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Host-controlled Restriction of T-even Bacteriophages: Relation of Endonuclease I and T-even-induced Nucleases to Restriction

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Restriction of nonglucosylated T2 phage (T*2) as a function of bacterial growth state was the same for endonuclease I-containing and endonuclease I-deficient strains of *Escherichia coli* B. Furthermore, *E. coli* strains with various levels of restriction for T2 had comparable endonuclease I activities. It was also found that a T4 mutant temperature-sensitive for gene 46 and 47 functions was fully restricted at 42 C. It therefore appears that neither endonuclease I nor the phage-induced nucleases whose activities are blocked by mutations in genes 46 and 47 catalyze the initial event in restriction of nonglucosylated T-even phages.

The deoxyribonucleic acid (DNA) of T-even bacteriophages is unusual in that it contains hydroxymethylcytosine (HMC) in place of the normal cytosine. These HMC residues are glucosylated by phage-induced glucosyl transferases which employ bacterial uridine diphosphoglucose (UDPG) as glucosyl donor. Bacterial mutants lacking UDPG produce nonglucosylated progeny phage (T* phage) when infected with T2, T4, or T6 (7, 16, 26, 29). Phage mutants (T*gt* phage) that cannot induce glucosyl tranferases are nonglucosylated whether or not the host provides UDPG (12, 25).

This and the following paper (6) are concerned with the conditional lethality that loss of glucosylation confers upon T-even phages. Nonglucosylated phage are restricted in their ability to induce successful infection of *Escherichia coli* B. The DNA of such phages, upon injection into strain B, does not successfully direct the synthesis of progeny phage and is partially broken down to acid-soluble products (10, 15). Like T2 ghosts, T2*gt* kill only one-third of the bacteria to which they adsorb; yet, like intact T2, they induce early enzymes (albeit at a lower level; *see* 17).

It had been suggested that endonuclease I is responsible for hydrolysis of T* DNA during restriction (10, 22). However, recent experiments have shown that both endonuclease I-deficient mutants (6, 24, 30) and physiologically endonuclease I-deficient spheroplasts (24; B. Molholt, Ph.D. Thesis, Indiana Univ., Bloomington, 1967) are fully restrictive. In addition, nonrestricting strains are found to contain normal levels of endonuclease I (6, 24). The accompanying paper (6) shows that exonucleases I, II, and III are also found in normal levels in nonrestricting bacteria.

The initial lesion in restriction, therefore, does not appear to be catalyzed by any of the well-characterized deoxyribonucleases of *E. coli* (19). We have also examined another set of nucleases found in the T-even-infected cell, those nucleases controlled by T4 genes 46 and 47 which are active in the breakdown of host DNA (34). It was found that restriction occurs in the absence of gene 46 and 47 function.

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MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study are listed in Table 1.

Bacteriophages. All T2 strains used were derivatives of an *r*I mutant of T2L. T*2 was made by several cycles of growth on MT2002 or MT1102. The glucosyl transferaseless mutant, T2*gt*, was isolated by plating hydroxylamine-treated (28) T2 on a double indicator composed of Rst$^-$ and Rst$^+$ bacteria and selecting turbid plaques (25). T4*ts*L109 and T4*ts*L86, temperature-sensitive mutants of T4D in genes 46 and 47, respectively, were obtained from O. Sköld. The double mutant T4*ts*L86L109 was isolated according to the method of Wiberg (34). T4*r*II638 is a deletion mutant for the entire *r*IIB cistron, obtained from S. Benzer; T*4*r*II638 was grown on W4597. Phage ϕX174 was obtained from R. L. Sinsheimer.

Media. 3XD was as described in Fraser and Jerrel (9); D medium was one-third strength 3XD. L broth contained (grams per liter): tryptone (Difco), 10;

TABLE 1. *Bacterial strains*

Escherichia coli strain	Source (reference)	Characteristics[a]
MT2001	Sinsheimer, his strain C (14)	Wild type is Rst⁻ *galU*⁺
MT2002	Spontaneous T4-resistant, galactose-negative mutant of MT2001	Rst⁻ *galU*⁻
MT1100	Arber, his strain Bc251 (2)	*rst*⁺ *galU*⁺
MT1101	Nitrosoguanidine mutagenesis of MT1100	*rst*⁻ *galU*⁺ (*rst-1*)
MT1102	Spontaneous T4-resistant, galactose-negative mutant of MT1101	*rst*⁻ *galU*⁻
AC2519	Boyer (4)	*rst*⁺ *galU*⁺
MT1111	Nitrosoguanidine mutagenesis of AC2519	*rst*⁻ *galU*⁺ (*rst-2*)
MT1500	Benzer, his strain B (3)	*rst*⁺ *galU*⁺
MT1501	Nitrosoguanidine mutagenesis of MT1500	*rst*⁺ *galU*⁺, low level of restriction for T*2
MT1502	Nitrosoguanidine mutagenesis of MT1500	*rst*⁺ *galU*⁺, high level of restriction for T*2
MT1540	Hoffmann-Berling, his strain B41	*rst*⁺ *galU*⁺ *dnsA*⁻, *lac*⁻, (F′ *lac*⁺).
W4597	Fukasawa (11)	*rst*⁺ *galU*⁻

[a] Rst⁻ is the phenotype indicating absence of restriction of nonglucosylated T-even phage. The genotypes *rst* and *galU* refer to genes controlling restriction and modification of T-even phages. Mutant *rst-2* maps near *his* in *E. coli* B (*unpublished data*); *galU* is the gene for UDPG synthetase (31). The gene involved in the synthesis of endonuclease I is designated *dnsA*.

yeast extract, 5; glucose, 1; and NaCl, 10; it was adjusted to *p*H 7.0 with NaOH. Basal agar plates were either L agar (L broth plus 1.5% agar) or nutrient agar (grams per liter): Nutrient Broth (Difco), 8; NaCl, 5; agar, 12. Soft agar was the same as nutrient agar, but with 0.65% agar. T2 adsorption buffer contained (grams per liter): Na_2HPO_4, 2.9; KH_2PO_4, 1.5; NaCl, 4.0; K_2SO_4, 5.0; $MgSO_4$, 0.12; $CaCl_2$, 0.01; gelatin, 0.01; and chloramphenicol, 0.02. For adsorption of T4, 20 μg of L-tryptophan per ml was added.

Nitrosoguanidine mutagenesis. *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine was obtained from Aldrich Chemical Co., Milwaukee, Wis., and was used as described by Adelberg, Mandel, and Chen (1).

Restriction assays. A 0.2-ml amount of bacteria at a concentration of 10^8 to 10^9 cells per ml was incubated with 0.1 ml T*2 at 10^8 particles per ml for 10 min at 37 C in 3XD or L broth. Infectious centers were then assayed, either by dilution into soft agar seeded with *E. coli* B or by adding 2 ml of B-seeded soft agar directly to the adsorption tube. The yielder frequency (ratio of infectious centers on a given host to infectious centers on MT2001) is used as a quantitative expression of restriction.

Preparation of φX174 DNA. The hot phenol technique of Guthrie and Sinsheimer (14) was used to isolate infectious φX174 DNA from phage grown on MT2001 in 3XD medium. Routinely, 10^8 infectious DNA molecules per ml were obtained from φX174 stocks at 10^{11} phage per ml.

Endonuclease I preparation. The enzyme was prepared as the crude supernatant fraction after centrifugation of spheroplasts made by the lysozyme-ethylenediaminetetraacetate (EDTA) method of Guthrie and Sinsheimer (14). Endonuclease I is released quantitatively by this treatment (5, 23). Spheroplast supernatant fluids were dialyzed in the cold against two changes of 100 volumes 0.15 M tris(hydroxymethyl)-aminomethane (Tris)-buffered saline (*p*H 8) to remove sucrose and EDTA.

Endonuclease I assay. Assay specificity depends upon the inhibition of endonuclease I by soluble ribonucleic acid (20) and loss of φX174 DNA infectivity through endonucleolytic conversion of circles to linear molecules (8). Crude extracts displayed no activity against φX174 DNA until pretreated with ribonuclease I (see control curve, Fig. 3). For the assay, 0.03 ml of 10 mg/ml ribonuclease I (Worthington Biochemicals Corp., Freehold, N. J.) was incubated with 3 ml of dialyzed spheroplast supernatant fluid for 30 min at 37 C. Of the resultant preparation, 0.2 ml was added to 1.8 ml of φX174 DNA (10^{11} phage equivalents per ml) at 37 C, and 0.4-ml portions were removed at timed intervals to 0.4 ml of MT2001 spheroplasts. After 10-min incubation at 37 C, 3.2 ml of prewarmed PAM (14) was added, incubation was continued for 5 min, and the resulting infectious centers were assayed on MT2001. Controls consisted of (i) untreated φX174 DNA plus spheroplasts, (ii) φX174 DNA plus untreated spheroplast supernatant fluid plus spheroplasts, and (iii) φX174 DNA plus 10 μg/ml of ribonuclease I plus spheroplasts.

*Breakdown of ultraviolet-irradiated ^{32}P-labeled T*2 DNA.* MT1102 grown in Tris-glucose medium (18) supplemented with 2.5 μc/ml of inorganic ^{32}P (Nuclear Science and Engineering, Pittsburgh, Pa.) was infected with T2. The crude T*2 lysate was purified by dialysis and diethylaminoethyl-cellulose chromatography and was irradiated at 40 cm from a Westinghouse Sterilamp for 4 min to give 100 lethal hits. The phage were then adsorbed to 3 ml of MT1100 or MT1101 in T2 adsorption buffer at an input ratio of 1.0. After 3 min, the infected cells were sedimented, washed in cold adsorption buffer (without chloramphenicol), and resuspended in 1 ml of cold adsorption buffer (without chloramphenicol). The suspension was brought to 37 C and diluted with 2 ml of 37 C 3XD (time-zero). Samples (0.5 ml) were withdrawn

at timed intervals, diluted in 2 ml of cold D medium, and centrifuged at 7,000 × g for 15 min; the supernatant fractions were assayed for acid-soluble ^{32}P after precipitation with cold 10% trichloroacetic acid. Samples were counted in a Packard model 3003 liquid scintillation spectrometer.

RESULTS

Endonuclease I. Luria (21) has shown that the fraction of T*2 restricted upon infection of a Rst^+ strain depends on the physiological state of the host. The dependence of restriction in MT1100 on growth state is shown in Fig. 1. Restriction of T*2 decreases 1,000-fold as cells enter stationary phase. Upon dilution into prewarmed medium, complete restriction is restored during the lag before cells regain exponential growth. This loss of restriction for T*2 during the stationary phase suggested the involvement of endonuclease I, since the specific activity of this enzyme decreases markedly in the stationary phase (27).

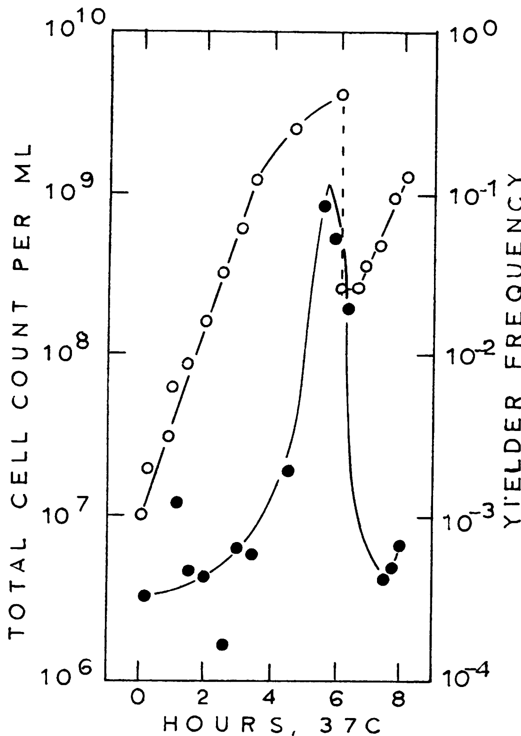

FIG. 1. *Effect of aging on restriction of MT1100 for T*2. An exponential culture of MT1100 was diluted to 10^7 cells/ml in 3XD at 37 C, and 2-ml samples were withdrawn at various times for determination of total cell count and yielder frequency (infectious centers per infected cell) for 10^8 T*2 per ml. At 6 hr (stationary phase), the culture was diluted 20-fold into fresh 3XD at 37 C (broken line). Symbols: ○ = total cell count; ● = yielder frequency for T*2.*

Endonuclease I levels of four strains of *E. coli*, each with a unique level of restriction for T*2, were examined to detremine whether a significant correlation does indeed exist between this enzyme and restriction. Since restriction depends upon growth state (Fig. 1), yielder frequencies of these four strains are presented as a function of growth state (Fig. 2). The C strain MT2001 is phenotypically Rst^- whereas B strains MT1500, MT1501, and MT1502 are Rst^+, but with three distinct patterns of restriction which vary over 100-fold in exponential-phase cells. If endonuclease I were the restricting factor, then a hierachy of endonuclease I levels, MT1502 > MT1500 > MT1501 > MT2001, would be expected. No such hierachy of endonuclease I levels was found in extracts of exponential-phase cells (Fig. 3).

Endonuclease I-deficient mutants have been found to retain restriction for nonglucosylated T-even phages (6, 24, 30). However, these *dnsA*⁻ mutants contain a residual amount of the enzyme (J. Eigner and H. Hoffmann-Berling, *personal communications*), and it is possible that if this residuum were strategically placed in the cell (viz., at T-even receptor sites) it could still effect restriction. To test this hypothesis, control of restriction by growth state in an endonuclease I-deficient mutant (MT1540) was studied. If endonuclease I was a critical factor in restriction, depletion of the residual amount of enzyme would

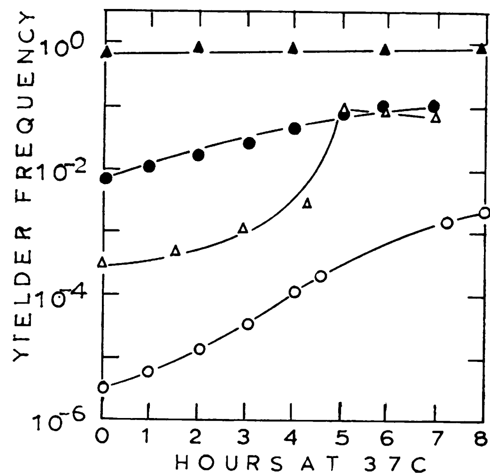

FIG. 2. *Effect of aging on restriction of four strains of Escherichia coli for T*2. Yielder frequencies were determined by assaying infectious centers on MT1100. At time-zero, each strain was diluted to 10^7 cells/ml. Symbols: ▲ = MT2001; ● = MT1501; △ = MT1500; ○ = MT1502.*

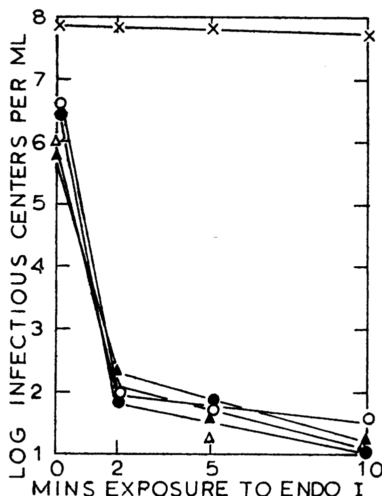

FIG. 3. *Endonuclease I assay of hosts with various capacities for restriction. Endonuclease I was released from the four strains of Fig. 2 and assayed as ribonuclease-released activity against infectious φX174 DNA. Symbols: × = control curve, MT1500 endonuclease I without ribonuclease pretreatment; ▲ = MT2001; ● = MT1501; △ = MT1500; ○ = MT1502.*

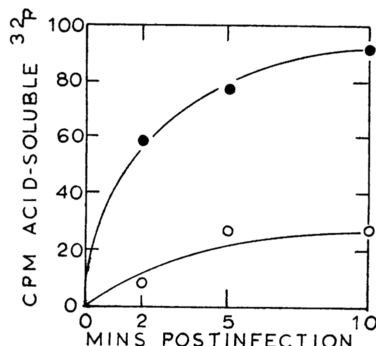

FIG. 4. *Breakdown of ultraviolet-inactivated T*2. MT1100 (rst+) and MT1101 (rst−) were infected at time-zero with 32P-labeled T*2 which had sustained over 100 lethal ultraviolet hits. Symbols: ● = MT1100; ○ = MT1101.*

be expected to occur rather early in stationary phase *dnsA*⁻ cells, resulting in an earlier loss of restriction than seen in aging *dnsA*⁺ bacteria. However, cultures of *dnsA*⁻ and *dnsA*⁺ cells were found to lose restriction upon aging, and to regain it upon dilution, with identical kinetics (*data not shown*). It was concluded that the level of endonuclease I is unimportant in restriction of nonglucosylated T-even phage.

T-even-induced nucleases. Two experiments have been conducted to assess the role of T-even-induced nucleases in restriction, since restriction could be a "self-digestion" occurring when T-even DNA has lost its protection (glucosylation) against enzymes active in degrading host DNA.

The first experiment examined breakdown of ^{32}P-labeled T*2 DNA which had been heavily irradiated with ultraviolet light. Each particle was subjected to an average of 100 lethal hits to preclude phage-specific enzyme synthesis during infection. Figure 4 shows that the DNA of such particles was broken down to acid-soluble products upon infection of MT1100 (*rst*⁺), whereas much less breakdown occurred with the *rst*⁻ mutant MT1101. These results indicate that phage-induced enzymes are not involved in the breakdown of T* DNA.

Genes 46 and 47 in phage T4 control nucleases which catalyze the breakdown of bacterial DNA during T4 infection (34). For a direct measure of involvement in restriction of the nucleases controlled by genes 46 and 47, a double temperature-sensitive mutant, T4*ts*L86L109, was isolated from a cross between T4*ts*L86 (*ts* mutation in gene 47) and T4*ts*L109 (*ts* mutation in gene 46). The double mutant was then grown for one cycle on a *galU*⁻ host, W4597, resulting in the formation of T*4*ts*L86L109. If the nucleases controlled by genes 46 and 47 are active in restriction, the nonglucosylated double *ts* mutant should *not* be restricted on a Rst⁺ strain at the nonpermissive temperature. It is shown in Table 2 that restriction occurred normally in strain MT1100 at 42 C. Thus, we concluded that the nucleases controlled by genes 46 and 47 are not involved in restriction of nonglucosylated T-even phages.

DISCUSSION

The findings that *dnsA*⁻ mutants of *E. coli* B and K-12 restrict nonglucosylated T-even phages (6, 24, 30; this report) and that *rst*⁻ mutants contain wild-type levels of endonuclease I (6, 24; this report) render unlikely the involvement of endonuclease I in the process of restriction. The residuum of endonuclease I in *dnsA*⁻ mutants does not appear to catalyze restriction, since the kinetics of restriction loss upon entering stationary phase was found to be the same for *dnsA*⁻ and *dnsA*⁺ cells.

None of the three well-characterized exonucleases of *E. coli* (19) can be implicated in the primary control of restriction (6, 24). However, these nucleases may act in a secondary fashion to further degrade DNA after restriction has occurred. Such digestion may be responsible for the loss in early enzyme-synthesizing capacity seen in T2*gt*-infected cells in the absence of protein synthesis (17).

It is possible that T-even phages have adapted

TABLE 2. *Relation of gene 46 and 47 function to restriction*[a]

Phage strain	Glucosylation	46 and 47 nucleases at 42 C	Yielder frequencies			
			On MT1101 (*rst*$^-$)		On MT1100 (*rst*$^+$)	
			At 30 C	At 42 C	At 30 C	At 42 C
T4*ts*L86L109	+	−	1	1	1	1
T*4*r*II638	−	+	10^{-1}	10^{-1}	3×10^{-3}	6×10^{-3}
T*4*ts*L86L109	−	−	10^{-1}	10^{-1}	8×10^{-3}	9×10^{-3}

[a] T4 strains were adsorbed to 2×10^9 cells/ml in T2 adsorption buffer plus chloramphenicol plus 20 μg of L-typtophan per ml at a multiplicity of 0.1 for 10 min at 30 or 42 C. The cells were then sedimented, resuspended at 2×10^8 per ml in 3XD at 30 or 42 C, and infectious centers were assayed on MT1100 after a further 20 min of incubation. The yielder frequency is the number of infectious centers formed per infected cell. Burst sizes of both T4*ts*L86L109 and T*4*ts*L86L109 were much smaller on both MT1100 and MT1101 at 42 C than at 30 C.

to utilize the restricting factor to catalyze the high recombination rate characteristic of T-even infection. According to this model, upon infection of Rst$^+$ bacteria, parental T* DNA would look like progeny DNA (which initially lacks glucose; 7) and undergo inactivation by polynucleotide scissions accompanying recombination. However, this possibility appears to be unlikely since the rate of recombination for T-even phages is the same in Rst$^+$ and Rst$^-$ hosts (*unpublished data*).

It should be pointed out that breakdown of T-even DNA may be a result, rather than the cause, of restriction. Although a rapid release of up to 50% of nonglucosylated T-even DNA as acid-soluble material has been found to accompany restriction in *E. coli* B (10, 15), T*6 DNA may not be broken down upon restricted infection of *E. coli* BB (26), and Pizer (*personal communication*) found that T2 is excluded without detectable breakdown of T2 DNA by *Shigella* lysogenic for P2, as well as by certain strains of *E. coli* lysogenic for the P2-related prophage, ϕW.

Hattman found that nonglucosylated T-even DNA was made acid-soluble by Rst$^+$ bacteria pretreated with 100 μg of chloramphenicol per ml (15). It is reported here that breakdown of T*2 DNA is similarly unaffected by specifically inactivating the capacity of phage DNA for protein synthesis through heavy ultraviolet irradiation. Both results suggest a lack of involvement of any phage-induced enzymes in restriction. However, this conclusion is at best indirect, since DNA breakdown and restriction may not be causally related.

Evidence that the phage-induced nucleases controlled by genes 46 and 47 are not involved in restriction comes from the present studies in which gene 46 and 47 products were specifically inactivated. However, the experiments reported here do not rule out control of restriction by other enzymes synthesized during T4 infection. Warren and Bose (33) have demonstrated that T4 probably induces endonucleases that are active in degrading host DNA prior to exonucleolytic cleavage by the gene 46 and 47 controlled nucleases. Similar observations have been made by Kutter and Wiberg (*personal communication*). Since T4 mutants lacking endonucleolytic activity have not been identified, the specific effect of these enzymes on restriction has not been assessed. In addition to the ability to induce endo- and exonucleases active against host DNA, T4 appears to possess a gene (*ex*) that controls breakdown of T2 DNA during T2-T4 mixed infection (13). When 100% glucosylated, T2 is not excluded by the T4 *ex* gene product, indicating a substrate specificity based on glucosylation. T*4 is unlike T*2 in that it fails to undergo multiplicity activation on *rst*$^+$ hosts (15). Also, T*4 is generally more restricted than is T*2 by *rst*$^-$ mutants of *E. coli* B [6, 24; also, in Table 2 note the low yielder frequency (0.1) of T*4 on MT1101]. These observations implicate a T4-induced product, perhaps of the *ex* gene, which acts to restrict T*4, in addition to those restricting factors active during T*2 restriction. Perhaps an efficiency of plating of 1.0 by T*4 on *Shigella* is due to the loss of synthesis of some phage proteins on this host, specifically those which shut off host syntheses (32).

Nevertheless, it is presumed that restriction is a host-controlled process since changes in the physiological state of the host cell and loss of gene *rst* function drastically alter the restrictive response of *E. coli* B for nonglucosylated T-even phage, and since the breakdown of T* phage which accompanies restriction occurs in the absence of protein synthesis.

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