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Restriction Insensitivity in Bacteriophage T5

I. Genetic Characterization of Mutants Sensitive to *Eco*RI Restriction

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Unmodified bacteriophage T5 is able to to grow normally on bacterial hosts carrying three different *Escherichia coli* restriction systems, *Eco*K, *Eco*PI, and *Eco*RI. Under the same conditions, the plating efficiency of bacteriophage λ is less than 10^{-9}. At least in the case of *Eco*RI, this lack of in vivo restriction is not due to lack of restriction sites on the T5 DNA molecule. These observations suggest that bacteriophage T5 specifies one or more restriction protection systems. Mutants (*ris*) of T5 have been isolated which confer sensitivity to *Eco*RI restriction but not to *Eco*K or *Eco*PI. The mutations are located in the pre-early region of the genetic map but are too far apart to be alleles of a single gene. Complementation studies show that the *ris* mutants can be helped to grow on the *Eco*RI-restricting host by coinfection with $T5^+$. This result provides evidence for a restriction protection function but does not necessarily show that the *ris* mutants are defective in such a system.

The phenomenon of host-controlled restriction and modification has been elucidated using bacteriophage λ and *Escherichia coli* as a model system (1, 4). Restriction endonucleases recognize specific sequences on the DNA and cause cleavage of the molecule (16, 27). In the case of class II restriction enzymes (e.g. *Eco*RI), the site of recognition and the site of cleavage is identical (7) so that the sites can be located on the genome by physical (23) as well as genetical means (18). Class I restriction enzymes (e.g. *Eco*K and *Eco*PI) act by a different mechanism, since the site of cleavage is not the same as the site of recognition and they have different cofactor requirements (9, 17). In both cases, DNA not containing restriction recognition sites is insensitive to restriction in vivo and in vitro (18).

We investigated the in vivo restriction of bacteriophage T5 for both class I (*Eco*K and *Eco*PI) and class II (*Eco*RI) restriction systems and found, surprisingly, that T5 is insensitive to these systems individually or in combination. T5 DNA does not contain unusual bases (25), and it seems very unlikely that a DNA molecule of molecular weight 77×10^6 would contain no recognition sites for any of these three enzymes. In the case of *Eco*RI it is known that T5 DNA has six sites sensitive to *Eco*RI in vitro (5, 20).

A likely hypothesis is that T5 specifies one or more restriction protection systems such as have been found in bacteriophages T3, T7, and Mu 1 (6, 11, 22, 24). To test this possibility we isolated mutants of bacteriophage T5 sensitive to *Eco*RI, *Eco*K, and *Eco*PI restriction systems. This communication describes the genetic properties of mutants sensitive to the *Eco*RI restriction system.

Recently we have found that the *Eco*RI-sensitive phenotype of the *ris* mutants is due not to a defective restriction protection system but to the creation by mutation of new *Eco*RI sites located in the pre-early region of the DNA molecule, where *Eco*RI sites do not naturally occur.

MATERIALS AND METHODS

Media. L-broth contained 1% Difco tryptone broth, 1% NaCl, and 0.5% yeast extract, and for use with bacteriophage T5 it was supplemented with 1 mM $CaCl_2$. L-agar was made by the addition of 1.2% Difco agar to L-broth.

Bacterial and bacteriophage strains. *E. coli* strains are described in Table 1. Mutants of bacteriophage T5 (8, 13) were obtained from N. Kleckner or D. McCorquodale.

Transformation. Plasmid DNA was extracted and purified by the method of Smith et al. (21). Plasmids were transferred from one strain to another by transformation (15) followed by selection for resistance to ampicillin or immunity to colicin E1 (ColE1).

Mutagenesis. *E. coli* W3350 was grown in L-broth + 1 mM $CaCl_2$ to a density of 3×10^8 cells per ml. The culture was concentrated 10-fold by centrifugation and suspension in 10 mM Tris-hydrochloride (pH 7.4)-10 mM $MgCl_2$-1 mM $CaCl_2$ and infected with bacteriophage $T5^+$ at a multiplicity of infection of 10. After 20 min at room temperature, the culture was centrifuged, suspended in L-broth + 1 mM $CaCl_2$, and shaken at 37°C for 10 min. The bacteria were then centrifuged

TABLE 1. *Strains of E. coli*

Strain	Relevant genotype	Source or reference
W3350	*hsdR*$^+$ *hsdM*$^+$ *sup*^{0} *gal*	H. Echols
803	*hsdR*$^-$ *hsdM*$^-$ *metB supE*	N. Murray
ED8655	*hsdR*$^-$ *hsdM*$^+$ *metB supE*	N. Murray
NTP13 (1100)	ColE1, *Eco*RI R$^+$, *Eco*RI M$^+$/*endA thi*	N. Murray
NTP14 (1100)	ColE1, *Eco*RI R$^+$, *Eco*RI M$^+$ *ampR*/*endA thi*	N. Murray
NTP13 R$^-$M$^+$ (1100)	ColE1, *Eco*RI R$^-$, *Eco*RI M$^+$/*endA thi*	Yoshimori et al. (26)
NTP14 (W3350)	ColE1, *Eco*RI R$^+$, *Eco*RI M$^+$ *ampR*/*hsdR*$^+$ *hsdM*$^+$ *sup*^{0}	J. Davison[a]
NTP14 (ED8655)	ColE1, *Eco*RI R$^+$, *Eco*RI M$^+$ *amp*^{r}/*hsdR*$^-$ *hsdM*$^+$ *supE*	J. Davison[a]
ColE1 (ED8655)	ColE1/*hsdR*$^+$ *hsdM*$^+$ *supE*	J. Davison[a]
NTP13 R$^-$M$^+$ (ED8655)	ColE1, *Eco*RI R$^-$, *Eco*RI M$^+$/*hsdR*$^-$ *hsdM*$^+$ *supE*	J. Davison[a]
W3350 (PI)	*hsdR*$^+$ *hsdM*$^+$ *sup*^{0} (*Eco*PI R$^+$, *Eco*PI M$^+$)	J. Davison
NTP14 (W3350[PI])	ColE1, *Eco*RI R$^+$, *Eco*RI M$^+$/*hsdR*$^+$ *hsdM*$^+$ *sup*^{0} (*Eco*PI R$^+$, *Eco*PI M$^+$)	J. Davison[a]
JD197 (λBh*cI*$^-$*rex*$^+$)	λ*rex*$^+$	Pastrana and Davison (19)
JD197 (λBh*cI*$^-$*rex*$^-$)	λ*rex*$^-$	Pastrana and Davison (19)

[a] Strains were constructed in this laboratory by transformation using purified plasmid DNA obtained from other strains listed.

and suspended in 100 mM sodium citrate buffer (pH 5.5) containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co.) at a concentration of 1 mg/ml. After 30 min at 37°C, the culture was washed, suspended in L-broth + 1 mM $CaCl_2$, and shaken at 37°C until lysis.

Isolation of *Eco*RI-sensitive mutants of bacteriophage T5. Isolated plaques of mutagenized T5$^+$, growing on W3350, were transferred to bacterial lawns of W3350 and NTP14(W3350) (carrying the ColE1 type plasmid coding for *Eco*RI restriction and modification). Those plaques able to grow on W3350 but not on NTP14(W3350) were repurified by two sequential single-plaque isolations on W3350 and investigated as possible restriction-sensitive mutants.

Genetic mapping. ED8655 was grown to a density of 3×10^8 cells per ml and infected with each mutant of bacteriophage T5 at a multiplicity of infection of 5 phage per cell. After adsorption for 20 min at room temperature, the culture was diluted 20-fold into L-broth + 10 mM $CaCl_2$ and shaken at 37°C until lysis. The number of wild-type recombinants was measured by plating on a strain on which neither parent alone is able to grow.

RESULTS

Restriction insensitivity of bacteriophage T5. The results given in Table 2 show the relative plating abilities of unmodified λ$^+$ and T5$^+$ phages on various restricting hosts. It is clear that whereas bacteriophage λ is sensitive to all the *Eco*K, *Eco*PI, and *Eco*RI restriction systems, T5 is virtually insensitive even to the three systems in combination. It is unlikely that the T5 DNA molecule (77×10^6 daltons) completely lacks restriction sites for all three restriction systems, and in the case of *Eco*RI it is known that T5 DNA has six *Eco*RI cleavage sites (5, 20). It therefore seemed more probable that restriction insensitivity results from protection caused by a T5 coded gene product, and to identify such a protection system a search was made for a mutant in this hypothetical gene.

TABLE 2. *Efficiency of plating of bacteriophage T5$^+$ on various restricting hosts*[a]

Host	Restriction system	Efficiency of plating of bacteriophage	
		λ	T5
ED8655	None	1.0	1.0
NTP14 (ED8655)	*Eco*RI	3×10^{-4}	7×10^{-1}
ED8655 (PI)	*Eco*PI	1×10^{-4}	9×10^{-1}
W3350	*Eco*K	2×10^{-3}	9×10^{-1}
NTP14 (W3350)	*Eco*RI, *Eco*K	3×10^{-6}	7×10^{-1}
W3350 (PI)	*Eco*K, *Eco*PI	1×10^{-8}	9×10^{-1}
NTP14 (W3350 [PI])	*Eco*K, *Eco*PI, *Eco*RI	$<10^{-9}$	5×10^{-1}

[a] Bacteriophage grown on a host lacking a restriction-modification system were plated on hosts carrying combinations of the *Eco*RI, *Eco*PI, and *Eco*K systems.

T5*ris* mutants are sensitive to in vivo restriction by *Eco*RI. After mutagenesis, mutants (*ris*) were isolated that were unable to grow on strains carrying the plasmids NTP13 or NTP14, which are ColE1-type plasmids specifying the *Eco*RI restriction-modification system (21). The properties of three independent *ris* mutants are shown in Table 3. The mutants make plaques of normal size on hosts lacking the *Eco*RI system but, on restricting hosts, tiny plaques and normal-sized plaques are found at a 10^{-2} and 10^{-4} frequency, respectively. This behavior of forming plaques of two different sizes contrasts with the classical behavior of

TABLE 3. *Effect of EcoRI restriction on T5ris mutants*

Phage	Plating on host carrying EcoRI restriction system[a]			
	None	R^-M^+	R^+M^+ (tiny plaques)	R^+M^+ (normal plaques)
λ	1.0	7×10^{-1}		3×10^{-4}
T5	1.0	1.2		7×10^{-1}
T5*ris*1	1.0	7×10^{-1}	6×10^{-3}	4×10^{-5}
T5*ris*2	1.0	1.0	2×10^{-2}	1×10^{-4}
T5*ris*3	1.0	6×10^{-1}	5×10^{-2}	5×10^{-4}

[a] The results given represent the plating ability of $T5^+$ and its mutants on strains carrying (i) no restriction system [ED8655 or ColE1 (ED8655)]; (ii) a mutant of NTP13 that lacks *Eco*RI restriction but not *Eco*RI modification [NTP13 R^-M^+ (ED8655)]; (iii) and (iv) the complete *Eco*RI restriction modification system carried by plasmid NTP13 or NTP14 [NTP13 (1100) or NTP14 (ED8655)].

bacteriophage λ, which forms only normal plaques at low efficiency on the restricting host. The properties of large-plaque "revertants" of T5*ris* mutants are described in the accompanying paper (3).

Evidence that the *Eco*RI restriction system is responsible for the low plating efficiency of *ris* mutants is provided by the observation (Table 3) that *ris* mutants form plaques with normal efficiency on a strain carrying a mutant of plasmid NTP13 which lacks the *Eco*RI restriction endonuclease but which is not defective for modification (26). The *ris* mutants are not temperature sensitive or suppressible by *supE*.

T5*ris* mutants are insensitive to in vivo restriction by *Eco*K and *Eco*PI. To test whether *ris* mutants had also acquired sensitivity to in vivo restriction by *Eco*K and *Eco*PI, the mutants were grown on a host lacking the appropriate restriction-modification system and were then tested for their ability to form plaques on the restricting host. The results (Table 4) demonstrate that *ris* mutants are not sensitive to *Eco*K and *Eco*PI restriction, showing that they are not defective in a generalized restriction protection system.

Sensitivity to the λ*rex* product of bacteriophage λ. It was found that one of the three mutants (*ris*1) was also unable to grow on a strain lysogenic for bacteriophage λ (Table 5). To investigate the λ gene responsible for the inhibition, T5*ris*1 was tested for its ability to grow on a cryptic λ lysogen, in which the *rex* gene product is the only gene of λ expressed, and on an isogenic control strain in which the *rex* gene is inactivated by mutation (19). These experiments showed that the inhibition of T5 is due to the *rex* gene product of bacteriophage λ. Mutants (*lr*) of bacteriophage T5 that are unable to grow on a λ lysogen have been described previously (10) and map within the A1 gene located in the pre-early region of the T5 genetic map. The mechanism by which λ product inhibits the growth of T5*lr* mutants is not known.

To ascertain whether the *ris*1 mutation was responsible for both the *Eco*RI- and the λ-sensitive phenotypes, revertants were studied that had been selected for insensitivity to either *Eco*RI or to λ*rex* product. Of 21 independent λ*rex*-insensitive revertants, 5 had simultaneously become insensitive to *Eco*RI. Similarly, among 21 revertants selected for insensitivity to *Eco*RI, 11 were able to grow on λ lysogens. These results show that the *ris*1 mutation is responsible for both phenotypes and that revertants can acquire insensitivity to each independently. It is likely that those revertants that simultaneously become insensitive to both *Eco*RI and λ are true back mutations at the same site as the original mutation. Two new mutants, isolated as unable to grow on a λ lysogen, were not *Eco*RI sensitive (Table 5), showing that the two effects are not normally correlated.

Genetic mapping of *ris* mutations. Bacteriophage T5 is unusual in that, although it has a single DNA molecule, it behaves genetically as though it has four linkage groups which recom-

TABLE 4. *Effect of K and PI restriction on T5ris mutants*[a]

Phage	Plating on host carrying restriction system:		
	None	K	PI
λ	1.0	2×10^{-3}	1×10^{-4}
$T5^+$	1.0	8×10^{-1}	9×10^{-1}
T5*ris*1	1.0	7×10^{-1}	1.0
T5*ris*2	1.0	6×10^{-1}	7×10^{-1}
T5*ris*3	1.0	7×10^{-1}	1.0

[a] Bacteriophage grown on a host carrying no restriction system were plated on bacteria containing the *Eco*K (W3350) or *Eco*PI (ED8655[PI]) restriction system.

TABLE 5. *Growth of T5ris mutants on lysogens of bacteriophage λ*[a]

Phage	Plating ability on bacterial host:		
	0	(λ)	*Eco*RI (λ)
$T5^+$	1.0	1.0	0.73
T5*ris*1	1.0	3×10^{-6}	7×10^{-7}
T5*ris*2	1.0	1.0	5×10^{-5}
T5*ris*3	1.0	1.0	8×10^{-4}
T5*lr*	1.0	3×10^{-6}	3×10^{-6}

[a] Bacteriophage were plated on W3350, W3350 (λ), and NTP14 (W3350[λ]). T5*lr* is a newly isolated mutant that is unable to grow on a λ lysogen but is insensitive to *Eco*RI restriction (data not shown).

bine maximally with each other (8). Preliminary data showed that the *ris* mutations belong to the A linkage group since they show maximal recombination with members of linkage groups B, C, and D. The A linkage group has only two known essential genes, A1 and A2, and genetic recombination studies between mutants in these genes and the *ris* mutants gave the genetic map shown in Fig. 1. The recombination frequencies given in Fig. 1 are highly reproducible between different experiments but tend to be nonadditive over long distances. A similar phenomenon can be seen in the data for the genetic map of Hendrickson and McCorquodale (8).

The *ris*1 mutation (which also confers sensitivity to λ *rex* product) is closely linked to the A1*am*27 mutation. This is in agreement with previous observations (10) that certain A1 gene mutations show a λ-sensitive phenotype. The λ-sensitive mutants isolated in this laboratory showed close linkage to both A1*am*27 and to *ris*1 (Davison and Brunel, unpublished data).

Although the *ris*1, *ris*2, and *ris*3 mutations are located within the A linkage group, they are not closely linked within this linkage group. The fact that the A2*am*231 mutation is located between *ris*1 and *ris*3 makes it impossible that the *ris* mutations are different alleles of the same gene. In the accompanying communications, it will be shown that the *ris* mutations are in fact new *Eco*RI-sensitive sites located in the pre-early region of the DNA molecule (3; Brunel and Davison, J. Mol. Biol., in press).

Another pre-early gene, A3, is responsible for the inability to grow on a host harboring the colicinogenic factor ColIb. Mutants in this gene allow T5 to grow on this host (13). The precise mapping of such mutants is difficult, since wild-type recombinants cannot be specifically selected. When crossed with A1*am*27, A2*am*231, *ris*2, and *ris*3, the mutant A3*h*12 gave 34, 2, 2, and 21 wild-type recombinants, respectively, among 720 progeny tested for each cross. These limited data suggest the tentative location of A3*h*12 between A2*am*231 and *ris*2. This assignment is consistent with the results of Beckman et al. (2).

Protection of *ris* mutants by T5$^+$. If, as suggested, T5$^+$ specifies a restriction protection function, it may be expected that this could prevent restriction of the *ris* mutants in trans during mixed infection. To test this point the yield of T5*ris* progeny was measured after infecting an *Eco*RI restricting host and also after coinfection of the same host by both T5$^+$ and T5*ris*. Under these conditions, an increase in the yield of *ris* progeny in the coinfection would indicate that T5$^+$ was able to protect the *ris* mutant from *Eco*RI restriction. In the experiment the mutant T5 A1*am*27 was used as the wild-type helper, since the A1*am*27 mutation is suppressed on the *supE Eco*RI host used for complementation. Scoring the progeny is thus facilitated, because the helper can be removed by plating on a *sup*^{0} host. Moreover, recombination between A1*am*27 and *ris*1 is low, due to close linkage.

The results of such an experiment are given in Table 6. The assays on ED8655, W3350, and NTP14(W3350) represent total phage, *ris* phage plus wild-type recombinants, and wild-type recombinants, respectively. It is clear that the presence of the suppressed A1*am*27 mutant allows the *ris* mutant to grow on the restricting host, whereas it cannot do so in the absence of helper. In view of the fact that the *ris* mutations represent new *Eco*RI sites (3), the explanation of this complementation result is not clear, but a possible explanation will be given in the next section.

DISCUSSION

The observation that bacteriophage T5 is insensitive in vivo to three different restriction

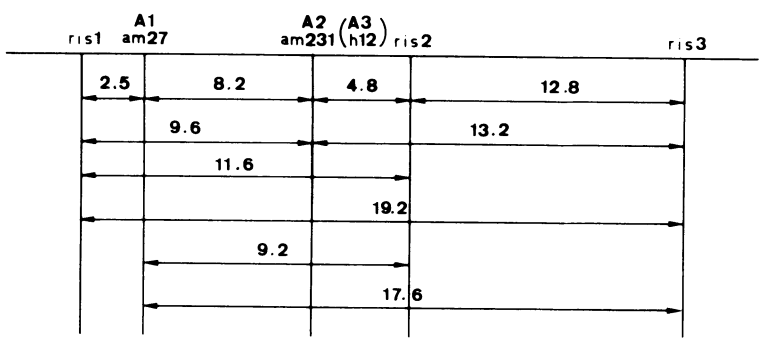

FIG. 1. *Genetic mapping of T5ris mutants. Genetic crosses between the various mutants were performed as described in the text. The figures given represent percentage of recombination and were calculated as twice the ratio between the number of plaque-forming phages on NTP14(W3350) and ED8655.*

TABLE 6. *Complementation of T5ris by T5*$^{+a}$

Infection by	Complementation on	Plaque-forming ability when assayed on:		
		sup$^+$	*sup*^{0}	*sup*^{0}, *Eco*RI
*ris*1	*sup*$^+$	6×10^9	6×10^9	1×10^5
A1*am*27	*sup*$^+$	4×10^9	3×10^4	2×10^4
*ris*1 + A1*am*27	*sup*$^+$	3×10^9	4×10^8	1×10^7
*ris*1	*sup*$^+$, *Eco*RI	4×10^6	4×10^6	4×10^4
A1*am*27	*sup*$^+$, *Eco*RI	2×10^9	8×10^3	8×10^3
*ris*1 + A1*am*27	*sup*$^+$, *Eco*RI	3×10^9	2×10^8	4×10^7

[a] Complementation tests were carried out by infection of NTP14 (ED8655) (*amp*$^+$, *Eco*RI), at a density of 3×10^9 cells per ml, with T5 A1*am*27 (multiplicity of infection = 5) and T5*ris* (multiplicity of infection = 1). On this host T5 A1*am*27 behaves as wild type, but T5*ris*1 is restricted. As a control the same experiment was carried out on ED8655, on which both mutants grow normally. Progeny phage were assayed on ED8655 (*sup*$^+$, no restriction), W3350 (*sup*^{0}, no restriction) and NTP14 (W3350) (*sup*^{0}, RI restriction), respectively.

systems of *E. coli* (*Eco*K, *Eco*PI, and *Eco*RI) suggests that it may have evolved one or more restriction protection mechanisms. This is supported by the knowledge that the DNA contains only the four usual bases (25) and, at least in the case of *Eco*RI, carries six cleavage sites (5, 20). Restriction protection functions have been described for bacteriophages T3, T7, and Mu I (6, 11, 22, 24).

We have attempted to isolate mutants of T5 defective in the hypothetical restriction protection system, and, as a first approach to this problem, have isolated *ris* mutants that are unable to grow on *Eco*RI restricting hosts. The mutants remain insensitive to *Eco*K and *Eco*PI restriction, indicating that they are not defective in a general protection system which confers insensitivity to all restriction systems. The map location of the *ris* mutants shows that they are too widely spaced to lie within a single cistron, though all lie within the pre-early linkage group A. One of the mutations, *ris*1, lies within a previously recognized essential gene, A1, since it is closely linked to the A1*am*27 mutation and exhibits the sensitivity to λ*rex* gene product characteristic of certain A1 gene mutants (10).

These observations are consistent with the finding that the *ris* mutations represent new *Eco*RI cleavage sites located in the terminally redundant ends of the molecule (4; Brunel and Davison, in press). The new *Eco*RI sites generated by the *ris* mutations presumably result from a base change in a sequence already resembling the 6-base pair *Eco*RI cleavage site (7). In the case of the *ris*1 mutant, the base change has resulted in a second phenotype, sensitivity to λ*rex* gene product, probably caused by an amino acid change in the A1 protein. The two phenotypes can revert together or separately. This is best explained by suggesting that when they revert together the original base sequence is restored. In contrast, revertants to λ*rex* insensitivity alone probably result from a pseudo-reversion inducing a compensatory amino acid change elsewhere in the A1 protein but not changing the new *Eco*RI site. Similarly, the *Eco*RI-insensitive, λ*rex*-sensitive revertants may be caused by a change in one of the other base pairs in the *Eco*RI cleavage site.

The complementation data that show that T5$^+$ can protect T5*ris* from *Eco*RI restriction are difficult to explain in light of the knowledge that the *ris* mutants create new restriction sites located in the terminally redundant, pre-early region of the DNA molecule. The most probable explanation is that infection is not highly synchronous, so that in some cases the *ris* mutant enters a cell that has already been infected by T5$^+$ and in which the protection function has already been expressed. Alternatively, the complementation may be due to protection of the *ris* mutation located at the other end of the DNA molecule, followed by marker rescue by recombination, which is very efficient in T5. More evidence is needed to test the validity of these hypotheses, but the fact that the *ris Eco*RI sites can escape restriction and appear in the progeny on coinfection with T5$^+$ provides strong evidence that T5 specifies a restriction protection function.

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