

The Effect of *recA* Mutation on the Expression of *EcoKI* and *EcoR124I hsd* Genes Cloned in the Multicopy Plasmid

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ABSTRACT. Type I restriction-modification (R-M) endonucleases are composed of three subunits - HsdR, required for restriction, HsdM and HsdS, which can produce a separate DNA methyltransferase, and of which HsdS is required for DNA recognition. In this paper we describe the effect of the cloned *EcoKI* and *EcoR124I* *hsd* genes on the resulting R-M phenotype. The variability in the expression of the wild type (w.t.) restriction phenotype after the cloning of the w.t. *hsd* genes in the multicopy plasmid in *Escherichia coli* *recA*⁺ background suggests that the increased production of the restriction endonuclease from pBR322 is detrimental to the cell and this leads to the deletion of the cloned *hsd* genes from the hybrid plasmid and/or inactivation of the enzyme. The effect of a mutation in *E. coli* *recA* gene on the expression of R-M phenotype is described and discussed in relation to the role of the cell surface and the localization of the restriction endonuclease in the cell.

The type I R-M systems are the most complex so far discovered. The main enzymes are the three-subunit proteins containing the products of the *hsdR*, *hsdM* and *hsdS* genes. *R.EcoKI* and *R.EcoR124I* are the members of the type IA and IC groups of restriction and modification enzymes (for reviews see Wilson and Murray 1991, Bickle and Krüger 1993, Redashi and Bickle 1996). These multifunctional enzymes are able both to cleave and methylate double-stranded DNA, and are potentially important models for studying protein-DNA and protein-protein interactions. Genetic complementation tests have shown that the HsdS polypeptide dictates DNA sequence specificity (Boyer and Roulland-Dussoix 1969, Glover and Colson 1969, van Pel and Colson 1974). The products of all three genes are required for restriction (Hubáček and Glover 1970), whilst for modification, the products of the *hsdS* and *hsdM* genes are sufficient (Boyer and Roulland-Dussoix 1969, Glover and

Colson 1969, Hubáček and Glover 1970). The gene order is *hsdR*, *M*, *S*, the *hsdR* gene has its own promoter P_{res} and the *hsdM* and *hsdS* genes are transcribed from a single promoter P_{mod} situated between the *hsdR* and *hsdM* genes (Gough and Murray 1983).

The temperature-sensitive (ts) mutations in chromosomal *EcoKI hsdM* and *hsdS* genes were found to be determined by single-base pair transitions in DNA sequence resulting in single amino acid changes in the critical regions of the domains important for enzyme assembly or interaction of the enzyme with DNA target site (Hubáček and Glover 1970, Zinkevich et al. 1990, Weiserová et al. 1993). However, temperature-sensitive restriction phenotype is not expressed when the *EcoKI hsdS_{ts-1}*, *R* and *M* genes are cloned in multicopy plasmid (Weiserová et al. 1993, 1994).

We also published previously that the ability to isolate temperature-sensitive mutations of *EcoR124I* depends on the physiological state of the bacteria. Long term storage at 4°C or growth in the presence of nonmutagenic solvent dimethyl sulfoxide (Me₂SO) as well as ethyl methanesulfonate treatment, results in a transient state of restriction-deficiency. However the genotype of such cells is not altered. This phenomenon hampered the isolation of mutations by conventional means. These observations with *EcoR124I* are in contrast to the situation with *EcoKI*, where Me₂SO treatment or low temperature have no effect on the R-M phenotype. (Hubáček et al., 1994).

We attempted, therefore, to isolate ts mutants in the *E. coli* K strain with the deleted w.t. chromosomal *hsd* operon but reintroduced on a multicopy plasmid. We expected that if we succeeded in the isolation of the ts mutations in the *hsd* genes located directly on the multicopy plasmid they would differ in the molecular mechanism of the mutation and the expression of the R-M phenotype. The results obtained in *EcoKI* R-M system were compared with that obtained with *EcoR124I hsd* genes cloned in multicopy plasmid.

MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages. The *E. coli* strains and plasmids used in this study are described in Table I. The *E. coli* QR47-3 Δ (*hsdR-S*) strain was prepared by two-step P1-mediated cotransduction of QR47-1 *thr* strain using phage lysates of *E. coli* Cb *serB* (P1) in the first step and of *E. coli* C Δ (*hsdR-S*) P1 lysogen in the second step. Recombinant plasmids used in this work are derivatives of the vector pBR322 (Bolivar et al. 1977) or pACYC184 (Chang and Cohen 1978). The virulent mutant of phage λ (Jacob and Wollman 1954) was used for the *in vivo* testing of restriction and modification. P1 lysates were prepared by thermal induction of lysogens with the thermoinducible mutant P1CM*chl*100 phage (Rosner 1972).

Media and microbial methods. Phage buffer, complex LB medium, M9 medium, L-amino acids, vitamin supplements were as described by Hubáček and Glover (1970). The solid medium is LB or M9 with agar added at 1.5 %. Soft agar overlay is LB with agar added at 0.6 %. Ampicillin and chloramphenicol were used at a concentration of 100 μ g/ml and 30 μ g/ml, respectively. Preparation of F' plasmid and the construction of F' merodiploid were described previously (Hubáček and Glover, 1970). Transduction was done according to Rosner (1972). Transformations were carried out as described by Maniatis et al. (1982).

Tests for restriction and modification. Cultures were grown overnight in LB at 30 °C and 42 °C, or in the case of strains harbouring F' plasmid in M9 medium. Spot tests were as those described in Colson et al. (1965). Restriction was measured as described by Hubáček and Glover (1970). Modification specificity K was estimated as the EOP of phage λ produced by the tested strain and measured on *E.coli*.K(C600) and C indicator strains. Modification specificity R124 indicates the EOP of phage λ produced by the tested strain measured on *E.coli* C[pCP10005] and *E.coli* C indicator strains.

DNA for ethidium bromide - CsCl equilibrium density gradient centrifugation and for rapid small scale isolation was prepared by the alkaline lysis method (Birnboim and Doly 1979). Restriction enzyme digest, ligations, gel electrophoresis and elution of DNA fragments from agarose gels by direct extraction were performed as described by Maniatis et al. (1982).

Construction of hybrid plasmids. (i) pVM27. The *Hind*III fragment from pBg3 isolated from an agarose gel was further digested with *Sma*I, the *Hind*III cohesive end was filled in using the Klenow fragment of *E. coli* DNA polymerase I. The small fragment with *hsdM* gene was ligated to pACYC184 cut at the *Hind*III site and blunt ends were produced by treatment with the Klenow fragment. (ii) pACWR93. The large *Hind*III - *Sma*I fragment from pBg3 carrying the *hsdR* gene was ligated to pACYC184 opened in sites *Hind*III and *Eco*RV. (iii) pACYCW1 and pACYCX3. The *Hind*III fragment from pCP1005 carrying the entire *R.Eco*R124I *hsd* operon or the deleted *Hind*III - X fragment, respectively, were cloned in *Hind*III site of pACYC184.

RESULTS

Temperature-sensitivity of *Eco*KI restriction phenotypes in *E. coli* transformed with multicopy hybrid plasmid

In our previous work we described the phenotypic variability of *Eco*K R-M system when expressed from multicopy plasmid pVMC3 in *E. coli* C strain with spontaneously deleted *hsd* region (Weiserová et al. 1994). Therefore in this study we decided to use *E. coli* K strain, which is the natural host of *Eco*KI R-M system. For this purpose the chromosomal *hsd* genes had to be removed. This was achieved by introduction of the *hsdRMS* deletion from *E. coli* C into *E. coli* K (QR47) using two - step P1- mediated transduction. The recombinant strain QR47-3 was transformed with plasmid pVMC3 carrying the w.t. *hsd* operon cloned in pBR322. The following R-M phenotypes were observed among Ap^R transformants: R⁺

phenotype, E.O.P. of $\lambda vir.0$ was 0.0002-0.0003 at 30 °C with one order of magnitude lower at 42 °C (the representative colony in Table 2, lines 1 and 2), R^+_{ts} phenotype, E.O.P. of $\lambda vir.0$ in individual colonies was 0.01-0.0001 at 30 °C with two to three orders of magnitude lower at 42 °C (the representative colony in Table 2, lines 3 and 4), and R^- phenotype, E.O.P. of $\lambda vir.0$ about 1.0 at both temperatures (the representative colony in Table 2, lines 5 and 6). The structurally unchanged pVMC3 plasmid DNA was detected in a cross-section of phenotypes tested with the exception of two R^- colonies in which the *HindIII* fragment carrying the *hsd* genes was lost (data not shown). This indicates that the phenotypic heterogeneity is not caused by genetic instability of cloned fragment in *recA*⁺ host cells, nevertheless this fact made the mutagenesis of cloned *hsd* genes impossible.

Complementation analysis of the $R^+_{ts}M^+$ phenotype confirmed that the *hsdR*, *M* and *S* genes were unchanged, as after transformation of QR47-3 (pVMC3) $R^+_{ts}M^+$ clone with the compatible plasmid pACWR93 (pACYC184 carrying *hsdR_K*), pVM27(pACYC184 carrying *hsdM_K*) or after infection with the F⁻-47 *hsdS*, *hsdM* plasmid, the restriction and modification phenotypes of the cells remained $R^+_{ts}M^+$.

In some cases the $R^+_{ts}M^+$ phenotype spontaneously changed to $R^-M^+_{ts}$ phenotype (Table 2, lines 7 and 8). The $R^-M^+_{ts}$ colonies were also observed when the pVMC3-DNA, isolated from the QR47-3 (pVMC3) $R^+_{ts}M^+$ strain was transformed into *E. coli* *recA* (C3-6) strain; 50 % of tested transformants were of R^+M^+ phenotype and 50 % were $R^-M^+_{ts}$.

To investigate the influence of RecA protein on the expression of R-M phenotype, the *recA* mutation was introduced directly into $R^+_{ts}M^+$ QR47-3 (pVMC3) clone (Table 2, lines 3 and 4). The strain was made *thy*⁻ and conjugated for 30 min in interrupted mating with HfrJC 5088 strain to select *Thy*⁺*RecA*⁻ recombinants. From 18 colonies of recombinants tested 4 colonies were *recA*⁻. 14 colonies *RecA*⁺ were of the original $R^+_{ts}M^+$ phenotype but the *recA*⁻ colonies were of a stable R^+ phenotype and

exhibited an increased level of restriction (Table 2, lines 9 and 10) compared to the E.O.P. of phage $\lambda_{vir.0}$ on standard $R^+_{\kappa}M^+_{\kappa}$ *E. coli* strain.

The effect of *recA* mutation on the expression of R124 restriction and the stability of hybrid plasmid in the presence of dimethyl sulfoxide

The effect of *recA* mutation on the expression of *EcoKI* restriction phenotype together with the fact that *recA* mutation changes the composition of the cell membrane (Inouye 1971), turned our attention to study the role which the localization of restriction endonuclease (in the cell surface) could play in the expression of the restriction function using the dipolar aprotic surfactant Me_2SO .

E. coli C strain transformed with multicopy plasmid pCP1005, carrying the w.t. *hsd* gene coding for *EcoR124I* R-M system was used. These transformants were found to be temperature-sensitive in restriction (the E.O.P. of unmodified phage λ was 0.000003 at 30°C and 0.1 at 42°C) or shifted from R^+ to R^- phenotype after several week storage at 4°C, but after special treatment reverted back to the restriction-proficient phenotype (Hubacek et al. 1994). After cultivation in M9 synthetic medium in the presence of Me_2SO up to 3 % (V/V) concentration of Me_2SO the low efficiency of $\lambda_{vir.K}$ plating is not changed, in the range of 3-5 % plating ability of $\lambda_{vir.K}$ increases about three orders of magnitude, however, all the cells in the population are ampicillin-resistant and carry plasmid pCP1005 (Fig. 1, compare curves 1 and 4). At the concentration of 5-7 % of Me_2SO the E.O.P. of $\lambda_{vir.K}$ increases further from 10^{-4} to 1.0 and the number of Δp^R colonies decreases: approximately only 1.0 % and 0.01 % of cells, respectively, retained the plasmid and remained $\Delta p^R Me_2SO^R$ (Fig. 1, curve 4).

The plasmid DNA isolated from these colonies was designated as pCP1005-X DNA and analyzed further. After transformation into *E. coli* JM109(DE3) strain the transformants were found to be $R^-_{124} M^-_{124}$ (Table 3, line 1). The *HindIII* digestion revealed that pCP1005-

X DNA consists of the fragment corresponding to the plasmid vector (2.8 kb) and 3.8 kb segment of the originally cloned 14.1 kb *Hind*III fragment. For complementation analysis the deleted fragment X has been cloned into plasmid pACYC184 compatible with plasmids carrying the individual *hsd* genes under the control of T7 gene 10 promoter. Fragment 3.8 kb, designated as *Hind*III-X DNA, was cloned in pACYC184 (plasmid called pACYCX3) and transformed into *E. coli* JM109(DE3). The transformants were found to be R⁻₁₂₄M⁻₁₂₄ (Table 3, line 2).

Introduction of *hsd*S_{PT7} on pJS491 plasmid does not complement either restriction or modification (Table 3, line 3). Only M⁺₁₂₄ phenotype (Table 3, line 4) was detected when the plasmid pJS4M with the *hsd*S_{PT7} and *hsd*M_{PT7} genes was transformed into *E. coli* JM109 (DE3), [pMW50], which indicates that the expression of w.t. methylase does not complement the restriction function.

The R₁₂₄ phenotype is sensitive to Me₂SO only when the *Eco*R124I *hsd* genes are cloned in multicopy plasmid pCP1005 and the plasmid is transformed into *E. coli* *recA*⁺ strain. The R₁₂₄ phenotype is less sensitive when the *hsd* genes cloned in multicopy plasmid are transferred into *E. coli* C carrying *recA* mutation (Fig. 1, compare the curves 1 and 3) and is not sensitive at all when the cell carries the natural plasmid R124 (Fig. 1, curve 2) or when the *Hind*III fragment with the *Eco*R124 *hsd* genes is cloned in the low-copy number plasmid pACYC184 (plasmid pACYCW1) (Fig. 1, curve 5).

In conclusion, the R₁₂₄ phenotype is sensitive to Me₂SO in concentration 3-5 % and the cells, carrying the hybrid multicopy plasmid, become restriction-less. At the concentrations of this solvent higher than 5% either the whole plasmid is eliminated or at least the *hsd* operon is deleted. The sensitivity of R₁₂₄ phenotype is dependent on the number of copies of *hsd* genes in the cell and the *recA* mutation.

DISCUSSION

The ability of *E. coli* and *Salmonella* strains to restrict unmodified phages was found to be heat-sensitive when the bacteria were heated before infection for a few minutes at temperatures up to 44 °C. The protection against the effects of heat obtained in hypertonic media was explained to be due to plasmolysis of the cells which prevents the release and inactivation of the cell surface-localized restriction enzyme (Schell and Glover 1966). In our experiments the clones with the temperature-sensitive R-M phenotype were obtained even at 42 °C after the cloning of the w.t. *EcoK hsdR, M* and *S* complex gene in multicopy plasmid. The complementation experiments have not shown any change in the *hsd* complex gene. The important role of a mutation in *recA* gene on the efficiency of restriction was observed under these conditions.

RecA protein protects unmodified DNA in the process of *EcoKI* restriction when the *recA* gene is cloned in pBR322 (Koukalová et al. 1985). This protein could play a role in the repair of damaged DNA and protect it from degradation. The protection by RecA protein against the action of RecBC deoxyribonuclease, after a double-strand break has occurred, has been discussed (Williams et al. 1981). However, the *recA* and *recB* mutant strains are not more sensitive to *EcoRI* scissions and it was shown that although these double-strand breaks induce the SOS response, neither this induction nor recombination are required for repair (Heitman et al. 1989). It was further demonstrated that the *E. coli*K strain with the chromosomal *recA* mutation has a level of restriction comparable with that of the original *recA*⁺ strain and 29 % of clones tested showed rather a tendency to reduce the restriction of nonmodified phage λ (Koukalová et al. 1985). The role of single-stranded DNA binding protein, which could substitute for the RecA protein in the protection of gapped DNA against the action of some nucleases, could explain this phenomenon (Lieberman and Witkin 1983).

In our experiments the introduction of chromosomal *recA* mutation increases the level of *EcoKI* restriction at 30 °C and especially at 42 °C, so that the temperature-sensitive restriction

phenotype is lost (Table 2). The increased stability of the hybrid plasmid DNA in *recA* background plays an important role in the increased level of restriction. The structural instability of endonuclease plasmid and hereby the phenotypic variability is not only the problem of *recA*-dependent recombination (Weiserová et al. 1993, 1994). The mechanism by which the RecA protein protects unmodified DNA in the process of restriction seems to be more complicated. We have observed the temperature-sensitive restriction phenotype also in *E. coli*C(pCP1005) carrying the high copy number plasmid with the cloned *EcoR124I hsd* w.t. genes. We described the restriction deficiency in this strain after the exposition to nonmutagenic solvent Me₂SO, which is caused by some kind of inactivation of R.*EcoR124I* restriction enzyme at the lower concentration of Me₂SO (up to 5 % V/V) and elimination of hybrid plasmid or deletion of *hsd* operon at the higher concentration of Me₂SO. The mechanism of action of this compound is related to the phospholipid composition and the organization of the cell membrane (Tapiero et al. 1983). The effect of Me₂SO on restriction in *E. coli*C(pCP1005) was not so expressive in *E. coli*C(pCP1005) strain with a mutation in *recA* gene (Fig. 1, curve 3). This could indicate that the changes in the composition of the cell membrane, related to the effect of *recA* mutation (Inouye 1971), could stabilize and activate the cell surface-localized (membrane-bound) restriction endonuclease and thus affect the expression of R-M phenotype *in vivo*.

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Table I. Bacterial strains and plasmids used

Bacterial strains and plasmids	Genotype	Restriction phenotype	Reference
Escherichia coli			
C	prototroph $\Delta(\text{hsdR-S})$	R^-M^-	Bertani and Weigle 1953
Cb	$\text{serB } \Delta(\text{hsdR-S})$	R^-M^-	Hubáček 1973
C3-6	recA56 derivative of E. coliC	R^-M^-	Weiserová et al. 1993
C600	thr leu thi	R^+M^+	Appleyard 1954
QR47	prototroph	R^+M^+	Zinkevich et al. 1992
QR47-1	lac thr	R^+M^+	EMS-mutagenesis of QR47
QR47-2	lac serB	R^+M^+	P1-transduction of QR47-1
QR47-3	$\text{lac } \Delta(\text{hsd-R-S})$	R^-M^-	P1-transduction of QR47-2
JM109(DE3)	$\text{F}' \text{ tra}\Delta 36, \text{ lacI}, \Delta(\text{lacZ})\text{M15}$ $\text{proAB}/\text{recA1}, \text{ endA1},$ $\text{gyrA96 (Nal}^{\text{R}}), \text{ hsdR17},$ $\text{mcrA}, \text{ relA1}, \text{ supE}, \text{ sbcBC},$ $\text{thi-1}, \Delta(\text{lac-proAB}) \lambda(\Delta\text{E3})$		Promega
HfrH47	thi hsdS hsdM	R^-M^+	Hubáček 1973
HfrJC5088	thr ilv recA56	R^+M^+	Hubáček and Weiserová 1980
Plasmids			
pBg3	hsdM hsdR		Sain and Murray 1980

pVMC3	hsdS _K hsdM hsdR on pBR322	Weiserová et al. 1993
pCP1005	hsdS ₁₂₄ hsdM hsdR on pUR51	Firman et al. 1985
F'-47	hsdS hsdM	this paper
pVM27	hsdM on pACYC184	this paper
pACWR93	hsdR on pACYC184	this paper
pACYCW1	hsdS ₁₂₄ hsdM hsdR on pACYC 184	this paper
pACYCX3	HindIII-X fragment on pACYC184	this paper
pJS491	hsdS _{P17}	Patel et al. 1992
pJS4M	hsdS _{P17} hsdM _{P17}	Patel et al. 1992
pACYC184	Cm ^R , Tc ^R , ori p15A	Chang and Cohen 1978
R124	Tc ^R , IncFIV, hsdM,S,R	Hedges and Datta 1972

Table II. The diapazon of R-M phenotypes in the population of *E. coli* QR4-3 transformants with hybrid plasmid DNA^a

Line	<i>E. coli</i> strain	Temperature °C	Restriction	Modification	R _K -M _K phenotype
1	QR47-3(pVMC3)	30	0.0002	0.7	R ⁺ M ⁺
2	..	42	0.001	0.8	R ⁺ M ⁺
3	..	30	0.0005	0.7	R ⁺ M ⁺
4	..	42	0.5	0.7	R ⁻ M ⁺
5	..	30	0.8	0.8	R ⁻ M ⁺
6	..	42	0.9	1.0	R ⁻ M ⁺
7	..	30	1.0	0.4	R ⁻ M ⁺
8	..	42	1.0	0.003	R ⁻ M ⁻
9	QR47-3 (pVMC3) recA ⁻	30	0.00002	1.1	R ⁺ M ⁺
10	..	42	0.00002	0.9	R ⁺ M ⁺

^aRestriction indicates the E.O.P. of λ *vir*.0 relative to λ *vir*.K at 30 °C and 42 °C

Modification indicates fraction of λ *vir*.K in lysates of the transformants at 30 °C and 42 °C

Table III. The analysis of R124I restriction deficiency in the hybrid plasmid pCP1005 after the action of Me₂SO^a

Line	Plasmid	Restriction EOP of λ vir.K	Modification fraction of λ vir.K,124	R ₁₂₄ M ₁₂₄ phenotype
1	pCP1005-X	0.8	0.00003	R ⁻ M ⁻
2	pACYCX3	0.9	0.00003	R ⁻ M ⁻
3	pACYCX3 pJS491	1.0	0.00001	R ⁻ M ⁻
4	pACYCX3 pJS4M	0.7	0.8	R ⁻ M ⁻

^aRestriction indicates the E.O.P. of λ vir.K relative to λ vir.K,124

Modification indicates fraction of λ vir.K,124 in lysates of transformants

All assays were carried out in JM109(DE3)

Fig. 1. The effect of Me₂SO (%) on the expression of *Eco*R124I restriction phenotype in *E. coli*C strain (EOP of λ vir.K). Curve 1 - *E. coli*C(pCP1005), curve 2 - *E. coli*C(pR124), curve 3 - *E. coli*C *recA*(pCP1005), curve 4 - fraction of Ap^RMe₂SO^R colonies (Ap^R), curve 5 - *E. coli*C(p Δ CYCWI)

