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Genetics of host-controlled restriction and modification in *Escherichia coli*

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1. INTRODUCTION

In recent years many aspects of the processes of restriction and modification which characterize the host controlled modification (HCM) of bacteriophages in *Escherichia coli* have been clarified (reviewed by Arber (1965*a*) and Klein (1965)).

Modification is a process which acts directly on DNA and may take the form of specifically altering certain base sequences by methylation (Arber, 1965*b*; Klein & Sauerbier, 1965; Arber & Smith, 1966). Therefore, DNA synthesized in a particular strain may carry a characteristic pattern.

When a cell is infected with bacteriophage DNA which does not bear an acceptable modification, phage growth is restricted and the DNA may be rapidly degraded shortly after entry at a site near the cell surface (Dussoix & Arber, 1962; Schell & Glover, 1966).

In several laboratories mutants have been isolated which either have lost the ability to restrict but are still able to modify DNA or have lost both the ability to restrict and to modify DNA. If the wild type is represented as r^+m^+ then these two classes of mutants can be represented as r^-m^+ and r^-m^- respectively (Glover, Schell, Symonds & Stacey, 1963; Wood, 1966; Lederberg, 1966). An important feature of all these investigations is that among the mutants of phage P1, *E. coli* K and *E. coli* B selected for their r^- phenotypes there are roughly equal numbers which are m^+ and m^-. That is, mutations leading to the double phenotypic change r^-m^- occur as frequently as mutations leading to the single change, r^-m^+. The genetic location of both these mutations has been roughly determined in *E. coli* K and *E. coli* B (Colson, Glover, Symonds & Stacey, 1965; Wood, 1966) and similar conclusions have been reached by Boyer (1964) and by Hoekstra & de Haan (1965) from genetic crosses between *E. coli* K and *E. coli* B.

In this paper we describe: (i) the results of genetic experiments which locate the sites of these mutations close to the *serB* locus, (ii) the results of genetic crosses between different HCM mutants, and (iii) the results of experiments designed to elucidate the number of functional units involved in the control of HCM and the relationships between them.

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2. METHODS

Bacteria. E. coli K 12. The following strains were employed: C600 (Appleyard, 1954); HfrH (Hayes, 1953). *E. coli* B. This was the derivative B251 which is able to adsorb phage λ (Arber & Dussoix, 1962). *E. coli* C (Bertani & Weigle, 1953).

Bacteriophages. Phage λ and a virulent mutant λ*v* (Jacob & Wollman, 1954); phage P1 (Lennox, 1955).

Phage techniques. The general phage techniques are as described by Adams (1950). Special techniques relating to λ are those described by Arber (1958, 1960).

Tests for restriction and modification. Restriction was scored with λ*v*.B λ*v*.K and λ*v*.C by the method described by Colson *et al.* (1965). Modification was scored using standard indicator strains B, K and C by the methods of Colson *et al.* (1965).

Transduction. P1 lysates were prepared and transduction carried out following the procedure of Glover (1962).

Conjugation. Hfr × F^- crosses were performed following the methods described by Colson *et al.* (1965).

Mutagenesis. Log phase cultures, approximately 5×10^8 bacteria per ml., were centrifuged and resuspended in fresh broth at pH 7·4. *N'*-*N*-methyl-*N*-nitroso-guanidine (NTG) was added to a final concentration of 60 μg./ml. and the suspension incubated at 37 °C for 15 min. NTG was removed by twice washing in fresh broth and the cells were finally resuspended in 10 × the volume of broth and incubated at 37 °C to permit expression of induced mutations.

Zygotic induction. The HfrH $(\lambda)^+$ donor culture was washed twice in fresh broth and treated with anti-λ serum for 15 min. at 37 °C to inactivate free λ particles in the culture. Equal volumes of donor and F^- $(\lambda)^-$ *str-r* recipient cultures were mixed together and mated for 50 min. at 37 °C. The mixture was diluted 10^{-4} into fresh broth containing 0·01 M-$MgSO_4$ and 250 μg./ml. streptomycin. Free phage was assayed by the chloroform method and infective centres assayed at zero time plus 10 min. after the end of mating. Zygotic induction was complete after 90 min. aeration at 37 °C and the burst was assayed after chloroform treatment.

3. RESULTS

(i) *The linkage of r^-m^+ and r^-m^- mutations to* thr *in Hfr crosses*

We have previously shown that in *E. coli* K12 the mutations r^-m^+ and r^-m^- map close to *thr* and on the opposite side of it to *leu* (Colson *et al.* 1965). Table 1 summmarizes the results of crosses in which the donor strain used was HfrH, but instead of carrying the normal host specificity genes of K it carried host specificity genes from *E. coli* B (Table 1, cross 3) or an *E. coli* B r^-m^+ mutant (Table 1, crosses 1 and 2).

Clearly the r^-m^+ mutation in the donor is closely linked to *thr*, and since selection against the inheritance of thr^+ considerably reduces the inheritance of r^-m^+ while selection against the inheritance of leu^+ has little effect (cross 1) it can be concluded that, as in K, *thr* lies between r^-m^+ and *leu*. The reciprocal cross 2 confirms this result and cross 3 shows that r^-m^- also maps close to *thr*.

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The crosses listed in Table 2 illustrate that the genes controlling K host specificity are homologous with those controlling B host specificity. The recombinants express either the donor or the recipient type of host specificity and not an additive host specificity combining characteristics from K with characteristics from B. When the donor is K and the recipient B the majority of *thr*$^+$ recombinants express the donor K type of host specificity. Conversely, when the donor is B and the recipient K the majority of *thr*$^+$ recombinants express the donor B type of host specificity.

Table 1. *Linkage of* thr$^+$ *and host specificity in crosses with* E. coli *B*

Host specificity of parent strains			Donor type host specificity among selected recombinants (%)		
Cross	Hfr	F$^-$	*thr*$^+$ *leu*$^+$	*thr*$^+$ *leu*$^-$	*thr*$^-$ *leu*$^+$
1	Br$^-$m$^+$	Br$^+$m$^+$	78·0	69·0	15·0
2	Br$^+$m$^+$	Br$^-$m$^+$	81·0	71·0	14·0
3	Br$^-$m$^+$	Br$^-$m$^-$	73·0	63·0	28·0

In each cross equal volumes of donor HfrH *thi* bacteria were mixed with F$^-$ *thr leu isl str-r* bacteria and mated at 37° for 1 hr. Suitable dilutions were then plated on selective media and the different recombinant classes scored by replica plating. At least 100 of each class of selected recombinants after purification were scored for restriction and modification.

Table 2. *Host specificity of selected recombinants from crosses between* E. coli *K and* E. coli *B*

Host specificity of parent strains			Host specificity among selected recombinants (%)		
Hfr	F$^-$		*thr*$^+$ *leu*$^+$	*thr*$^+$ *leu*$^-$	*thr*$^-$ *leu*$^+$
Kr$^-$m$^+$	Br$^-$m$^+$	Like donor	74	85	8
		Like recipient	26	15	92
Kr$^-$m$^+$	Br$^-$m$^-$	Like donor	66	85	21
		Like recipient	34	15	79
Kr$^-$m$^-$	Br$^+$m$^+$	Like donor	55	64	9
		Like recipient	45	36	91
Br$^+$m$^+$	Kr$^+$m$^+$	Like donor	82	75	8
		Like recipient	18	25	92

Experimental procedure as in Table 1.

(ii) *Cotransduction of host specificity,* serB *and* thr *with phage P1*

The linkage of host specificity and *thr*$^+$ was confirmed by transduction with phage P1. The results of these experiments, summarized in Table 3, show that among selected *thr*$^+$ transduced colonies about 3% also carry the host specificity of the donor. This figure is comparable to that found for the cotransduction of *thr*$^+$ and *leu*$^+$ (Lennox, 1955) and is in agreement with conclusions drawn earlier from the results of conjugation experiments (Colson *et al.* 1965; Wood, 1966; Hoekstra & de Haan, 1965) that *thr* was roughly equidistant between *leu* and

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host specificity. As expected no cotransduction of *leu*+ and host specificity was detected.

Attempts to increase the precision of mapping the genes controlling host specificity by utilizing the gene *R try*, a regulatory gene controlling tryptophan synthesis and conferring resistance to 5-methyl tryptophan which maps in the *thr* region (Cohen & Jacob, 1959) were not successful. Nor was any advantage gained from using strains carrying a gene, *val-rB*, conferring valine resistance in K12 which maps close to *thr* (Glover, 1962).

In *Salmonella* two loci concerned with the synthesis of serine are known. The first, *serA*, determines the synthesis of 3-phosphoglycerate dehydrogenase and is not cotransduced with the second locus, *serB* which determines the synthesis of phosphoserine phosphatase. The *serB* locus is however cotransduced with *thr* (Umbarger, Umbarger & Siu, 1963). In *E. coli* only the *serA* locus had been identified by Umbarger *et al.* (1963), although the enzyme phosphoserine phosphatase had been identified in *E. coli* by Smith, Shuster, Zimmerman & Gunsalus (1956). We, therefore, isolated *ser* mutants after mutagenesis with NTG, followed

Table 3. *Cotransduction of host specificity and* thr

Expt no.	Recipient strain	No. of *thr*+ tested	No. with donor host specificity
1	K r^-m^- *thr*	99	1
2	K r^-m^- *thr*	100	3
3	*E. coli* C *thr*	69	2
4	K r^-m^- *thr*	760	28
	Total	1028	34

Log phase cultures of recipient bacteria were centrifuged and resuspended at 4×10^8 cells per ml in broth containing 10^{-2} M $CaCl_2$. A donor lysate was prepared on K r^+m^+ *thr*+ bacteria and added to a multiplicity of *c.* 0·5 and allowed to adsorb for 90 min. at room temperature. The suspension was centrifuged, resuspended in buffer containing 0·5% (w/v) sodium citrate and suitable dilutions were plated on selective media containing citrate. Host specificity was scored after purification of the colonies on selective media.

by penicillin selection. Ten independent *ser* mutants were isolated from Hfr H and 14 from strain C600. Each mutant was used either as donor or recipient in a conjugation experiment and its linkage to *thr* and *leu* was measured. None of the 14 *ser* mutants isolated in C600 was linked to *thr*, but of the 10 *ser* mutants isolated in HfrH one was very closely linked to *thr*. The location of this *ser* mutation was confirmed by P1 transduction experiments which are summarized in Table 4. Clearly this *ser* mutation is located between the genes controlling host specificity (cotransduction about 20%) and *thr* (cotransduction about 50%). We therefore conclude that the *ser* locus involved is analogous to the *serB* locus of *Salmonella*.

Further experiments not listed in Table 4 have shown that r^-m^+ and r^-m^- mutations isolated in *E. coli* B also show about 20% cotransduction with the *serB* locus.

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One additional feature concerning this *ser* mutant must be mentioned. It was isolated in a derivative of HfrH which already carried a host specificity mutation r^-m^+. After isolation and purification this strain was found to be r^-m^-. However, the change from r^-m^+ to r^-m^- is completely independent of the *ser* mutation. Reversion to *ser*$^+$ occurs quite frequently and all the *ser*$^+$ reversions scored were r^-m^-. In addition, in both conjugation and transduction experiments recombinants can be obtained between *ser* and r^-m^-. We conclude that the second change from r^-m^+ to r^-m^- was the result of a second independent NTG induced mutation. Subsequently, we have isolated further *serB* mutants after mutagenesis without observing concomitant alterations in host specificity.

Table 4. *Cotransduction of* serB, thr, leu *and host specificity by Phage P1*

P1 lysates prepared on	Recipient strains	Selected markers	Nos. tested	No. with co-transduced marker	Frequency of cotransduction (%)
K *thr*$^+$ *leu*$^+$ *ser*	K *thr leu ser*$^+$	*thr*$^+$	860	27 (*leu*$^+$)	2·1
		leu$^+$	1140	24 (*thr*$^+$)	3·1
		leu$^+$ *thr*$^+$	41	1 (*ser*)	2·4
		leu$^+$	1140	0 (*ser*)	< 0·1
		thr$^+$	1230	632 (*ser*)	51·4
Kr^+m^+ *ser*$^+$	Kr^-m^- *ser*	*ser*$^+$	100	20 (r^+m^+)	20·0
Kr^-m^+ *ser*$^+$	Kr^+m^+ *ser*	*ser*$^+$	185	44 (r^-m^+)	23·8

Experimental procedure as in Table 3.

(iii) *Transduction experiments between host-specificity mutants*

In the absence of a satisfactory outside marker on the opposite side of host specificity to *serB*, transduction experiments were carried out between *thr serB*$^+$ donor strains and *thr*$^+$ *serB* recipients in which selection was made for the inheritance of *serB*$^+$ and against the inheritance of *thr*. In other words, recombination was forced between *thr* and *serB*. In this way it was hoped that the effect of negative interference (if any) would act to increase the amount of recombination in the adjacent region controlling host-specificity (Maccacaro & Hayes, 1961). Strains for these experiments were constructed by introducing the *serB* marker into *thr* strains either by conjugation or transduction and selecting for *thr*$^+$ *serB* recombinants. P1 lysates were then prepared on each of the donor strains and used to transduce the *serB thr*$^+$ recipients. In each experiment *ser*$^+$ *thr*$^+$ transductants were selected, purified and then tested for host-controlled restriction using λ.C, λ.K and λ.B and the host specificity of the phage produced by each tested colony was scored by plating on *E. coli* K, B and C. In this way, donor type HCM as well as any recombinant type of HCM could be detected.

Table 5 summarizes the results of these experiments. A total of 4059 *ser*$^+$ *thr*$^+$ transductants was tested among which donor type HCM was present in 590. This gives an average cotransduction frequency with *serB* of 11·7 % excluding Expt. 10 in which donor and recipient HCM could not be distinguished. A small

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number of colonies were found which expressed neither the donor nor the recipient type of HCM but a different one. Experiment 1 yielded one K r^+m^+ transductant in a cross between an r^-m^+ donor with an r^-m^- recipient and the reciprocal cross (Expt. 2) yielded 6 Kr^+m^+ transductants. These r^+m^+ colonies are unlikely to be reversions for the following reasons. First, after treatment of r^-m^+ strains with the mutagen NTG more than 3000 colonies were scored for HCM properties and no reversions to r^+m^+ were obtained. Secondly, the proportion of r^+ to r^- bacteria in

Table 5. *Transduction between HCM mutants of* E.coli *B and* E. coli *K*

(In each experiment the donor was *serB*$^+$ *thr* and the recipient *serB thr*$^+$.)

Expt. no.	Donor HCM	Recipient HCM	No. of *ser*$^+$ *thr*$^+$ tested	No. with donor HCM	No. with non-parental HCM
1	K r^-m^+	K r^-m^-	510	70	1 K r^+m^+
2	K r^-m^-	K r^-m^+	498	95	6 K r^+m^+
3	B r^-m^+	B r^-m^-	407	89	2 B r^+m^+
4	K r^-m^+	B r^-m^-	420	60	1 K r^+m^+
5	B r^-m^-	K r^-m^+	300	16	1 K r^+m^+
6	B r^+m^+	K r^-m^+	500	28	4 K r^+m^+
7	K r^+m^+	B r^-m^+	398	53	3 K r^-m^+
8	K r^+m^+	K r^-m^- 2-step	106	26	1 K r^-m^+
9	K r^+m^+	K r^-m^- 2-step	400	153	2 K r^-m^+
10	K r^-m^-	K r^-m^- 2-step	520	—	0
		Total	4059	590	21

Experimental procedure as in Table 3.

artificial mixtures of two strains can be enriched more than 100-fold by treatment with λv.C, which kills r^- bacteria more readily than r^+ bacteria yet no r^+ reversions have been obtained among several hundred colonies scored after λv.C treatment of r^-m^+ strains even when the selection was carried out on mutagenized cell populations. The reversion rate from r^-m^+ and r^-m^- to r^+m^+ is thus less than 1 in 10^4. Thirdly, the efficiency of plating (e.o.p.) of phage λ obtained by induction from r^-m^- λ-lysogenic bacteria on r^+m^+ bacteria does not differ significantly from the e.o.p. of λ.C on restricting hosts. Even after mutagenesis followed by induction, the λ obtained from r^-m^- strains does not plate better on r^+m^+ hosts than does λ.C. The e.o.p. of λ from m^- bacteria on r^+ hosts is approximately 4×10^{-4} and an increase in e.o.p. of fivefold would very easily have been detected by this method. Therefore, an upper limit can be set for reversion from r^-m^- to r^+m^+ at less than 10^{-3}. Rejecting then reversion as an explanation for the origin of these r^+m^+ transductants we conclude that they arise by recombination between the parent strains. This implies that the mutational site responsible for the r^-m^- phenotype is separable by recombination from the site responsible for the r^-m^+ phenotype. So that either recombination has taken place between two mutational sites in the same gene, one mutation producing the r^-m^+ phenotype the other producing the r^-m^- phenotype; or the two mutations are in different genes. If the latter is true, which

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seems more probable, it implies that in K r^-m^- there is an unexpressed r^+ gene. The same situation also applies in *E. coli* B since the cross between B r^-m^+ and B r^-m^- (Expt. 3) yielded 2 B r^+m^+ transductants. Transduction experiment 4 between K r^-m^+ and B r^-m^- yielded, in addition to the normal frequency of colonies transduced to donor type HCM, one colony which was K r^+m^+. It seems unreasonable to imagine that this could arise by spontaneous mutation in the recipient B r^-m^- strain, and control experiments described above make it unlikely that it could be due to P1 transduction from a K r^+m^+ reversion in the donor population. We conclude that it is due to recombination between K r^-m^+ and B r^-m^-. It is important to note that not only does this recombinant express the modification property of the K donor but it also expresses fully the normal restriction property of wild-type K r^+m^+. If, as has been said, the K r^+ property of the recombinant does not come from the donor strain what kind of recombination event could account for its origin? One can suppose that it arises by recombination between the K r^- mutation and the B r^- mutation such that the recombinant structure, in spite of being a hybrid between K and B, functions as K r^+ and not as B r^+ nor as an intermediate between the two. If a hybrid structure is produced then it would appear to function always like K r^+ and never like B r^+, since the recombinant obtained from the reciprocal transduction (Expt. 5) was also K r^+m^+. Alternatively, it could be that B carries unexpressed, i.e. recessive, K host specificity but that K does not carry unexpressed B host specificity. Recombination between B and K host specificity mutants could then restore normal K HCM functions but never normal B HCM functions. This, however, seems to be very unlikely since the B r^-m^- mutants do not express any restriction or modification properties characteristic of K.

The most likely explanation for the origin of the K r^+m^+ recombinants from crosses between K r^-m^+ and B r^-m^- would seem to be that a third function is involved which can confer K or B specificity to an otherwise non-specific and presumably, therefore, non-functional *r* gene product. This third function could be either associated with the m^+ gene or specified by a third gene. The B r^-m^- mutant would, on this hypothesis, have lost the ability to specify the function of r^+ due either to a mutation in m or to a mutation in a third gene, the normal function of which is to confer B specificity to both r^+ and m^+. The K r^-m^+ mutant would, on the other hand, carry an intact specificity-determining gene, but have a defect in the r gene. By recombination between the two mutants a recombinant structure can be obtained which would carry intact K specificity from the K r^-m^+ parent and the r^+ gene from the B parent, and would consequently express K specificity in both restriction and modification in spite of having inherited r^+ and perhaps m^+ from the B parent. This hypothesis provides a satisfactory explanation for the origin of the recombinants obtained in Expts 4 and 5 and also in Expts 6 and 7, the K parent in each case contributing intact K-specificity to the recombinants. Whether this K specificity is associated with the m^+ gene from K or constitutes a third gene cannot be distinguished from the recombinant phenotypes obtained.

In Expts 8–10 another class of HCM mutants was employed. The phenotype of this class is r^-m^- but they were derived from wild type r^+m^+ strains by two in-

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dependent mutational steps. First, an r^-m^+ mutant was obtained by selecting for the r^- phenotype and then this mutant was made lysogenic for phage λ. After mutagenesis, surviving colonies were then screened to determine the host-specificity of the λ they produced. About 1 % of the survivors after treatment of K r^-m^+ (λ) with NTG produced λ which did not carry K specificity. These mutants have been designated K r^-m^- 2-step to distinguish them from the r^-m^- mutants obtained in 1 step from the r^+m^+ wild-type. In Expts. 8 and 9 the donor was K r^+m^+ and the recipient K r^-m^- 2-step. A total of three recombinants was obtained, each of which was K r^-m^+.. Clearly then we have separated the two mutations present in the recipient by recombination with the wild-type parents.

According to the hypothesis suggested to explain the origin of these recombinants three types of two-step r^-m^- mutants are possible at the genetic level depending on whether the specificity of r is conferred by the m gene itself or by a third gene determining the specificity of both *r* and *m*. In the first case one would expect every r^-m^- two-step mutant to be a true m^- while in the second case the r^-m^- phenotype could be accounted for either by a mutation in the gene determining r and m specificity or by a mutation in the m gene. Each of these three genotypes for 2-step r^-m^- mutants can yield r^-m^+ recombinants when crossed with wild-type. Therefore, on the basis of these experiments we cannot determine the true genotype of the two-step mutants. In Expt. 10 where the donor was K r^-m^-, a one-step mutant, and the recipient was a K r^-m^- two-step mutant, non-parental recombination phenotypes could only have been obtained if the suggestion that K specificity is determined by a third gene was true and if the second mutation in the recipient strain involved the m gene only. In that case two types of recombinant are possible. First, the recombinant could inherit the K specificity determinant from the recipient together with r^+ and m^+ from the donor to produce a wild-type recombinant. Secondly, an r^-m^+ recombinant could be produced by combining the K specificity and r^- from the recipient with the m^+ gene from the donor. On the other hand, if the genetic basis of the two-step mutant parent was a mutation affecting K-specificity then no recombinants distinguishable from the r^-m^- phenotypes of the two parents could be produced, since both parents would contribute mutant forms of the K specificity determinant. As shown in Table 5 no recombinants were obtained among 520 tested transductants from this cross. The evidence, therefore, favours marginally the idea that the two-step mutation in this strain involves the loss of the ability to confer K specificity with or without impairing the m gene itself.

In addition to the recombinants listed in Table 5 one other recombinant was obtained in crosses. This recombinant, B r^+m^+ was found among thr^+ recombinants obtained from the cross between Hfr B r^-m^+ and F^- B r^-m^- listed in Table 1.

In summary, then, we can say that the non-parental recombinants obtained from crosses between HCM mutants cannot be satisfactorily exlained either by reversions in the parent strains, or on a simple two-gene model in which the specificity of restriction is determined solely by the r gene and the specificity of modification is determined solely by the m gene. Instead it is necessary to invoke

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an independent function controlled either by the m gene or by a third gene, which confers host specificity upon otherwise non-specific r and perhaps also m proteins. Clearly evidence of complementation between mutants is necessary to establish this hypothesis.

(iv) *Zygotic induction as a test for complementation*

To demonstrate complementation between two different mutants it is necessary to establish some kind of partial diploid. The system *par excellence* for the demonstrations of complementation in *E. coli* is the F′ heterogenote. However, a suitable F′ carrying the chromosomal region concerned with HCM was not available. Instead, it was decided to look for complementation in merozygotes produced by conjugation between Hfr and F^- bacteria. This system is limited to looking for complementation between mutants both of which were unable to confer normal host modification. Previous experiments have shown that the ability of zygotes to carry out host restrictions is impaired (Glover & Colson, 1965) and also the kinetics of expression of restriction appear to be slower than the expression of modification which is quite rapid (Arber & Dussoix, 1962). Control crosses between restricting donor bacteria and non-restricting recipient F^- bacteria made it quite clear that expression of restriction in zygotes was not efficient, in fact, the zygote population did not restrict λ more efficiently than the unmated F^- parent. To measure the capacity of zygotes to modify λ the host specificity of λ produced by zygotic induction after mating Hfr $(\lambda)^+$ with non-lysogenic F^- recipients was tested. Experiment 1 in Table 6 shows that zygotes produced by mating K × K produce, after zygotic induction, a burst consisting entirely of λ.K particles. In order to test for complementation between non-modifying mutants it was necessary to show that the genes conferring host specificity could be satisfactorily transferred to zygotes and express themselves rapidly enough to confer host specificity on λ produced by zygotic induction in the cytoplasm of a non-modifying mutant. Experiments 2 and 4 (Table 6) show that the burst of λ obtained from zygotes after mating contains a large fraction of phage particles carrying the host specificity of the donor strain. Thus the time interval between the entry of the genes determining host specificity, which are transferred early by HfrH some 18 min before λ, combined with the time required to produce mature λ particles is sufficient to permit efficient modification of a large fraction of the burst. Conversely, if instead of HfrH the donor is Hfr-13, which transfers the genes controlling HCM late very few λ particles in the burst carry the host specificity of the donor (Expts. 5–7).

According to the hypothesis suggested to account for non-parental recombinants in crosses, complementation would be possible between one-step and two-step r^-m^- mutants as well as between different two-step mutants only if a genetically independent function confers specificity on both r and m. In that case two-step mutants can be of two kinds genetically. One class would be mutant in the gene conferring host specificity as well as carrying the original r^- mutation; the other class would have a mutation in the m gene as the second step. Complementation should therefore be possible between mutants of one class and the other. In addi-

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tion, the class of two-step mutants with a mutation in the m gene should be able to complement one-step r^-m^- mutants, which have a normal m^+ gene and are mutant only in the gene determining host specificity. Conversely, if specificity is controlled by the m gene itself no complementation would be expected between m^- mutants since only one such class of mutants could arise. A total of eleven two-step mutants have been isolated in K. All of them have been tested in the

Table 6. *Zygotic induction complementation tests between HCM mutants*

	Hfr $(\lambda)^+$ donor HCm	F^- recipient HCM	Percentage burst able to form plaques on		
			E. coli C	*E. coli* B	*E. coli* K
1	HfrH K r^+m^+	K r^+m^+	100	—	100
2	HfrH K r^+m^+	K r^-m^-	100	—	16
3	HfrH K r^-m^-	K r^+m^+	100	—	100
4	HfrH B r^-m^+	C	100	50	—
5	Hfr-13 K r^+m^+	K r^+m^+	100	—	100
6	Hfr-13 K r^+m^+	C	100	—	0·17
7	Hfr-13 K r^+m^+	B r^+m^+	100	100	0·5
8	HfrH K r^-m^- 2·13	K r^-m^- 2·184	100	—	0·05
9	HfrH K r^-m^- 2·25	K r^-m^- 2·184	100	—	0·01
10	HfrH K r^-m^- 2·617	K r^-m^- 2·184	100	—	0·25
11	HfrH K r^-m^- 2·582	K r^-m^- 2·184	100	—	0·05
12	HfrH K r^-m^- 2·249	K r^-m^- 2·184	100	—	0·1
13	HfrH K r^-m^-	K r^-m^- 2·184	100	—	0·02
14	HfrH K r^-m^- 2·13	K r^-m^-	100	—	0·06
15	HfrH K r^-m^- 2·1	K r^-m^-	100	—	0·02

The donor culture was washed twice in buffer and resuspended in broth containing anti-serum to neutralize free phage particles and then mixed with an equal volume of the recipient culture. Mating was terminated after 50 min. at 37° and the mixture diluted 10^{-4} into fresh broth containing 10^{-2} M-$MgCl_2$ and streptomycin to kill the donor. The zygotes were aerated for 90 min and the burst assayed after chloroform treatment on *E. coli* C, K, and B. A sample was taken after mating to assay the amount of residual free phage and the number of infective centres.

zygotic induction complementation tests against at least one other two-step r^-m^- mutant and some of them have been tested against one another and against an r^-m^- one-step mutant. The results of a number of typical experiments are shown in Table 6 (Expts. 8–15). None of the two-step mutants yielded a significant amount of λ.K in the burst after zygotic induction from crosses with other two-step mutants. It would seem then that all of these two-step mutants are of the same class genetically. None of the two-step mutants yielded a significant amount of λ.K from crosses with one-step mutants (Expts 14 and 15) from which it can be concluded that the second mutation in the two-step mutants is the same as that in the one-step mutants, thus precluding the possibility of complementation. Thus, either we failed to isolate a mutation in the m gene itself in a set of eleven independent r^-m^- two-step mutants and obtained only the class which has lost the ability to confer K specificity to both r and m or the K specificity is genetically indistinguishable from m.

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4. DISCUSSION

The starting-point in several investigations into the genetic control of host modification has been the isolation of non-restricting mutants by various selective techniques. The mutants isolated in this way can be classified into two groups according to whether they are able to confer normal host specificity upon λ DNA or not. The result obtained in several different laboratories is that among non-restricting r^- mutants there are roughly equal numbers of modifying m^+ and non-modifying m^- types, (Glover *et al.* 1963; Wood, 1966; Lederberg, 1966). In other words, the single change from r^+m^+ to r^-m^+ occurs about as frequently as the double phenotypic change to r^-m^-. This perplexing problem has arisen in each of the three HCM systems, which have been examined genetically.

The mapping experiments reported here, as well as the result of earlier investigations, agree in showing that both the mutation leading to the r^-m^+ phenotype and the mutation leading to the r^-m^- phenotype map close to *thr* in *E. coli* K12 and *E. coli* B (Colson *et al.* 1965; Wood, 1966). Further experiments described above locate the position of these mutations close to the *serB* locus and on the opposite side of it to *thr*. The absence of satisfactory outside markers and more importantly the lack of adequate quantitative selective techniques for the recovery of recombinants between HCM mutants precludes for the time being fine structure genetic analysis. However, the transduction experiments we have described yielded a total of 21 transductants which had non-parental HCM properties. We have shown that these cannot be accounted for by reversion of the recipient nor by reversions in the donor population from which the P1 lysates were prepared. We, therefore, regard these non-parental transductants as recombinants between the two parents.

Several different genetic models can be constructed which would account for the origin of these recombinants. First, it can be supposed that there is a single gene which specifies a single protein, and this protein has two functions, restriction and modification. Since the substrate for both restriction and modification appears to be DNA which lacks a particular modification it can be assumed that the protein carries a site for recognizing unmodified sites on DNA. Which of these two functions the protein carries out may depend upon the location of the enzyme, or its configuration or whether or not it is charged with methyl groups. Some mutations then affect one function leading to the r^-m^+ phenotypes other mutations affect both functions leading to the r^-m^- phenotype. The situation may be analogous to the *hisB* gene in *Salmonella typhimurium* which determines two functions: (i) imidazole glycerol phosphate phosphatase and (ii) L-histidinol phosphate phosphatase. Some mutants in the *hisB* locus lack only one function while others lack both. (Loper *et al.* 1964). The recombinants we have obtained can be accounted for on this model by recombination between two different mutant sites in the same gene. No difficulties arise in accounting for recombinants between mutants of K in K × K crosses nor for mutants of B in B × B crosses, but in the case of K × B crosses it is necessary to make the assumption that the mutations do not involve the sites

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conferring specificity on the enzyme, so that by recombination either donor or recipient type of host specificity can be recovered.

An alternative model can be constructed based upon two distinct genes r and m specifying restriction and modification respectively. One mutation to r^-m^+ is easily explained on this model, but to account for the second class of mutants, r^-m^-, it must be supposed that the two genes are adjacent and the mutations are deletions or that the two genes form an operon and the mutation is polar. The recombinants from K × K or B × B crosses can be accounted for by recombination between two mutational sites one of which may be polar, or between one mutation and a non-overlapping deletion. But a serious difficulty arises in considering the nature of recombinants obtained from K × B crosses. On this two-gene model the specificity of restriction must be determined by the r gene itself and the specificity for modification must be determined by the m gene.

To account for recombinants possessing K specificity from K r^-m^+ × B r^-m^- crosses the recombinant structure must, in spite of being a hybrid between K and B, expess K specificity alone and not some behaviour partly that of K and partly that of B. This model clearly predicts that recombinants with K specificity for restriction and B specificity for modification should occur. No such recombinants have been obtained as a result of many such crosses between K and B, although it can be argued that such recombinants would not be viable.

The most satisfactory model is based upon a specificity-determining function. We have supposed that there is a gene r which determines the synthesis of a protein essential for restriction, a gene m which determines the synthesis of a protein essential for modification, and a third function which involves the conferring of strain specificity upon the products of the r gene and also, but not necessarily, upon the m gene as well. In fact, whether the third function confers specificity to the r protein alone and is the m protein itself or, on the other hand, the third function confers specificity to both r and m proteins and is determined by a third gene cannot be distinguished by the experiments presented here. All non-parental recombinants can be accounted for by assuming that a specificity function is present in the parent which expresses the r^-m^+ phenotype. The recombination event then occurs either between the third gne and the other two, or between r and m.

In addition, the model predicts that recombinants from K and B crosses will express either K or B specificity depending solely upon whether the specificity determinant is inherited from the K or from the B parent. They will not have mixed properties intermediate between K and B.

At the molecular level, one way in which this model could operate would be through oligomeric enzymes. The protein conferring specificity could form a subunit of the enzyme essential for restriction, the other subunit being the r protein. The specificity-determining protein could also be a subunit of the enzyme essential for modification, the second subunit in this case being the m protein. On the alternative model the m protein would provide a specificity-determining function when combined as a subunit with the r protein, but when not so combined would alone

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determine the specificity of modification. At the purely genetic level it is not a simple matter to distinguish between the proposed models. Crosses between mutants will merely serve to order the sites of mutations, giving no information about the number of functionally distinct cistrons involved in the genetic control of HCM. The results of the complementation tests by zygotic induction are not conclusive, and some reasons for this have been suggested in the previous section. Another important point that must not be lost sight of is that mutants deficient only in their ability to modify solely due to a mutation in the m gene have not been unambiguously identified. It may be that none of the 2-step r^-m^- mutants which were used in the complementation tests carries such a mutation but rather all of them are mutants in r and in the third gene. If this is so then complementation would not have been possible in any of the experiments. Furthermore, genetic experiments with these mutants cannot therefore contribute any information about the location of the m gene and, in any case, unless m^+ was transferred to the zygotes during mating, complementation was clearly not possible. Other more satisfactory methods for measuring complementation are at present being tested in this laboratory. Preliminary experiments (Glover, 1968) indicate that F′ heterogenotes of the structure Kr^+m^+/Br^-m^+ restrict λ.C, λ.K and λ.B and produce phage able to plate on *E. coli* B, K and C indicating that complementation between K and B can occur.

SUMMARY

The sites of mutations affecting host-controlled modification (HCM) have been mapped in *E. coli* K and *E. coli* B by conjugation and transduction experiments between mutants. These mutations all map close to the *serB* locus on the opposite side to the marker *thr*. Non-parental HCM has been observed among colonies obtained from P1 transduction experiments between HCM mutants. Control experiments have shown that these non-parental recombinants can not be accounted for by reversion of either parent and must result from recombination between mutants. Several genetic models are suggested which could account for these recombinants and an attempt is made to distinguish between various models by testing for complementation between mutants in a zygotic induction complementation test.

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