6	0.8[d]

a. Defined relative to the Δ*hsdRMS* strain NM679; the data are the average of three experiments.
b. The e.o.p. of modified phages was always close to 1 (data not shown).
c. pNK3, a derivative of pACYC184.
d. The substitution F269Y results in a wild-type phenotype and serves as a positive control.

Enzyme		None		HsdM (WT)				HsdM (F269G)				HsdM (G177D)			
Methylation		.0	.K	.0	.0	.0	.K	.0	.0	.0	.K	.0	.0	.0	.K
Time (mins)	M	60	60	1	15	60	60	1	15	60	60	1	15	60	60

Fig. 2. Endonuclease activity of modification-deficient *Eco*KI. The HsdM subunit of the modification enzyme used in the *in vitro* assembly of *Eco*KI is identified at the top. The substrate DNA was plasmid pBRsK1, a plasmid with one unmodified (.0) or modified *Eco*KI target (.K). The incubation times are indicated in minutes. M identifies marker DNAs from 10 to 2.5 kb in length. oc, open circular; lin, linear; ccc, covalently closed circular plasmid DNA.

a mutation (F269G) that blocks modification activity of the *Eco*KI complex is not lethal despite residual competence of the *Eco*KI complex in the restriction reaction.

Mutations in hsdR *that affect the degradation of HsdR*

Both ClpX and ClpP have been shown to be essential for the acquisition of genes specifying *Eco*KI and for the degradation of HsdR in response to 2-aminopurine (2-AP) (Makovets *et al.*, 1998; 1999). Data presented by Makovets *et al.* (1999) show that the transfer of *hsdR$^+$* to an *hsdR$^-$ hsdM* (F269G) *hsdS$^+$* recipient is lethal in the absence of ClpX (Makovets *et al.*, 1999). A similar result (data not shown) was obtained in the absence of ClpP.

The ClpXP-dependent degradation of HsdR associated with RA was prevented by a missense mutation in *hsdR*. Similarly, a wild-type HsdR polypeptide was shown to evade degradation in the absence of HsdM and HsdS. It was suggested that the substrate required for proteolysis was a functional *Eco*KI complex (Makovets *et al.*, 1999). The degradation of HsdR was first detected in response to treatment with 2-AP, but it was found to occur when modification activity was impaired by the F269G substitution in motif IV. The constitutive degradation of HsdR required for the viability of a restriction-proficient, modification-deficient bacterium (*hsdM* F269G), like the temporary RA after treatment with 2-AP, is prevented by a missense mutation in *hsdR* (Makovets *et al.*, 1999). The *hsdM* F269G mutation provides a convenient means of activating RA and is used here to investigate the properties of *Eco*KI required to make HsdR susceptible to proteolysis by ClpXP.

Recent analyses characterize two classes of *hsdR* mutants (Davies *et al.*, 1999b). In one, substitutions affecting any of the seven DEAD-box motifs identify sequences essential for the ATP-dependent translocation activity that precedes DNA breakage. In the other, mutations identify a motif essential for endonuclease activity; conservative substitutions in this motif impair endonuclease activity but are without effect on ATPase activity and DNA translocation (see Fig. 1).

The present experiments take advantage of the F269G substitution to provoke RA (Fig. 3A, lane 10) and monitor

the effects of amino acid substitutions in HsdR on the degradation of this polypeptide (Fig. 3A and B). The missense mutation analysed by Makovets *et al.* (1999) affects DEAD-box motif III (A619V). The experiments illustrated in Fig. 3A examine the effects of substitutions in each of the six previously untested DEAD-box motifs. The Western blots indicate that each 'DEAD-box mutation' (Fig. 3A, lanes 2–8) prevents the degradation of HsdR, the normal response (Fig. 3A, lane 10) to unmodified target sequences. The data from the DEAD-box mutants contrast with those for conservative mutations that impair endonuclease activity but leave the *Eco*KI complex with normal ATPase and translocation activities. The behaviour of one such mutant (D298E) in the absence and the presence of the *hsdM* mutation is shown (Fig. 3B, cf. lanes 2 and 3). The HsdR polypeptide, like that of *hsdR$^+$* (Fig. 3B, lane 9), remains susceptible to proteolysis. Similarly, a conservative change at position 312 (E312D) has no effect on the degradation of HsdR (data not shown). A less conservative change at position 312 (E312H), which results in an enzyme with much reduced ($\approx$10%) ATPase activity (Davies *et al.*, 1999b), was also examined. Some loss of HsdR is apparent (Fig. 3B, lanes 4 and 5); the effect of this substitution on proteolysis appears to be intermediate to that seen for the ATPase-proficient mutant in lane 3 and that seen for the ATPase-deficient DEAD-box mutant in lane 7. The endonuclease-deficient mutants (see Fig. 3B, lanes 2 and 4) produced normal levels of HsdR in the presence of a wild-type HsdM but, in the absence of methyltransferase activity, the HsdR polypeptide of a translocation-proficient complex was degraded. These experiments using the two classes of *hsdR* mutants show that the *Eco*KI complex becomes sensitive to ClpXP-dependent proteolysis before the final step in the restriction pathway (Fig. 1). The sensitivity to proteolysis correlates with the ability to hydrolyse ATP, consistent with a conformational change associated with DNA translocation.

The residual restriction activity is not effective against all foreign DNA

Under the conditions that result in RA, the bacterial

even in the absence of RecA-dependent DNA repair, an analogous mutation in the modification gene of a type II R–M system is predicted to lead to cell death, given that the concomitant loss of the restriction and modification genes of some type II R–M systems has been shown to lead to cell death (Kulakauskas *et al.*, 1995; Kusano *et al.*, 1995). Kobayashi and coworkers, in particular, have documented very fully the demise of bacteria when the type II R–M genes are lost (for a review, see Kobayashi, 1998). Under these conditions, residual endonuclease activity attacks the bacterial chromosome (Handa *et al.*, 2000). Methyltransferase-deficient mutants have been isolated for the *Bcg*I system, an atypical R–M system of *Bacillus coagulans* that shares characteristics with both type I and type II restriction systems. The complex *Bcg*I enzyme, like *Eco*KI, requires AdoMet for both its modification and its restriction activities (Kong and Smith, 1997). A mutation affecting motif IV in the active site for methyltransferase activity blocks enzymatic activity but permits AdoMet binding and generates an enzyme that is proficient in restriction and defective in modification. Under the conditions of the reported *in vivo* experiments, in which the genes for the *Bcg*I system are present in plasmids maintained in *E. coli*, the mutation conferring a defect in modification, in contrast to that obtained in the *hsdM* gene of *E. coli* K-12, confers a lethal phenotype (Kong, 1998). *Bcg*I, like other type II R–M systems, may not be susceptible to RA.

Analyses of RA have focused mainly on systems in *E. coli*, and therefore any generalizations must be made with caution. Nevertheless, it is tempting to speculate that RA is associated with R–M systems in which DNA translocation is an integral part of the restriction pathway. This correlation with DNA translocation is consistent with the present analyses of mutations in *hsdR* that interfere with the restriction reaction. Our data suggest that the HsdR subunit of *Eco*KI becomes susceptible to ClpXP after the conformational change that is dependent on the cofactor ATP, as all the mutations in *hsdR* that prevent DNA translocation permit this conformational change (Davies *et al.*, 1998; 1999b), yet HsdR is not degraded in any of these mutants. HsdR is degraded in restriction-deficient mutants that permit ATP-dependent DNA translocation. Our present results are consistent with the idea that exposure of the target sequence within HsdR requires the hydrolysis of ATP, or even ATP-dependent DNA translocation. Irrespective of which steps in the restriction pathway are necessary for HsdR to become sensitive to ClpXP, the mechanism by which restriction activity is controlled requires the enzyme to bind to unmodified target sequences and initiate the restriction pathway. The remarkable effectiveness of RA in the preservation of an unmodified bacterial chromosome indicates that active enzyme is extinguished efficiently before its activity

culminates in DNA breakage. The complex restriction pathway of type I systems provides an opportunity for control that is absent for simpler restriction endonucleases. Our current understanding of the control process does not necessarily explain the low level of HsdR in cells under conditions of RA. The residual level of HsdR in cells treated with 2-AP is influenced by growth rate; conditions of very active growth enhance the depletion of HsdR (Makovets *et al.*, 1999). Under these conditions, replication and mutagenesis will be enhanced.

A critical question that remains to be answered is why the translocating complexes of *Eco*KI are attacked by ClpXP when the unmodified target sequences are in the bacterial chromosome, but not when they are within an infecting λ genome. It was noted by Makovets *et al.* (1999) that the two DNA substrates differ in both their location and their association with other proteins. Indirect evidence for 'surface-localized' restriction activity was presented many years ago (Schell and Glover, 1966). Recent direct assays for the subunits of *Eco*KI have been reported to support this idea (Holubová *et al.*, 2000). These authors present evidence that *Eco*KI is associated with the inner membrane, and they suggest that this correlates with the restriction activity that remains under conditions of RA. Our experiments used a different fractionation procedure from that of Holubová *et al.* (2000), one that includes a DNase and RNase treatment at an early step (de Maagd and Lugstenberg, 1986). In these experiments (Fig. 4), *Eco*KI was detected in both the cytoplasmic and the membrane fractions of wild-type cells but was preferentially retained in the membrane fraction under the conditions of restriction alleviation. This retention of HsdR in the membrane fraction is consistent with the residual restriction activity detected when double-stranded, but not single-stranded, phage genomes enter the cell under conditions of RA (Table 2).

The preferential depletion of HsdR from the cytoplasmic fraction under conditions of RA may explain the residual restriction activity of the modification-deficient (*hsdM* F269G) strain, but it does not provide an understanding of the basic mechanism of how the bacterial chromosome is distinguished from foreign DNA in the absence of the imprint of modification. In the infection experiments using λ*hsd* phages, the production of *Eco*KI is dependent upon transcription of the phages. Yet, in these infections, unmodified phage genomes are susceptible to *Eco*KI in the presence of ClpXP (Tables 1 and 3) as well as in its absence (Table 3). In contrast, even modified λ*hsd* phages have a low e.o.p. in the absence of ClpXP if the host chromosome is unmodified (Table 3). The degradation of the bacterial chromosome and consequent loss of bacterial functions appears to impair the propagation of phages.

The experiments with λ*hsd* phages (Table 3) imply that

unmodified phage genomes are sensitive to restriction when they are replicating within a restriction-proficient Clp$^+$ cell. Apparently, ClpXP alleviates restriction of the bacterial chromosome but fails to protect λ DNA in the cytoplasm (Table 3). Similarly, the Clp phenotype has no or very little effect on the e.o.p. of unmodified M13 phages or on the transfer of unmodified plasmid DNA by conjugation. In both these examples, the DNA enters in a single-stranded form and must be replicated before it becomes a target for *Eco*KI. The double-stranded DNA that is made after the transfer of unmodified chromosomal DNA by conjugation was shown to be sensitive to restriction (Boyer, 1964), but Glover and Colson (1965) noted a drop in the restriction proficiency of the recipient cell 30–60 min after the initiation of DNA transfer. Our preliminary evidence shows that this alleviation of restriction is ClpX dependent. What is the stimulus for RA after conjugation? Does it depend on the incorporation of unmodified DNA into the recipient chromosome? If so, unmodified targets in the DNA fragments would become protected from restriction after their assimilation into the bacterial genome. For type I, in contrast to type II, R–M systems, the DNA fragments produced by restriction will generally include unmodified targets. The nature of the nucleoprotein complex in the bacterial chromosome could affect the efficiency of DNA translocation and the accessibility of HsdR to ClpXP. Attempts to mimic RA *in vitro* have failed (L. M. Powell, personal communication). This may indicate that it has not yet been possible to simulate the appropriate protein–DNA substrate *in vitro*. Currently, we conclude that unmodified DNA target sequences within the nucleoid are distinguished from those in newly acquired phage or plasmid genomes, but the molecular basis for this distinction remains to be determined.

Experimental procedures

Bacterial strains

These are listed in Table 4.

Phages and plasmids

λ*hsdM*⁻*S*⁺ *imm*^{21} phages (NM1326, NM1327, NM1329, NM1330 and NM1332) reported by Willcock *et al.* (1994) include mutations that impair methylation (see Table 1). λNM1394, a *cl*857 derivative of an integration-deficient λ*hsdM*(F269G)*S*⁺ phage, was used to transfer the *hsdM* mutation to the chromosome of *hsdR* strains (Makovets *et al.*, 1999). λ*hsdR*⁺*M*⁺*S*⁻ (NM1128) and λ*hsdR*⁺*M*⁺*S*⁺ (NM1129) are isogenic derivatives isolated after the lysogenization of the *hsdS* strain WA803 with λNM1050 (Sain and Murray, 1980).

The standard phage for monitoring restriction was λ*vir*, but others with fewer *Eco*KI targets were the lambdoid phages, NM150 (*h*^{82} *imm*^{λ} with four targets), NM106 (phage 82 with two targets), NM105 (*h*^{80} *imm*^{λ} with two targets) (Murray *et al*, 1973), phage T7 D104 Δ1.3–7.2 with four targets (Garcia and Molineux, 1999) and mpAT38, a derivative of mp18 that includes a DNA fragment shown by sequence analysis to include two *Eco*KI targets (A. J. B. Titheradge, personal communication).

pNK3 is a derivative of pACYC184 including the *hsdR* gene of *E. coli* K-12 (Makovets *et al.*, 1999). pBRsK1 is a derivative of pBR322 with one target for *Eco*KI (G. P. Davies, personal communication). pOX38Km is a derivative of the F factor Chandler and Gallis (1985).

Microbial methods

Media and general methods have been described previously (Makovets *et al.*, 1998). P1 transductions were performed according to the methods of Miller (1972). Integration-deficient λ*hsdcl*857 phages were used to transfer *hsd* alleles to bacterial chromosomes. Lysogens were selected as immune colonies at 32°C using λ*b2cl*⁻ and *h*^{82} *b522-cl*⁻ phages, and cured derivatives were selected at 42°C. Tests for restriction alleviation in response to 2-AP were determined as described by Makovets *et al.* (1999).

Analysis of proteins

Components of *Eco*KI were purified and analysed as described by Dryden *et al.* (1997). Polypeptides were separated by electrophoresis through 7.5% SDS–polyacrylamide gels (Laemmli, 1970). Protein concentrations were estimated by a modification of the Lowry method (Peterson, 1979). Western blots used rabbit antisera against *Eco*KI and the chemiluminescence detection system (POD) from Roche.

Subcellular fractionation and enzyme assays

Late logarithmic phase cells (an OD of 0.6 at A_{600}) were harvested and washed in 0.9% NaCl. All subsequent steps were carried out at 4°C. Cells were converted to spheroplasts by lysozyme–EDTA treatment as described by de Maagd and Lugtenberg (1986). Conversion to spheroplasts was monitored by phase-contrast microscopy. Spheroplasts were frozen at −70°C. Cells were disrupted by repeated freezing and thawing in the presence of *p*-phenylsulphonylfluoride (Sigma) to inhibit proteases, and the debris was then removed by centrifugation at 10 000 *g* for 1 h. The supernatant was subjected to centrifugation (60 000 *g*) for 1 h to separate the membrane (pellet) and cytoplasmic (supernatant) fractions. After washing with 10 mM Tris-HCl (pH 8.0), the membrane fraction was resuspended in buffer (10 mM Tris-HCl, pH 8.0, 10 mM dithiothreitol, *p*-phenylsulphonylfluoride) and applied to a Qiagen DNA-binding column before clarification by centrifugation. Beta-galactosidase activity was assayed as described by Miller (1972), using the substrate *o*-nitrophenol-β-galactoside. NADH oxidase activity was assayed as described by Osborn *et al.* (1972), using the substrate β-nicotinamide adenine dinucleotide, reduced form (NADH).

Table 4. Bacterial strains.

E. coli K-12 strains	Relevant genotype or phenotype	Reference
C600	hsd_k	Makovets et al. (1999)
WA803	hsdS	Wood (1966)
NM679	ΔhsdRMS	King and Murray (1995)
NK301	rac⁻ gyrA lacY thr leu thi	Makovets et al. (1999)
NK303[a]	clpP::cat	Makovets et al. (1999)
NK304[a]	clpX::kan	Makovets et al. (1999)
NK308[a]	recA::cat	Makovets et al. (1999)
NK311[a]	Δhsd	Makovets et al. (1999)
NK312[a]	Δhsd clpX::kan	Makovets et al. (1999)
NK379[a]	ΔhsdR	Makovets et al. (1999)
NK380[a]	ΔhsdR clpX::kan	Makovets et al. (1999)
NK386	hsdM(F269G)	Makovets et al. (1999)
VC301[a]	lac⁺	This study
VC3017[a]	lac⁺ hsdM(F269G)	This study
AB1157	hsd_k⁺ rpsL	Bachmann (1972)
VC1[b]	hsdM(F269G)	This study
VC797[b]	hsdR(H577D) hsdM(F269G)	This study
VC799[b]	hsdR(A619V) hsdM(F269G)	This study
VC801[b]	hsdR(K477R) hsdM(F269G)	This study
VC892[b]	hsdR(G177C) hsdM(F269G)	This study
VC893[b]	hsdR(D502Y) hsdM(F269G)	This study
VC898[b]	hsdR(R826H) hsdM(F269G)	This study
NM904[b]	hsdR(D298E)	This study
VC904[b]	hsdR(D298E) hsdM(F269G)	This study
NM908[b]	hsdR(E312H)	This study
VC908[b]	hsdR(E312H) hsdM(F269G)	This study
NM890[b]	hsdR(F629Y)	This study
VC890[b]	hsdR(F629Y) hsdM(F269G)	This study

a. Derivatives of NK301.
b. Derivatives AB1157.

Note added in proof

We find that the restriction of λhsdM(F269G)S⁺ in Clp⁺ Δ(hsdRMS) bacteria carrying pNK3 (i.e. hsdR⁺) is strain dependent (see Table 1) In contrast, the e.o.p. of λhsdM(F269G)S⁺ is low on every clpXΔ(hsdRMS) pNK3 strain we have tested, irrespective of whether the phage is modified or unmodified.

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