

Complementation Analysis of Temperature-sensitive Host Specificity Mutations in *Escherichia coli*

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A selection procedure was devised for the isolation of temperature-sensitive mutants of *Escherichia coli* K12 unable to restrict foreign DNA. Many of the non-restricting mutants isolated also displayed temperature sensitivity in the modification of DNA. The mutations were shown to map in the *hs* cluster of genes which determine the host specificity of DNA in *E. coli*. The kinetics of inactivation of restriction showed that a short exposure to high temperature was sufficient to impair restriction of phage λ DNA and that after shift to low temperature restriction did not return to the wild-type level until a period of growth had occurred. One of the mutants was used as a starting strain from which further mutants were then selected for their inability to host-modify DNA. Many of the mutants thus isolated, in addition to being impaired in modification, were found to be non-restricting at both high and low temperatures. A complementation analysis of the mutants was carried out using an F' donor strain derived from *E. coli* B and carrying the host specificity genes *hssB*⁺ *hsr*⁻ *hsm*⁺. In all but one of the F' merodiploids constructed between this F' and the temperature-sensitive host specificity mutants of *E. coli* K the temperature sensitivity of the mutant phenotype was complemented and the merodiploids displayed K- and B-specific restriction and K- and B-specific modification. From these results it is concluded that all of the temperature-sensitive mutants carry mutations in the *hsm* gene, and are *hssK*⁺ *hsr*⁺ *hsm*^{ts}. In addition it is argued that an *hsm*-directed polypeptide is required for restriction in addition to polypeptides directed by *hssK* and *hsr*. These results are discussed in terms of models based on the interaction of subunits to form oligomeric enzymes.

1. Introduction

Many strains of bacteria can recognize DNA from other so-called foreign strains. One result of this recognition is that the foreign DNA molecule can be degraded by a strain-specific endonuclease which produces a number of double chain scissions at defined sites along the DNA molecule (Meselson & Yuan, 1968). If the DNA molecule is a phage genome, for example phage λ , then phage growth may be prevented and the phage is said to be restricted. A small fraction of bacteria infected with such a phage prepared on a foreign strain fail to restrict the phage and these produce bursts of progeny phages which are host-modified so that they are no longer restricted. This host modification is a process which acts directly on DNA and for phage fd in *Escherichia coli* B takes the form of altering specifically the base adenine by methylation (Arber & Smith, 1966).

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Genetic analysis of this phenomenon in *E. coli* strains K, K(P1) and B has revealed that mutants deficient in restriction but not in modification, and mutants deficient in both processes can be readily isolated (Glover, Schell, Symonds & Stacey, 1963; Wood, 1966; Lederberg, 1966). Mutants deficient in both restriction and modification have also been isolated from the former class as a result of a second-step mutation (Glover & Colson, 1969). Functional analysis of these mutants by complementation tests in merodiploids constructed using F' donor strains reveals that at least two complementation groups are involved in restriction, and at least two complementation groups are involved in modification (Boyer & Roulland-Dussoix, 1969; Arber & Linn, 1969; Glover, 1968, 1970). The simplest explanation of these results and the results of crosses between mutants (Glover & Colson, 1969) is that three genes are concerned in the control of host modification. Arber & Linn (1969), have defined the symbols *hss* for a gene which determines the synthesis of a polypeptide responsible for site recognition on DNA; *hsr* for a gene responsible for the synthesis of a polypeptide involved in endonuclease activity and *hsm* for a gene responsible for the synthesis of a polypeptide involved in modification. The mode of interaction of these polypeptides which are thought to interact as subunits of oligomeric enzyme(s) is not known. Four simple modes of interaction can account for the results so far obtained. (1) Two oligomers are formed, one having restriction activity and one having modification activity and both enzymes would have one common polypeptide—the site recognition polypeptide which is responsible for enzyme substrate complexes. (2) Restriction activity resides in an oligomer composed of only two types of subunit as in the first model, but modification activity resides in an oligomer composed of all three types of subunit. (3) Conversely to model (2), restriction activity resides in an oligomer composed of all three types of subunit while modification is due to an oligomer composed of two types of subunit only as in model (1). (4) Both restriction and modification activities reside in an oligomer composed of all three types of subunit.

As a first step towards distinguishing between these four alternatives, it is necessary to establish in which of the three genes mentioned above any newly isolated mutation is located. This obviously cannot be inferred immediately from the phenotype of the mutant since it is clearly possible to have mutations in *hss* which would differentially affect the interaction of the site recognition subunit with the other two types of subunit. Thus for example, an r^-m^+ phenotype could be due to a mutation in *hss* which simply affected the binding of the site recognition subunit to the *hsr*-directed subunit but did not alter its binding to the *hsm*-directed subunit.

As a second step it is necessary to isolate conditional mutants in which it is likely that a polypeptide chain alteration has been produced, and thus the interaction of normal (or almost normal) and mutant subunits can be studied in the same strain. Temperature-sensitive mutants fulfil these requirements, and in this paper we describe the isolation and properties of a number of temperature-sensitive restriction-deficient mutants of *E. coli* K, and examine their behaviour in complementation experiments with F' donor strains.

2. Materials and Methods

(a) *Bacteria*

E. coli K12 strain C600 (Appleyard, 1954) and host specificity mutants derived from it (Colson, Glover, Symonds & Stacey, 1965); strain HfrH (Hayes, 1953); X8011 kindly provided by Dr J. Scaife.

E. coli B strain B251 (Arber & Dussoix, 1962) and host specificity mutants derived from it (Glover & Colson, 1969).

E. coli C (Bertani & Weigle, 1953).

(b) *Bacteriophages*

Phage λ ; λCI ; $\lambda CI857.h80$ (obtained from strain X8011); λvir ; phage P1.

(c) *Media*

Buffer for bacteria (g/l.) KH_2PO_4 , 3.0; Na_2HPO_4 , 7.0; NaCl, 4.0; $MgSO_4 \cdot 7H_2O$, 0.2. Phage buffer (g/l.) KH_2PO_4 , 3.0; Na_2HPO_4 , 7.0; NaCl, 5.0; $MgSO_4$ (0.1M), 10.0; $CaCl_2$ (0.001M) 10.0; gelatin (1%) 1.0. M9 buffer refers to M9 medium without glucose.

M9 medium (g/l.): Na_2HPO_4 , 7.0; KH_2PO_4 , 3.0; NaCl, 0.5; NH_4Cl , 1.0; $MgSO_4$ (0.1M), 10.0; glucose, 0.02. M9 medium was solidified with 1.5% Davis New Zealand agar. VB salts ($\times 20$): K_2HPO_4 , 300g; $NaNH_4HPO_4 \cdot 4H_2O$, 105g; $MgSO_4 \cdot 7H_2O$, 6.0 g; citric acid $\cdot H_2O$, 60g; H_2O , 1.332 l. VB agar: 1 in 20 dilution of VB salts in water solidified with 1.5% Davis New Zealand agar. Difco agar (g/l.) Oxoid Tryptone, 10.0; NaCl, 8.0; glucose, 1.0; Difco Bacto agar, 10.0. L-broth (g/l.) Difco Tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0. L-agar was L-broth solidified with 1.5% Difco Bacto agar. L-agar for soft agar overlays was solidified with 0.6% Difco Bacto agar. Oxoid broth (g/l.): Oxoid no. 2 nutrient broth, 25.0. Oxoid agar was Oxoid broth solidified with 1.25% Davis New Zealand agar. Water-soft agar was 0.6% Difco Bacto agar. L-Amino acid supplements were added to minimal media at 20 $\mu g/ml$; thiamin at 10 $\mu g/ml$. and streptomycin at 200 $\mu g/ml$.

(d) *Phage techniques*

The general techniques were as described by Adams (1950) and special techniques relating to λ were those described by Arber (1958,1960).

(e) *Spot tests for restriction and modification of λ*

Restriction was scored with $\lambda vir.K$, $\lambda vir.B$ and $\lambda vir.C$ by the method described by Colson *et al.* (1965). Modification was scored using standard indicator strains of *E. coli* K, B and C by the method of Colson *et al.* (1965).

(f) *Efficiency of plating determinations on temperature-sensitive mutants*

Bacteria (0.25 ml.) grown in L-broth or M9 overnight were suspended in 3 ml. water-soft agar with 0.01 M- $MgSO_4$ and mixed with standard dilutions of $\lambda vir.K$, $\lambda vir.B$, $\lambda vir.C$ and the contents of the tubes poured onto Difco agar plates. Bacteria grown at 30°C were plated immediately after mixing with phage; bacteria grown at 42°C were mixed with phage for 30 min at 42°C before plating. This modification of the standard technique was used to avoid temperature shifts during the penetration and restriction of λ DNA.

In experiments in which phage was pre-adsorbed to bacteria for 15 min at the appropriate temperature no significant differences in efficiency of plating were observed.

(g) *Transduction*

P1 lysates were prepared and transductions carried out following the procedure of Glover (1962). Introduction of host specificity mutations into new strains was accomplished by P1-mediated cotransduction with *serB* (Glover & Colson, 1969).

(h) *Ethylmethane sulphonate mutagenesis*

E. coli K strain X8011 was grown in 10 ml. L-broth to about 1×10^8 bacteria/ml., centrifuged and resuspended in 1 ml. M9 buffer containing 0.4M-ethylmethane sulphonate (Eastman Kodak Co.) at 37°C for 30 min. Then 0.3 ml. of the suspension was diluted 50-fold in M9 buffer and the viable count was determined (about 1% survival). The remainder of the suspension was centrifuged, resuspended in 10 ml. L-broth and grown to about 1×10^8 bacteria/ml. and stored at 4°C. For the isolation of mutants and measurements of mutation frequency 1.0-ml. samples were diluted 10-fold into L-broth and grown at 42°C to about 1×10^8 bacteria/ml. The proportion of non-restricting bacteria in the cultures was estimated directly by measuring the efficiency of plating of $\lambda vir.C$ on the cultures.

(i) Construction of *F'* merodiploids

A *serB* derivative of HfrH was transduced with P1 grown on *E. coli* $r^-_B m^+_B serB^+$ and from among the *serB*⁺ transductants an HfrH $r^-_B m^+_B serB^+$ colony was selected. The partial diploid $F' r^-_B m^+_B serB^+ thr^+ leu^+ / F^- r^+_K m^+_K thr leu arg pro his thi str-r recA$ was constructed by crossing HfrH $r^-_B m^+_B serB^+$ with AB2463 *thr leu arg pro his thi str-r recA* F^- and selecting for the early markers *thr*⁺ and *leu*⁺ which selects for colonies arising as a result of infection with *F'* factors spontaneously produced in the Hfr population (Low, 1968). The partial diploid was sensitive to male-specific phages MS2 and fd and transfers *thr*⁺ and *leu*⁺ at high frequency. The *F'* merodiploid $r^-_B m^-_B (2) ser^+ thr^+ leu^+ / F^- r^-_B m^-_B (2) met thi str-s$ was constructed by Dr Claire Berg from an $r^- m^-$ mutant of *E. coli* B isolated in this laboratory after mutagenesis of an $r^-_B m^+_B$ mutant and designated $r^-_B m^-_B (2)$. These and other merodiploids have been extensively used in a complementation analysis of conventional host specificity mutants (Arber & Linn, 1969; Glover, 1968, 1970) and were used here to complement host specificity temperature-sensitive mutants. Merodiploids to be used as donors and the appropriate recipients were grown in supplemented M9 medium and mated at 37°C. *Thr*⁺ *leu*⁺ colonies were selected on M9 medium, purified and scored for restriction and modification with *λvir* at 30°C and 42°C.

(j) Preparation of strain X8011

E. coli K X8011 *lac ΔU169 per II str-s thi (λCI857.h80 dlac⁺; λCI857.h80)* contains heat-inducible prophage (Sussman & Jacob, 1962), and was cured of *λ* by growing the cells at 42°C, and a *lac*⁻ colony which was sensitive to *λ* was isolated. This strain was subsequently made resistant to streptomycin and used in the experiments reported here.

3. Results

(a) Isolation of temperature-sensitive host specificity mutants

Several different methods have been used to select restriction-deficient mutants (Glover *et al.*, 1963; Colson *et al.*, 1965; Wood, 1966; Lederberg, 1966; Glover & Colson, 1966). They rely on the selection of bacteria infected with restricted DNA from either phage *λ*, *λdg* or an *F'* factor. In the experiments reported here we used a selection method for isolating non-restricting mutants which consists of infecting a mutagenised culture of *E. coli* K with *λ.C* at 42°C. Non-restricting bacteria are readily lysogenised by the phage, whereas restricting wild-type bacteria are not. The non-lysogenic bacteria were then killed at 42°C with *λ CI.K* which does not lyse non-restricting mutants which have been lysogenized by *λ.C* and are thus immune. In principle this method should be able to select 1 in 10⁴ non-restricting bacteria; in practice, however it was considerably less efficient.

In preliminary experiments lysogenization with *λ.C* was satisfactory, but difficulties were encountered with the second stage because the mutagenized population of bacteria was remarkably resistant to lysis with *λCI.K* and even *λvir.K*. The nature of the resistant survivors is not known and was not investigated further. It was most likely due to inefficient adsorption by the survivors; and therefore a second phage, a hybrid between *λ* and *φ80*, *λCI857 h80.K*, was used. This phage, because it adsorbs to different receptors, will lyse *λ*-resistant bacteria. Therefore after lysogenisation with *λ.C*, a mixture of the two phages *λCI.K* and *λCI857 h80.K* was used to kill the non-lysogenic survivors.

The results in Table 1 show that this technique was not sufficiently effective to produce a yield composed entirely of non-restricting mutants. Therefore an additional selection step was applied at 42°C. The survivors from the first selection step were infected with *Flac*⁺ using a donor which did not confer K host specificity and *lac*⁺

colonies were selected. After this second selection step a population of bacteria was obtained composed almost entirely of non-restricting bacteria.

To select temperature-sensitive mutants which would be non-restricting at 42°C and restricting at 30°C it was necessary to apply a selection at 30°C designed to kill

TABLE I

Multi-step selection of restriction-deficient mutants after mutagenesis with ethylmethane sulphonate

Selection step	Exp. no.	No. of colonies tested	Restriction phenotype of mutants†		
			r ⁻	r [±]	r ^{ts}
Ethylmethane sulphonate mutagenesis	1	20	1	0	n.t.
Infection with λ CI.K and λ CI857.h80.K	1	20	10	0	n.t.
Infection with F ⁺ lac ⁺	1	30	28	1	n.t.
Infection with λ vir.C	1	75	6	3	2
	2	80	3	0	1
	3	150	6	6	1

E. coli K strain X8011 was cured of λ as described in Materials and Methods and grown to 1.0×10^8 bacteria/ml. in L-broth. After ethylmethane sulphonate mutagenesis the culture was diluted 1 in 10 into L-broth and grown at 42°C for 3 hr, when the titre had reached about 1.5×10^8 bacteria/ml. The efficiency of plating of λ vir.C on the culture before mutagenesis was 5×10^{-4} and after mutagenesis 1×10^{-2} . The culture was then infected with wild-type λ .C at a multiplicity of 5.0 by mixing equal volumes of phage and bacteria in the presence of 0.01 M-MgSO₄ for 20 min at 42°C. The mixture was then diluted twofold with pre-heated L-broth (2 × concn) at 42°C and aerated for 20 min to facilitate the injection of the phage DNA. The suspension was then centrifuged and the bacteria resuspended in 0.25 vol. of 0.01 M-MgSO₄. Equal volumes of bacteria and the mixture of killing phages λ CI.K (multiplicity of infection = 44) and λ CI857.h80.K (multiplicity of infection = 60) were mixed and adsorbed for 15 min at 37°C. The suspension was then diluted twofold in L-broth (2 × concn) and aerated for 15 min at 37°C. After a further 10-fold dilution in L-broth the infected bacteria were aerated at 37°C for 60 min to ensure lysis by λ CI.K and for a further 60 min at 42°C to ensure lysis by λ CI857.h80.K. Approximately 2×10^6 bacteria/ml. survived the infection and the suspension was concentrated by centrifugation about 100-fold and stored overnight at 4°C.

To continue the selection the concentrated suspension was grown for 3 hr at 42°C in L-broth to restore the restriction-deficient phenotype and mixed in equal volumes with the donor culture *E. coli* X8011 r⁻km⁻ str-s Flac⁺. The suspension was filtered on a membrane immediately after mixing and placed on the surface of Oxoid agar for 60 min at 42°C before resuspending in 1 ml. Oxoid broth. A sample was plated on M9 medium supplemented with thiamin and lactose to select lac⁺ colonies and containing streptomycin to eliminate the donor bacteria. About 75% of the recipient bacteria were infected with Flac⁺.

A 0.2-ml. sample containing about 1.6×10^8 /ml. Flac⁺-infected bacteria was suspended in 5 ml. L-broth containing streptomycin (200- μ g/ml.) to eliminate the donor culture and incubated at 30°C until the culture reached the original concentration. The suspension was then centrifuged and the bacteria resuspended in 0.01 M-MgSO₄. It was then mixed in equal volumes with λ vir.C (multiplicity of infection = 10) and 60 min at 30°C allowed for adsorption before diluting twofold with L-broth (2 × concn) and aerating for 40 min at 30°C. The suspension was then filtered and washed and finally resuspended in 1 ml. broth before plating on M9 supplemented with lactose, thiamin and streptomycin. About 2.3×10^7 bacteria/ml. survived the infection with λ vir.C. Colonies were then picked and incubated in 1 ml. L-broth at 30°C and 42°C and screened for restriction phenotype. The modification phenotype was rapidly screened by replica-plating colonies, all of which were lysogenic for λ , on to indicator lawns of *E. coli* K and *E. coli* C. Presumptive mutants were then carefully tested for restriction and modification at 30 and 42°C as described in Materials and Methods.

† n.t. = not tested; r⁻ = efficiency of plating λ vir.C 1.0; r[±] = efficiency of plating λ vir.C about 1×10^{-2} ; r^{ts} = temperature-sensitive restriction of λ vir.C; for details see Table 2.

non-restricting bacteria and permit the survival of restricting bacteria. The method was simply to infect cultures grown at 30°C with λ vir.C, which preferentially kills non-restricting bacteria. In practice about 90% of the non-restricting bacteria were lysed and out of a total of 305 colonies tested at the end of the procedure 15 were non-restricting, nine were only partially restricting and four displayed a temperature-sensitive restriction phenotype (Table 1).

All of the selected mutants were tested for restriction and modification of phage λ at 30 and 42°C. Table 2 lists the variety of different phenotypes obtained. A number of conventional non-temperature-sensitive mutants came through the selection procedure and only one example of each type is given in Table 2. What is surprising is that

TABLE 2
Restriction and modification phenotypes of mutants at 30°C and 42°C

Mutant no.†	Restriction Efficiency of plating‡				Modification Fraction K-plating phage produced§		Phenotype symbol	
	λ vir.C		λ vir.K		30°C	42°C	30°C	42°C
2-45	0.7	0.8	0.9	1.3	0.6	0.6	r ⁻ _K m ⁺ _K	r ⁻ _K m ⁺ _K
2-47	0.7	1.2	1.0	0.9	0.5	0.003	r ⁻ _K m ⁺ _K	r ⁻ _K m [±] _K
1-71	0.9	1.1	1.0	1.0	0.6	0.0004	r ⁻ _K m ⁺ _K	r ⁻ _K m ⁻ _K
1-63	1.1	0.9	1.2	0.7	0.05	0.008	r ⁻ _K m [±] _K	r ⁻ _K m [±] _K
3-83	0.6	0.5	0.9	0.5	0.06	0.01	r ⁻ _K m [±] _K	r ⁻ _K m [±] _K
3-122	1.2	0.9	1.2	1.2	0.005	0.004	r ⁻ _K m [±] _K	r ⁻ _K m [±] _K
3-79	0.5	0.6	0.8	0.5	0.0004	0.0005	r ⁻ _K m ⁻ _K	r ⁻ _K m ⁻ _K
3-116	0.008	0.007	1.0	0.9	1.0	0.7	r [±] _K m ⁺ _K	r [±] _K m ⁺ _K
1-60	0.02	0.8	0.7	1.2	0.5	0.7	r [±] _K m ⁺ _K	r ⁻ _K m ⁺ _K
2-5	0.002	1.0	1.0	0.5	0.6	0.007	r [±] _K m ⁺ _K	r ⁻ _K m [±] _K
3-28	0.0001	0.01	0.5	0.5	0.8	0.8	r ⁺ _K m ⁺ _K	r [±] _K m ⁺ _K
1-30	0.0004	1.2	1.0	1.0	1.0	0.008	r ⁺ _K m ⁺ _K	r ⁻ _K m [±] _K

† The mutant number combines an experiment number from Table 1 together with an identifying number.

‡ Where the efficiency of plating of λ does not differ from 1.0 by more than a factor of two, the difference is not regarded as significant.

§ Where the fraction does not differ from 1.0 by more than a factor of two, the difference is not regarded as significant. The fractions are calculated as a function of the efficiency of plating on *E. coli* C which is regarded as 1.0.

|| The symbols r[±] and m[±] indicate intermediate phenotypes.

although selection was made only for temperature-sensitive restriction phenotypes, several mutants displayed temperature-sensitive modification phenotypes, and were non-restricting at both temperatures.

(b) Location of the temperature-sensitive host specificity mutations

That the mutations induced were mutations in the host specificity genes was confirmed by phage P1-mediated co-transduction with *serB*. The temperature-sensitive mutations were co-transduced with *serB* about 16% (Table 3), in close agreement with the average figure of 12% for conventional host specificity mutations obtained by Glover & Colson (1969).

TABLE 3

Co-transduction of serB and temperature-sensitive host specificity mutations

Recipient	Mutant donor	No. of <i>ser</i> ⁺ colonies tested	No. of colonies with donor host specificity phenotype	Percentage co-transduction
4K <i>r</i> ⁻ _K <i>m</i> ⁻ _K (λ) <i>str-r</i> <i>thr leu thi serB</i>	1-30	51	7	14
	1-71	24	5	20
	2-5	24	4	17
	1-63	24	3	12
	2-47	24	4	17

P1 lysates were prepared on the mutants, and the recipient strain 4K transduced as described in Materials and Methods, and *serB*⁺ colonies on VB medium supplemented with threonine, leucine and thiamin were selected after 48 hr incubation at 37°C. Selected colonies were purified on the same medium and then transferred as a template to the same medium and incubated at 30°C. The colonies were then resuspended in 1 drop of bacterial buffer and spotted on to lawns of indicator bacteria to screen for the modification phenotype at 30°C. Colonies which showed the modification phenotype of the donor were picked and grown at 30 and 42°C and tested for restriction and modification as described in Materials and Methods.

TABLE 4

Growth of λ vir.C in temperature-sensitive mutant 1-30

	Temperature	
	30°C	42°C
Number of bacteria	5.1×10^8 /ml.	4.35×10^8 /ml.
Number of phage particles	1.4×10^7 /ml.	1.4×10^7 /ml.
Multiplicity of infection	0.02	0.009
Adsorption	73%	27%
Number of infective centres on K	2.39×10^3 /ml.	1.41×10^3 /ml.
Number of infective centres on C	1.78×10^3 /ml.	1.69×10^3 /ml.
Transmission	1.8×10^{-4}	0.5
Burst on K	2.4×10^4 /ml.	9.35×10^4 /ml.
Burst on C	4.75×10^4 /ml.	1.9×10^7 /ml.
Average burst size	27	11
Fraction of K-plating λ vir among burst	0.5	5×10^{-3}

Mutant 1-30 was grown in L-broth overnight at 37°C and from this culture two 5-ml. volumes of L-broth were inoculated with 0.5 ml. One culture was grown for 3 hr at 30°C, the other for 3 hr at 42°C. The cultures were filtered and resuspended in 2.5 ml. 2×10^{-2} M-MgSO₄ and incubated at 30 and at 42°C for a further 90 min. A sample was taken to assay the number of bacteria and then 1 ml. of each suspension was mixed with 1 ml. of λ vir.C and 25 min allowed for adsorption. The mixture was diluted twofold with 2× concn L-broth and aerated for a further 15 min. Unadsorbed phage was removed by filtration and rapid washing twice with 10 ml. of phage buffer. The infected bacteria were resuspended in 1 ml. L-broth and the number of infected bacteria assayed on standard indicators *E. coli* K and C. The amount of residual unadsorbed phage was negligible. A sample, 0.1 ml., of the infected bacteria was transferred to 10 ml. of L-broth and aerated for 90 min before the addition of chloroform. Finally, the progeny phage was assayed on *E. coli* K and C. This procedure was carried out at 30 and at 42°C and in each case care was taken to use pre-heated media to ensure that at all times the culture was exposed to the given temperature.

(c) *Measurement of in vivo restriction and modification of phage λ*

To ensure that the temperature-sensitive effect on the restriction and modification phenotypes of the mutants was in fact due to temperature sensitivity of the restriction and modification processes, and not due to trivial effects of temperature on adsorption and penetration of the phage or upon burst size, a single cycle of infection with $\lambda vir.C$ was carried out on mutant 1-30 at 30 and 42°C. The results given in Table 4 show that the temperature sensitivity of the restriction process is reflected in the 5000-fold difference in the transmission of $\lambda vir.C$ at 30 and 42°C. Modification at 30°C was measured only with the small fraction of spontaneously non-restricting bacteria, virtually all of which produced $\lambda vir.K$. The temperature sensitivity of the modification process is reflected in the 100-fold decrease in the amount of $\lambda vir.K$ obtained in the burst at 42°C. Adsorption and burst size at 30 and 42°C were not significantly different.

(d) *Kinetics of temperature-sensitive restriction in mutant 1-30*

To determine whether the temperature-sensitive phenotype of mutant 1-30 was due to temperature sensitivity of the restriction and modification enzyme(s) itself, or to a temperature sensitivity in protein synthesis, the kinetics of the loss of restriction after temperature shift from 30 to 42°C were measured. The results, shown in Figure 1,

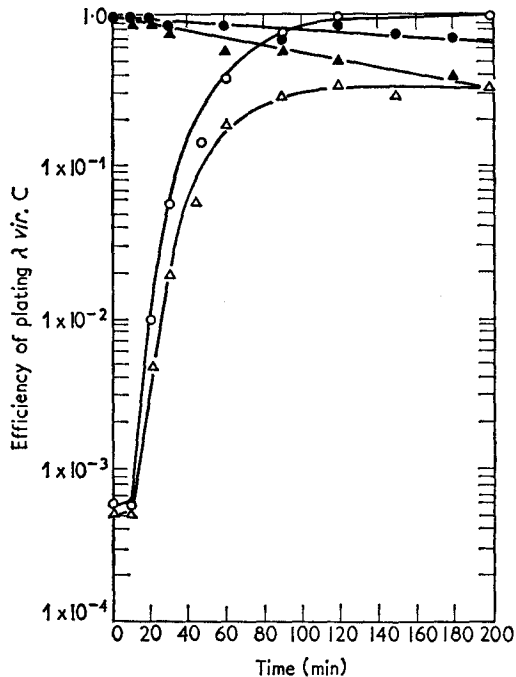


FIG. 1. An overnight L-broth culture of mutant 1-30 was inoculated into two 10-ml. vol. of M9 supplemented with threonine, leucine and thiamin; one culture was grown overnight at 30°C and the other at 42°C. The cultures were centrifuged and resuspended in 1 ml. M9 with added thiamin. 0.5-ml. samples of this suspension were transferred to 10 ml. M9 with thiamin and to 10 ml. fully supplemented M9. The suspensions were then aerated for 90 min at 30 and at 42°C. At this time the 30°C cultures were transferred to 42°C and the 42°C cultures transferred to 30°C. At intervals, samples were taken and the efficiency of plating of $\lambda vir.C$ scored on each sample.

—○—○—, Transfer from 30°C to 42°C in supplemented M9; —△—△—, transfer from 30°C to 42°C in absence of required amino acids; —●—●—, transfer from 42°C to 30°C in supplemented M9; —▲—▲—, transfer from 42°C to 30°C in the absence of required amino acids.

indicate that it is a temperature-sensitive enzyme which is involved since the non-restricting phenotype is rapidly expressed after transfer to 42°C. Furthermore, the recovery of the restricting phenotype on shift back from 42 to 30°C is clearly a very slow process and was not complete until new protein synthesis had been permitted and almost certainly requires growth of the cells.

Since the recovery of the restricting phenotype is very slow after transfer from 42 to 30°C the technique used to score restriction at 42°C was satisfactory, since the bacteria and phage were mixed together for 30 minutes at 42°C and then the short period at which the plates were at a temperature below 42°C while the soft agar overlay solidified will not have had any significant effect on the efficiency of plating of λ vir.C.

Similarly, since the kinetics of the loss of the restriction phenotype after transfer from 30 to 42°C requires several minutes before any effect can be measured, the technique employed to score restriction at 30°C was satisfactory, since the minor temperature rise caused by the addition of water-soft agar at 42°C for a very short period before plating will not have had any significant effect on the efficiency of plating of λ vir.C.

(e) *Isolation of modification-deficient mutants from temperature-sensitive mutant 1-30*

To obtain information about the role played by *hsm* in the expression of restriction and modification, it was necessary to attempt to induce mutations in the *hsm* gene itself, and to study the behaviour of such mutants in complementation tests.

Temperature-sensitive mutant 1-30 lysogenic for λ was grown in L-broth at 42°C and treated with ethylmethane sulphinate also at 42°C, otherwise following the procedure described in Materials and Methods. After a period of growth to permit the expression of mutations, surviving bacteria were plated to obtain single colonies on L-agar at 42°C. These colonies were then replicated on to Difco agar seeded with *E. coli* K and C soft agar overlays. Colonies which showed efficient lysis of the C indicator bacteria but no lysis of the K bacteria, were picked as presumptive mutants and streaked on L-agar. In experiment 1 (Table 5) all presumptive mutants were grown and tested for restriction and modification at 42°C to select r^-m^- phenotypes which survive at 42°C but which at 30°C could be r^+m^- and therefore potentially lethal. From the results of this experiment it is clear that no mutants of this type were isolated. Similarly no mutants which could even partially restrict unmodified DNA and which also produce unmodified DNA were isolated. The only mutants isolated were impaired in restriction either to the same extent as in modification or to an even greater extent, for example r^-m^- , at both temperatures or $r^\pm m^+$ at 30°C and r^-m^\pm at 42°C.

In the second experiment (Table 5) all presumptive mutants were grown at 30°C and only those which were r^- were tested for modification at 30 and 42°C. In all of the mutants isolated, the second mutation affected modification to extents varying from m^- at both temperatures to full temperature sensitivity, m^+ at 30°C and m^- at 42°C. In this experiment a total of 18 mutants was obtained from about 5000 colonies screened after mutagenesis.

(f) *Functional analysis of temperature-sensitive host specificity mutants*

The F' merodiploid selected as the donor in these experiments was $F'r^-_B m^+_B / F^- r^+_K m^+_K$ *thr leu arg pro his thi str-r recA*. The recipients all required threonine, leucine and thiamin for growth, and after mating, colonies were selected

TABLE 5
Restriction and modification phenotypes of modification-deficient mutants isolated from mutant 1-30

Exp. no.†	No. of presumptive mutants tested	Mutant no.	Restriction Efficiency of plating‡		Modification Fraction of K-plating §		Phenotype symbol	
			λ vir.C	λ vir.K	30°C	42°C	30°C	42°C
1	32	6	0.9	1.1	0.6	0.0002	r ⁻ k ⁻ m ⁻ k	r ⁻ k ⁻ m ⁻ k
		20	0.7	0.7	0.8	0.0001	r ⁻ k ⁻ m ⁻ k	r ⁻ k ⁻ m ⁻ k
		18	0.04	1.0	1.0	0.7	r [±] k [±] m [±] k	r ⁻ k ⁻ m ⁻ k
		22	0.02	0.9	0.5	0.01	r [±] k [±] m [±] k	r ⁻ k ⁻ m ⁻ k
		8	0.008	0.9	0.5	0.02	r [±] k [±] m [±] k	r ⁻ k ⁻ m ⁻ k
		2	0.002	0.8	0.5	0.01	r [±] k [±] m [±] k	r ⁻ k ⁻ m ⁻ k
		VII	1.0	1.0	0.002	0.001	r ⁻ k ⁻ m ⁻ k	r ⁻ k ⁻ m ⁻ k
		VIII	1.2	1.0	0.002	0.001	r ⁻ k ⁻ m ⁻ k	r ⁻ k ⁻ m ⁻ k
		X	0.9	1.0	0.008	0.001	r ⁻ k ⁻ m ⁻ k	r ⁻ k ⁻ m ⁻ k
		XI	1.1	1.0	0.001	0.0004	r ⁻ k ⁻ m ⁻ k	r ⁻ k ⁻ m ⁻ k
2	54	XII	0.6	1.0	1.0	0.001	r ⁻ k ⁻ m ⁻ k	r ⁻ k ⁻ m ⁻ k
		XIII	0.9	1.0	1.0	0.0004	r ⁻ k ⁻ m ⁻ k	r ⁻ k ⁻ m ⁻ k
					0.02			

† In experiment 1 the mutation 1-30 was present in the original strain X8011 in which it was first isolated. In experiment 2 the mutation 1-30 was first transduced by phage P1 into strain 4K.

‡ Where the efficiency of plating of λ does not differ from 1.0 by more than a factor of two the difference is not regarded as significant.

§ The fractions are calculated as a function of the efficiency of plating on *E. coli* C which is regarded as 1.0. Where the fraction does not differ from 1.0 by more than a factor of two the difference is not regarded as significant.

|| The symbols r[±] and m[±] indicate intermediate phenotypes.

TABLE 6

Restriction and modification properties of merodiploids

Exp. no.	Mutant no.	Temp. (°C)	Haploid phenotype Recipients	F'	λ <i>vir</i> .K	Restriction† λ <i>vir</i> .B	λ <i>vir</i> .C	Modification‡ K	Modification‡ B	Phenotype of merodiploid§
1	1-30	30	r ⁺ _K m ⁺ _K	r ⁻ _B m ⁻ _B (2)	0.0002	0.0002	0.0003	0.5	0.4	r ⁺ _{KB} m ⁺ _{KB}
		42	r ⁻ _K m [±] _K		0.1	0.2	0.2	0.5	0.3	r ⁻ _{KB} m ⁺ _{KB}
2	K	37	r ⁺ _K m ⁺ _K	r ⁻ _B m ⁺ _B	0.0001	0.0003	0.0001	1.0	1.0	r ⁺ _{KB} m ⁺ _{KB}
		37	r ⁻ _K m ⁺ _K	r ⁻ _B m ⁺ _B	1.0	1.0	1.0	1.0	1.2	r ⁻ _{KB} m ⁺ _{KB}
4	1-30	30	r ⁺ _K m ⁺ _K	r ⁻ _B m ⁺ _B	0.008	0.007	0.003	0.7	1.0	r ⁺ _{KB} m ⁺ _{KB}
		42	r ⁻ _K m [±] _K		0.009	0.009	0.006	0.7	1.1	r ⁺ _{KB} m ⁺ _{KB}
5	1-71	30	r ⁻ _K m ⁺ _K	r ⁻ _B m ⁺ _B	0.006	0.01	0.007	1.0	1.1	r ⁺ _{KB} m ⁺ _{KB}
		42	r ⁻ _K m ⁻ _K		0.006	0.01	0.008	1.0	0.9	r ⁺ _{KB} m ⁺ _{KB}
6	2-5	30	r ⁺ _K m ⁺ _K	r ⁻ _B m ⁺ _B	0.009	0.009	0.007	0.7	1.0	r ⁺ _{KB} m ⁺ _{KB}
		42	r ⁻ _K m [±] _K		0.02	0.02	0.007	0.9	0.8	r ⁺ _{KB} m ⁺ _{KB}
7	VIII	30	r ⁻ _K m ⁻ _K	r ⁻ _B m ⁺ _B	0.006	0.004	0.004	0.7	0.5	r ⁺ _{KB} m ⁺ _{KB}
		42	r ⁻ _K m ⁻ _K		0.006	0.005	0.003	0.9	0.9	r ⁺ _{KB} m ⁺ _{KB}
8	X	30	r ⁻ _K m ⁻ _K	r ⁻ _B m ⁺ _B	0.02	0.009	0.02	0.7	1.0	r ⁺ _{KB} m ⁺ _{KB}
		42	r ⁻ _K m ⁻ _K		0.02	0.02	0.01	0.7	0.8	r ⁺ _{KB} m ⁺ _{KB}
9	XI	30	r ⁻ _K m ⁻ _K	r ⁻ _B m ⁺ _B	0.0003	0.5	0.0003	0.00005	0.8	r ⁺ _B m ⁺ _B
		42	r ⁻ _K m ⁻ _K		0.003	0.5	0.002	0.0001	1.0	r ⁺ _B m ⁺ _B
10	XII	30	r ⁻ _K m [±] _K	r ⁻ _B m ⁺ _B	0.01	0.009	0.006	0.9	1.2	r ⁺ _{KB} m ⁺ _{KB}
		42	r ⁻ _K m ⁻ _K		0.008	0.01	0.005	0.7	1.3	r ⁺ _{KB} m ⁺ _{KB}

† Restriction indicates the efficiency of plating of λ *vir*.K, λ *vir*.B and λ *vir*.C on the partial diploids.

‡ Modification indicates the efficiency of plating of phage produced by the partial diploids on standard indicators *E. coli* K and *E. coli* B.

§ The symbol r⁺_K m⁺_K indicates restriction and modification of K and B specificities.

A colony of the F' donor strain was grown in supplemented M9 overnight at 37°C. The mutants were grown in L-broth at 37°C overnight and then sub-cultured in supplemented M9 and grown for 3 hr at 37°C and after centrifugation resuspended in buffer. Equal 0.1-ml. vol. of the recipient and serial 10-fold dilutions of the donor were mixed and plated on M9 supplemented with thiamin, to select *thr*⁺ *leu*⁺ colonies. Colonies were purified, grown at the appropriate temperatures and then tested for restriction on M9 containing thiamin at 30 and at 42°C and the phage produced tested for modification on Difco agar using standard indicator bacteria *E. coli* K, B and C.

on M9 supplemented with thiamin. Purified colonies were then tested for restriction and modification at 30 and at 42°C and the results of these tests are presented in Table 6.

In common with other observers (Boyer & Roulland-Dussoix, 1969) we noticed that partial diploids did not restrict λ as efficiently as the equivalent haploid strains. The first three experiments are controls which show that: (1) the temperature sensitivity of restriction and modification can be satisfactorily scored provided that no complementation occurs (experiment 1); (2) the r^- phenotype of the donor, $r^-_B m^+_B$, can be complemented by wild-type *E. coli* K to give normal B-specific restriction (experiment 2); and (3) the same donor cannot complement with 5K $r^-_K m^+_K$ (experiment 3).

From this evidence which will be discussed in detail we conclude that the donor, $r^-_B m^+_B$ carries *hssB*⁺ *hsr*⁻ *hsm*⁺ on the F' factor.

The merodiploids constructed between this F' and temperature-sensitive mutants 1-30, 1-71 and 2-5 (experiments 4, 5 and 6, Table 6) all displayed restriction of both K- and B-specificities and modifications of K and B specificity. Thus all of the mutants were able to complement the restriction deficiency in the donor $r^-_B m^+_B$ and were themselves complemented by the donor so that the temperature-sensitive restriction and modification phenotypes were lost and replaced by wild-type K-specific restriction and modification.

The merodiploids constructed between the same F' and the second-step mutants VIII, X and XII (experiments 7, 8 and 10, Table 6) similarly displayed wild-type restriction and modification of both K and B specificities. Thus these mutants, which presumably carry two host specificity mutations, are still able to complement the restriction deficiency in the donor F' $r^-_B m^+_B$ and can be complemented by the donor F' genes to restore wild-type K-specific restriction and modification. The merodiploid examined in experiment 9 (Table 6) constructed between the F' $r^-_B m^+_B$ and mutant XI displayed only wild-type B restriction and modification. Mutant XI was thus able to complement the restriction deficiency in the donor F', but there was no complementation of the $r^-_K m^-_K$ phenotype of the mutant.

In experiment 4 the merodiploid expressed normal K- and B-specific modifications; thus it seems most likely that the mutation in mutant 1-30 is in *hsm*. At 42°C the *hsm* function of mutant 1-30 is impaired and in the partial diploid it appears that this can be fully replaced by a product whose synthesis is directed by *hsm*⁺ on the F'. This product is not temperature sensitive; thus wild-type and not temperature-sensitive modification was observed. In addition the diploid expressed wild-type K- and B-specific restrictions from which we can conclude that *hsm* is involved in the expression of restriction. Furthermore it is extremely unlikely that the mutation in mutant 1-30 is in *hssK* for in that case we would expect to observe temperature-sensitive K-specific restriction and wild-type B-specific restriction in the merodiploid. Nor does it appear likely that the mutation in mutant 1-30 is in *hsr*, for in that case we would expect to observe that both K- and B-specific restrictions were temperature sensitive in the merodiploid.

In experiment 5 (Table 6) the F' merodiploid constructed with mutant 1-71 expressed wild-type K- and B-specific restrictions. The mutation is apparently not in *hsr* for in that case we would expect both K- and B-specific restrictions to be temperature sensitive and they are not. The mutation does not appear to be in *hssK* for in that case we would expect B-specific restriction to be present but K-specific restriction to

be impaired. Therefore we can conclude that as in mutant 1-30 the mutation in mutant 1-71 is in *hsm*. As a result of this mutation it seems that not only is the *hsm* function impaired but also it cannot interact with *hsr* since the phenotype of the mutant is r^- . The same mutation is also responsible for the temperature-sensitive modification phenotype of mutant 1-71. In the merodiploid the *hsm*⁺ function provided by the F' is able to replace the mutant gene function and restore wild-type K- and B-specific restrictions.

From the results of experiment 9 (Table 6) with mutant XI derived by a second mutation from mutant 1-30 and having an r^-m^- phenotype, it is clear that the second mutation must be located in *hssK* since in the merodiploid examined both K-specific restriction and modification are absent. This lends support to the notion that *hssK* is involved in the expression of *hsr* and *hsm*.

From the results of experiments 7 and 8 (Table 6) with second-step mutants VIII and X which also have r^-m^- phenotypes, it is clear that the second mutations are not in *hssK* because the partial diploid expressed K-specific restriction and modification. Rather the second mutations appear, like the first mutation, to be in *hsm*, and as a result of these mutations both restriction and modification are completely impaired in the mutants. This result supports the previous conclusion that *hsm* is involved in the expression of restriction. It is noteworthy that the same phenotype r^-m^+ at 30°C and r^-m^- at 42°C was found among first-step mutants (mutant 1-71) and among second-step mutants (mutant XII) and that they both behave in the same manner in the complementation test. This observation will be discussed later.

4. Discussion

The selective procedure employed to isolate temperature-sensitive mutants, which was based upon the selection of mutants defective in restriction at 42°C but not at 30°C, yielded mutants which were temperature-sensitive in modification also. In previous studies designed to select restriction-deficient mutants (Glover *et al.*, 1963; Wood, 1966; Lederberg, 1966) two general classes of mutants have been regularly observed; those impaired in restriction but not in modification and those impaired to varying extents in both processes. The phenotypes of the temperature-sensitive mutants also fall into these two general classes as follows: $r^\pm m^+$ (30°C), $r^- m^+$ (42°C); $r^+ m^+$ (30°C), $r^\pm m^+$ (42°C) and $r^+ m^+$ (30°C), $r^- m^\pm$ (42°C); $r^\pm m^+$ (30°C), $r^- m^\pm$ (42°C); $r^- m^+$ (30°C), $r^- m^\pm$ (42°C); $r^- m^+$ (30°C), $r^- m^-$ (42°C). These mutants were either r^- or temperature-sensitive in restriction to varying extents but invariably the deficiency in restriction was greater than the deficiency in modification. In other words, no mutants were isolated which would be able to restrict the DNA they synthesised at either temperature, for example, $r^+ m^+$ (30°C), $r^+ m^-$ (42°C); $r^+ m^+$ (30°C), $r^+ m^\pm$ (42°C); $r^\pm m^+$ (30°C), $r^+ m^\pm$ (42°C); $r^\pm m^+$ (30°C), $r^\pm m^-$ (42°C); and $r^+ m^\pm$ (30°C), $r^+ m^-$ (42°C).

This remained true irrespective of whether single-step mutants were selected (Table 2) or whether second-step mutants specifically selected for defects in modification were isolated (Table 5).

From the kinetics of the temperature-sensitive restriction in mutant 1-30 and its very slow recovery (Fig. 1) it seems that the mutant protein structure once collapsed by exposure to 42°C cannot easily be reconstituted. This result is not surprising since it appears that the purified K-specific endonuclease isolated by Meselson & Yuan

(1968) has a high sedimentation coefficient and is probably a complex protein of considerable size consisting of several polypeptide subunits. It is relevant to point out that the *in vivo* kinetics of inactivation of the temperature-sensitive mutant protein are similar in form to those of the wild-type protein. The mutant protein loses almost all activity after about 60 minutes exposure to 42°C and the wild-type protein loses all activity after 60 minutes exposure to 50°C (Schell & Glover, 1966). Similarly, once the restriction activity of the wild type is impaired by exposure to high temperature, it does not recover this activity again at 37°C and new protein synthesis is necessary for the expression of restriction.

In the merodiploids constructed between an $F' r^-_B m^+_B$ donor and both the single-step and second-step temperature-sensitive mutants, the temperature sensitivity of both restriction and modification was not expressed. In all but one of these merodiploids wild-type K-specific restriction and modification were found and the $r^-_B m^+_B$ phenotype of the donor was complemented by the mutants to produce wild-type B-specific restriction and modification. The single exception (second-step mutant XI $r^-_K m^-_K$ (Table 6)) is easily explained if we suppose that the second mutation is in *hssK* and as a result of this mutation K-specific restriction and modification cannot be expressed and since the F' factor does not carry *hssK* this defect cannot be complemented in the merodiploid.

Before these results can be interpreted in terms of interaction between genes carried on the F' factor and genes on the recipient chromosome, it is necessary to establish the true genotype of the F' donor. The F' donor appears to carry wild-type *hssB* which can complement with *hsr* since the merodiploid $F' r^-_B m^+_B / F^- r^+_K m^-_K$ expresses wild-type B-specific restriction (experiment 2, Table 6). It also appears to carry wild-type *hsm* since B-specific modification is expressed in the haploid from which the F' was derived, and also in all merodiploids constructed with it. Furthermore in the merodiploid $F' r^-_B m^+_B / F^- r^-_K m^+_K$ (experiment 3, Table 6), the restriction deficiency carried by the recipient was not complemented by the donor; therefore we conclude that the mutation carried by the F' donor is in *hsr*.

As a control over the function of *hsm* carried by the F' a second F' donor was used $F' r^-_B m^-_B (2) / F^- r^-_B m^-_B (2)$ which carries mutations in *hsm* (Glover, 1970). In the merodiploids constructed between this F' and mutant 1-30 temperature sensitivity of restriction was retained (experiment 1, Table 6).

Therefore we conclude that *hsm* is involved in the expression of restriction and that the defects in restriction and modification observed in all of the mutants isolated whether temperature sensitive or not (except mutant XI) arise as a result of mutations in *hsm* which can be complemented by *hsm^+* carried by the donor $F' r^-_B m^+_B$. In support of this conclusion we would point out that no *hsm* modification-deficient mutants were isolated which were still r^+ . Such mutants would have been detected in experiments 1 and 2 (Table 5) in which modification-deficient mutants were selected at 42°C from mutant 1-30 $r^+_K m^+_K$ (30°C), $r^-_K m^+_K$ (42°C) so that if the phenotype, $r^+ m^-$, is lethal such mutants would have survived. It is interesting to contrast this situation with that which was obtained for previously isolated *hsr* mutants. Such mutants are identified from the observation that merodiploids constructed between *hsr* and *hss* mutants express restriction. All of the *hsr* mutants tested (Boyer & Roulland-Dussoix, 1969; Arber & Linn, 1969; Glover, 1968, 1970) are impaired in restriction only, from which we tentatively conclude that *hsr* is probably not needed for modification. We shall return to reconsider this point.

Mutants with temperature-sensitive phenotype r^-m^+ (30°C), r^-m^- (42°C) were isolated after ethylmethane sulphonate treatment of wild-type (mutant I-71) and after a second ethylmethane sulphonate mutagenic treatment of mutant I-30 to give mutant (XII). Both mutants behave in the same way in merodiploids with $F' r^-_B m^+_B$ in that the merodiploid expressed wild-type K- and B-specific restriction and modification (experiments 5 and 10, Table 6). In both mutants it is clear that *hssK* is not affected since K-specificity is properly expressed in the merodiploid. Apparently *hsr* is not affected either since the merodiploid has the phenotype r^+_K and this property could not be conferred by genes on the F' which is *hsr*⁻. The r^- phenotype of both these mutants can now be explained by supposing that, although the mutants are wild-type *hsr*⁺, the r^- phenotype arises because of a mutation in *hsm* as a result of which, since *hsm* is required for the expression of restriction, restriction is not expressed.

Previous experiments (Arber & Linn, 1969; Boyer & Roulland-Dussoix, 1969; Glover, 1968, 1970) have demonstrated that two other host specificity genes *hss* and *hsr* are also required for the expression of restriction. Thus we conclude that all three host specificity genes are required for the expression of restriction. One interesting consequence of the notion that *hsm* function is required for restriction is that mutants impaired in *hsm* will most likely be impaired in restriction; thus the mutant phenotype r^+m^- will not arise irrespective of whether we suppose such phenotypes could be lethal and, as has frequently been pointed out before, no such mutant phenotypes have been isolated. The requirement of *hsm* function for restriction is consistent with the finding of Meselson & Yuan (1968) that K-specific endonuclease depends upon the presence of *S*-adenosylmethionine for activity, though it does not shed light upon the role that *S*-adenosylmethionine plays in restriction.

At the molecular level the most likely way in which these three host specificity genes function is through the formation of oligomeric enzyme(s) composed of three different polypeptide subunits. While we have no information concerning the number of each type of polypeptide subunit involved, four general modes of subunit interactions were listed in the Introduction. These results enable us to rule out the first two of these models both of which involve only two types of subunit interacting to form the specific restriction enzyme.

There remain the other two models, between which it is very difficult to distinguish. The first of these supposes that only two types of subunit interact to form the modification enzyme, while the other model supposes that as for restriction all three subunits interact to form modification enzyme. The evidence presented here does not bear directly on this problem. However, from the behaviour of conventional r^-m^+ mutants, if *hsr* function is necessary for modification we must suppose that such mutants produce an inactive form of the *hsr*-directed subunit still able to combine with the *hsm*-directed subunit. This would be quite the contrary situation to the one described here where it appears that mutations in *hsm* more readily impair interaction with *hsr* than modification activity. It will clearly be necessary to isolate mutants defective in modification in which the mutation is unambiguously located in *hsr* in order to establish the correctness of the fourth model.

A possible alternative explanation could be that the mutations are in an additional fourth gene which regulates the functions of the other three genes. However, in this case we could expect such mutations unless they are operator or polar mutations to be *trans* dominant, and clearly in the merodiploids we have examined they are not. *Trans* dominant host specificity mutants have been isolated which map close to the

other host specificity genes (Boyer & Roulland-Dussoix, 1969). However, it is possible to accommodate these mutations within our model if we assume that they are mutations in *hsm* and that they direct the synthesis of altered subunits which bind more efficiently than wild type to form inactive oligomeric enzymes.

Less likely, if a fourth gene function is involved which in some way influences the temperature stability of the oligomers, then we would be obliged to conclude that all of the mutants we have isolated were mutated in that gene and that the $F' r_{-B}^- m_{+B}^+$ carried the wild-type allele and in addition conclude that the $F' r_{-B}^- m_{-B}^-$ (2) which did not complement with our mutants also carried a mutation in the postulated fourth gene.

There appears to be a gradient among the phenotypes expressed by ethylmethane sulphonate-induced mutations in *hsm* varying from impairment of restriction only at high temperature with only a 100-fold effect on modification (mutant 1-30) to the extreme case of impairment of restriction at low and high temperatures together with a 1000-fold effect on modification (mutant 1-71). On the assumption that relatively few amino acids will be directly involved in the active site of the oligomeric enzyme, most mutations in *hsm* will cause disturbance to the conformation of the *hsm*-directed subunit altering its binding in the oligomer. The results of interference with the allosteric interactions will range from disruption of functions dependent on the conformation of the entire oligomer to total loss of enzyme activity. We interpret the effect of *hsm* mutations on restriction to be due to an important role of the *hsm*-directed subunit in the oligomeric protein responsible for restriction. Since the effects of the mutations in the *hsm* gene appear to be less severe on modification than on restriction, the conformation of the *hsm*-directed subunit is probably of greater importance in the functioning of restriction than of modification. This may provide a clue to the structure of the enzymes.

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