Host-controlled Restriction of T-even Bacteriophages: Relation of Four Bacterial Deoxyribonucleases to Restriction

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Escherichia coli strains B and K-12, which restrict growth of nonglucosylated T-even phage (T* phage), and nonrestricting strains (*Shigella sonnei* and mutants of *E. coli* B) were tested for levels of endonuclease I and exonucleases I, II, and III, by means of in vitro assays. Cell-free extracts freed from deoxyribonucleic acid (DNA) were examined with three substrates: *E. coli* DNA, T*2 DNA, and T2 DNA. Both restricting and nonrestricting strains had comparable levels of the four nuclease activities and had similar patterns of preference for the three substrates. In addition, mutants of *E. coli* B and K-12 that lack endonuclease I were as effective as their respective wild types in restricting T* phage.

The accompanying paper by Molholt and Fraser (13) and the recent report of Revel (15) summarize current knowledge of the host-controlled modification of T-even phages and the indirect evidence which suggests that one or more bacterial deoxyribonucleases may be responsible for restriction of T-even phages whose deoxyribonucleic acid (DNA) is nonglucosylated (T* phage). This paper describes an introductory study of the enzymology of T-even restriction. Levels of four well-characterized deoxyribonucleases (8-10, 17) were measured in extracts of restricting (*rst*⁺) strains of *Escherichia coli*, of a naturally occurring nonrestricting (*Rst*⁻) strain of *Shigella*, and of *rst*⁻ mutants of *E. coli* B. [Genetic and phenotypic symbols follow those given in Table 1 of the accompanying paper (13).] For each extract, enzyme specificity was determined with glucosylated phage DNA (T DNA) and nonglucosylated phage DNA (T* DNA) as substrates. If one of the four activities was involved in restriction, it was expected that its level would be greatly reduced, or its substrate specificity would be drastically altered in the *rst*⁻ strains relative to the *rst*⁺ strains. Particular attention was given to the role of endonuclease I, in view of its high activity, its strategic "defensive" location between cell membrane and wall (14),

and the suggestion arising from several earlier studies that it might be a restriction enzyme (for review, see 13 and 15). Several endonuclease I-deficient (*dnsA*⁻) strains were therefore examined for restriction properties and enzyme levels.

While this study was in progress, a report (16) appeared on the action of the four highly purified deoxyribonucleases of *E. coli* B upon T DNA and T* DNA substrates. Two recent investigations (15, 20) closely paralleled some of the studies reported here, and the accompanying paper (13) furnishes additional information on the relation of nucleases to restriction.

MATERIALS AND METHODS

Bacteria. *Shigella sonnei* and *E. coli* B (Benzer strain) were from our departmental collection. *E. coli* B3 (*thy*⁻) was obtained from N. Melechen. *S. sonnei*/4₀ was a spontaneous mutant selected for resistance to phage T4 and inability to utilize galactose. *E. coli* K-12-1100, the *dnsA*⁻ strain of H. Hoffmann-Berling, was kindly provided by I. R. Lehman; its *dnsA*⁺ parent, *E. coli* K-12-1000, was obtained directly from Dr. Hoffmann-Berling. Strains ER21 and ER22 (*dnsA*⁻ *rst*⁺) and ER39 (*dnsA*⁺ *rst*⁻) were obtained from *E. coli* B (*dnsA*⁺ *rst*⁺) as described below. Strains AC2519 and MT1100 (*rst*⁻) and their derivatives MT1111 and MT1101 (*rst*⁻) were supplied by B. Molholt [see Table 1 of the accompanying paper (13)].

Bacteriophage. Phage T2H was obtained from N. Melechen; T4 and T6 were obtained from A. de Waard. Nonglucosylated phages T*2 and T*6 were obtained by growth of T2 and T6 on *S. sonnei*/4₀, a uridine diphosphoglucose-less (*galU*⁻) as well as

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Rst⁻ host (7). A stock of T*4, kindly furnished by B. Molholt, had been derived by one cycle of infection of an rIIB mutant of T4 on *E. coli* W4597 (*galU*⁻ *rst*⁺; reference 7).

Media. L broth contained 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose per liter, adjusted to pH 7.0 with NaOH. L agar consisted of L broth plus 1.5% agar.

Isolation of mutants. *E. coli* B was mutagenized by treatment (1) with *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (Aldrich Chemical Co., Milwaukee, Wis.).

For the isolation of *dnsA*⁻ derivatives of *E. coli* B, a slight modification of the method of Dürwald and Hoffmann-Berling (J. Mol. Biol., *in press*) was employed. Survivors of mutagenesis were grown out on L agar containing 0.01% acridine orange (Matheson, Coleman and Bell, East Rutherford, N.J.). After autolysis under toluene (24 hr at 37°C), the plates were dried and examined under ultraviolet light. Colonies of *dnsA*⁻ mutants fluoresced brightly, and corresponding colonies were isolated from replica plates not treated with toluene. Of 31 nuclease-deficient strains isolated, 20 had very low levels, 6 had intermediate levels, and 5 had wild-type levels of endonuclease I. None was lacking exonuclease I.

A *rst*⁻ mutant of *E. coli* B was isolated as described by B. Molholt (Ph.D. Thesis, Indiana Univ., Bloomington, 1967) and Revel (15) by plating mutagenized *E. coli* B with T* phage and selecting a "nibbled" colony (strain ER39, *rst*-3).

Determination of plating efficiency. The efficiency of plating (EOP) of a phage stock is the ratio of its titer on a given bacterial strain to its titer on the standard Rst⁻ host, *S. sonnei*. Bacteria for plating were taken from early logarithmic cultures (10⁸ to 4 × 10⁸/ml) grown in L broth.

Preparation of deoxyribonuclease substrates. To prepare ³H-labeled T2 DNA and T*2 DNA, T2 and T*2 phage labeled with ³H-thymidine (Schwarz Bio Research Inc., Orangeburg, N.Y.) were grown on *S. sonnei* and *S. sonnei*/4₀, respectively, as described by Richardson (16). After two cycles of high- and low-speed centrifugation, a final purification of the phage on layered CsCl gradients was performed (21). ³H-DNA was isolated from the phage concentrates by the method of Massie and Zimm (12). Two phenol extractions were performed, and the purified DNA was dialyzed against SSC/10 (0.015 M NaCl and 0.0015 M sodium citrate). Buoyant densities of 1.437 for the T2 DNA and 1.429 for the T*2 DNA were determined in cesium sulfate equilibrium gradients relative to *E. coli* DNA ($\rho_0 = 1.426$; reference 6) used as density standard. These values are consistent with a normal level of glucosylation for the T2 DNA sample and virtual nonglucosylation for the T*2 DNA preparation (4, 6). Sedimentation coefficients (*S*_{20,w}), measured as previously described (5), were 17.5S for the T2 DNA and 26S for the T*2 DNA, corresponding to molecular weights of 5 × 10⁶ and 15 × 10⁶, respectively (5).

³H-labeled *E. coli* DNA was isolated from ³H-labeled *E. coli* B3 by a procedure involving deproteinization with Pronase and phenol (22), followed by removal of RNA with pancreatic ribonuclease and

ribonuclease T1 (18). The *S*_{20,w} of the product was 20S, corresponding to a molecular weight of 7.5 × 10⁶ (5).

To denature DNA for use as exonuclease I substrate, samples were diluted to 120 µg/ml, heated at 100°C for 10 min, and placed immediately into an ice-water bath.

Bacterial extracts. Cell-free extracts were prepared by a combination of sonic treatment and phase extraction developed by Alberts (2) to yield a partially purified soluble enzyme fraction essentially free from nucleic acids. Bacteria grown in L broth at 37°C were harvested in exponential phase to yield 3 g of packed wet cells per liter. After a wash with 5 M buffer [5 M NaCl, 0.04 M tris(hydroxymethyl)amino-methane, pH 8, 10⁻⁴ M ethylenediaminetetraacetate, 0.05 M MgCl₂, and 0.05 M 2-mercaptoethanol], 2 ml of 5 M buffer containing 0.32 g of polyethylene glycol 6000 (molecular weight, 6,000 to 7,500, from Matheson, Coleman and Bell) and 2 ml of 5 M buffer containing 0.20 g of dextran 500 (limiting viscosity number, 0.50 dl/g, from Pharmacia) were added per g of packed cells. The cell suspension, in 5-ml portions, was sonically treated (four times, 1 min, 30 to 40% maximal power) at 0°C with a Bronwill Biosonic apparatus fitted with a 4-mm diameter probe. After centrifugation at 4,000 × *g* for 15 min, the resulting polyethylene glycol-rich upper phase was dialyzed overnight against 100 volumes of 0.5 M buffer (as above, but with 0.5 M NaCl replacing 5 M NaCl). The retentate was clarified by centrifugation at 12,000 × *g* for 10 min, after which it contained about 5 mg of protein/ml and <2 µg of DNA/ml. No attempt was made to remove the polyethylene glycol. Extracts were stored on ice and assayed within 1 or 2 days after preparation.

Deoxyribonuclease assays. The four well-characterized deoxyribonucleases of *E. coli* were assayed with only slight modifications of the procedure described by Shortman and Lehman (19). In this procedure, 3 µg of soluble ribonucleic acid (sRNA) is added to the endonuclease I blank assay, none to the exonuclease I assay, and 1 µg to the exonuclease II and exonuclease III assays. These amounts were used in the experiments summarized in Tables 2-4, but, for the experiments of Table 5, 25 µg of sRNA (Schwarz Bio Research Inc.) was used in each of these assays.

Incubation mixtures contained 20 µmoles of ³H-DNA nucleotide equivalents containing 50,000 to 100,000 counts/min. After acid precipitation in the presence of 2 mg of bovine albumin as carrier, half of the supernatant fluid was counted in a liquid scintillation spectrometer (Packard model 3003). All enzyme units are reported as millimicromoles of DNA nucleotides made acid-soluble in 30 min at 37°C.

Other procedures. Protein was determined by the method of Lowry et al. (11), with bovine plasma albumin (Fraction V crystals, Pentex, Inc., Kankakee, Ill.) as the standard. DNA was measured by Burton's modification of the diphenylamine reaction (3), with deoxyadenosine monophosphate (Calbiochem, Los Angeles, Calif.) as the standard.

RESULTS

Restriction by *dnsA*⁻ strains. Plating efficiencies of T and T* phage on various bacterial strains are presented in Table 1. Of the strains tested, ER21, ER22, and K-12-1100 had low or undetectable levels of endonuclease I (*see below*). These strains plated T* phage with the same low efficiencies as their *dnsA*⁺ parents, *E. coli* B and *E. coli* K-12-1000.

Properties of a *rst*⁻ mutant of *E. coli* B. Mutant strain ER39 was nearly as permissive as *S. sonnei* for T*6 and was partially permissive for T*2, but it restricted T*4 (Table 1). It required arginine and thiamine for growth; *arg*⁺ but not *thi*⁺ revertants were readily obtained.

Two other *rst*⁻ strains (13), supplied by B. Molholt, were also tested for enzyme activities (Table 5) since, in addition to their ability to plate T*4, they plated T*2 with somewhat higher efficiency (EOP of 0.3 to 0.4; B. Molholt, *personal communication*) than did ER39.

Characteristics of the bacterial extracts. In preliminary studies sonic extracts of cells were employed. Such extracts contained approximately 0.15 μ mole of DNA nucleotides per μ g of protein, so that dilution of the ³H-substrate DNA (20 μ moles/assay) became a problem if more than 30 μ g extract protein per assay was used. As a result, it was difficult to measure nucleases with specific activities in the crude extract much lower than about 30 units/mg of protein. This limitation affected the endonuclease I blank, exonuclease II, and exonuclease III assays for all strains, and it affected the endonuclease I assay of *dnsA*⁻ strains. Crude extracts were therefore prepared by a slight modification of Alberts' method (2), which separates the bulk of the cellular enzymes from nucleic acids and particulate material. In addition to its efficient removal of DNA, this fractionation led to a several-fold increase of nuclease specific activities over those of

E. coli B extracts prepared by sonic disruption, without altering the relative activities of the four deoxyribonucleases.

Endonuclease I activities. In the extracts prepared by the polyethylene glycol-dextran fractionation, as well as in conventional, nucleic acid-containing extracts of *E. coli* (10), endonuclease I activity was found to be about 95% inhibited by RNA. Endonuclease I activities were, therefore, determined in the presence of excess pancreatic ribonuclease in order to establish their uninhibited levels (19). In addition, parallel assays were performed with sRNA in place of the ribonuclease to determine the contribution of deoxyribonucleases which are not inhibited by sRNA (19). *E. coli* DNA, T*2, DNA, and T2 DNA were used as substrates.

Two of the strains, the *E. coli* B derivative ER22 and K-12-1100, had very low levels of the enzyme (Table 2). Mutant ER21 had a low but significant level of endonuclease I, about 1% that of *E. coli* B. *E. coli* K-12-1000 had substantially less activity than *E. coli* B, as found in several other comparisons between these strains (14, 15, 19).

Mixing experiments indicated that the low endonuclease activities of extracts of *dnsA*⁻ strains were not due to an inhibitor present in excess. For example, when 3.9 units of enzyme from an extract of B was incubated in the standard assay with ER22 extract containing 20 μ g of protein and 1.2 units of activity (all insensitive to sRNA inhibition), the resulting activity was 7.8 units, a 50% stimulation (this stimulation is at least partly due to stimulation of exonucleases by endonuclease I, examples of which are cited below). Nor were the low activities of *dnsA*⁻ strains due to leakage of enzyme during growth, harvest, or wash, since no endonuclease I activity could be detected in the cell-free supernatant fractions from these steps, whereas in the case of *E. coli* B these supernatant fractions had readily

TABLE 1. Plating efficiencies of T and T* bacteriophage

Strain	Phage					
	T2	T*2	T4	T*4	T6	T*6
<i>Shigella sonnei</i>	1.00	1.00	1.00	1.00	1.00	1.00
<i>Escherichia coli</i> B.....	0.52 ^a	1.9×10^{-5}	1.01	6.6×10^{-4}	0.87	4.1×10^{-5}
<i>E. coli</i> ER39.....	0.47 ^a	0.073	1.03	3.0×10^{-3}	0.90	0.50
<i>E. coli</i> ER21.....	0.80	1.6×10^{-4}	1.04	2.2×10^{-3}	0.87	1.1×10^{-4}
<i>E. coli</i> ER22.....	0.48 ^a	1.2×10^{-4}	1.00	1.3×10^{-3}	1.00	6.1×10^{-5}
<i>E. coli</i> K-12-1000.....	0.024 ^b	1.5×10^{-5}	0.91	5.4×10^{-4}	0.74	3.3×10^{-3}
<i>E. coli</i> K-12-1100.....	0.015 ^b	6.7×10^{-6}	0.89	4.6×10^{-4}	0.90	1.4×10^{-3}

^a In other experiments, these values were closer to 1.0.

^b T2 is partially restricted by *E. coli* K-12 (e.g., *see* 15).

TABLE 2. *Endonuclease I specific activities*^a

Extract	<i>rst</i>	³ H-DNA substrate			
		<i>Escherichia coli</i>	T*2	T2	
<i>Shigella sonnei</i>	—	2,260 (101)	2,190 (66)	934	(41)
<i>E. coli</i> B.....	+	3,570 (166)	4,270 (72)	2,020	(49)
<i>E. coli</i> ER39.....	—	2,510 (118)	2,460 (78)	1,210	(47)
<i>E. coli</i> ER21.....	+	51 (72)	47 (24)	14.4	(19.5)
<i>E. coli</i> ER22.....	+	0 (72)	8.8 (26.4)	1.8	(12.3)
<i>E. coli</i> K-12-1000.....	+	511 (82)	520 (44)	165	(28)
<i>E. coli</i> K-12-1100.....	+	0 (93)	0 (57)	0	(43)

^a Enzyme units (millimicromoles of DNA nucleotides solubilized in 30 min at 37 C) per milligram of protein. Each entry is the corrected endonuclease I activity, taken as the difference between the results of the standard endonuclease I and the endonuclease I blank assays. The result of the blank assay is given in parentheses.

detectable amounts of enzyme, amounting to several per cent of the total intracellular activity.

S. sonnei had an endonuclease I activity about half as large as that of *E. coli* B, as did ER39, the *rst*[—] mutant of B (Table 2). The somewhat higher specific activities of the *E. coli* B extract observed in this experiment are not believed to be significant, since in several comparisons with sonic extracts *S. sonnei* had 70 to 80% of the endonuclease I activity of *E. coli* B, and in one case, in which cell-free extracts were prepared by osmotically shocking cells in 0.02 M Mg⁺⁺, as described by Nossal and Heppel (14), the *Shigella* extract had a specific activity equal to that of *E. coli* B (see also Table 5).

The patterns of substrate preference by the various *dnsA*⁺ extracts were remarkably similar (Table 2). For *E. coli* DNA and T*2 DNA, the hydrolysis rates were nearly identical. The activities toward glucosylated T2 DNA were two to three times lower. This pattern of substrate preference was also found in pilot studies with crude sonic extracts of *E. coli* B and *S. sonnei*. The substrates tested were ³H-DNA from *E. coli* and ³²P-DNA from phages T6 and T*6. The same pattern was found with 12-fold purified extracts of both strains prepared by osmotic shock in 0.02 M MgCl₂ (14).

The residual endonuclease I activity of the ER22 extract showed a fivefold preference for T*2 DNA (Table 2). Repetition of this experiment with a fresh ER22 extract, but with 25 μg of sRNA in the blank assay, revealed a more striking preference for T*2 DNA (Table 5). In contrast, the extract of strain K-12-1100 showed no net endonuclease I activity with any of the substrates tested (Table 2). However, the blank values in all assays with *dnsA*[—] extracts were too great to permit highly accurate determinations of residual endonuclease I levels.

TABLE 3. *Exonuclease I specific activities*

Extract	<i>rst</i>	³ H-DNA substrate		
		<i>Escherichia coli</i>	T*2	T2
<i>Shigella sonnei</i>	—	368	532	384
<i>E. coli</i> B.....	+	580	755	531
<i>E. coli</i> ER39.....	—	519	696	493
<i>E. coli</i> ER21.....	+	96	189	196
<i>E. coli</i> ER22.....	+	123	238	293
<i>E. coli</i> K-12-1000....	+	236	354	299
<i>E. coli</i> K-12-1100....	+	138	201	247

Exonuclease I activities. Table 3 shows that *rst*⁺ and *rst*[—] strains have comparable levels of exonuclease I. It is also apparent that there is a strong positive correlation between the exonuclease I levels and the endonuclease I levels (Table 2), suggesting stimulation of the former enzyme by the latter in the standard assay. This possibility was confirmed by adding a small quantity of a *dnsA*⁺ extract to *dnsA*[—] extracts. A threefold stimulation of the exonuclease I activity of the *dnsA*[—] extracts resulted. Moreover, when 25 μg of sRNA was added to the standard exonuclease I assay to suppress endonuclease I activity, the exonuclease I activities of *E. coli* B and ER39 decreased to the levels of the *dnsA*[—] strains (compare Tables 3 and 5). It is, therefore, apparent that the exonuclease I values of Table 3 for *dnsA*⁺ strains are exaggerated several-fold. More reliable are the results with the three *dnsA*[—] strains, which indicate that exonuclease I has a twofold preference for both of the phage DNA substrates over *E. coli* DNA.

Exonuclease II and exonuclease III activities. The results of surveys for these activities are given in Table 4. Again, *rst*⁺ and *rst*[—] strains are seen

to have comparable enzyme levels, and, as was the case with exonuclease I, there appears to be some stimulation of these activities by the high endonuclease I levels in the *Shigella*, *E. coli* B,

and ER39 extracts, even though 1 μ g of sRNA was added in both assays. When 25 μ g of sRNA was added, the two activities for *E. coli* B and ER39 were markedly reduced (Table 5). *E. coli*

TABLE 4. *Exonuclease II and exonuclease III specific activities*^a

Extract	<i>rst</i>	³ H-DNA substrate		
		<i>Escherichia coli</i>	T*2	T2
<i>Shigella sonnei</i>	—	136, 84	82, 43	82, 32
<i>E. coli</i> B.....	+	148, 110	88, 102	66, 90
<i>E. coli</i> ER39.....	—	126, 108	85, 66	57, 74
<i>E. coli</i> ER21.....	+	57, 64	22.6, 27.4	16.2, 9.7
<i>E. coli</i> ER22.....	+	59, 64	21.9, 19.7	9.5, 12.7
<i>E. coli</i> K-12-1000.....	+	73, 64	47, 30	29, 16.7
<i>E. coli</i> K-12-1100.....	+	71, 75	48, 34	38, 13.1

^a First entry in each column is the exonuclease II activity; second entry is the exonuclease III activity.

TABLE 5. *Deoxyribonuclease specific activities in modified assays*^a

Assay	Extract	<i>rst</i>	³ H-DNA substrate	
			T*2	T2
Endonuclease I ^b	<i>E. coli</i> B	+	2,940 (8)	1,520 (7)
	ER39	—	2,990 (12)	1,560 (9)
	ER22	+	6.8 (40)	0.3 (20)
	AC2519	+	2,780 (23)	1,740 (10)
	MT1111	—	2,740 (23)	1,900 (11)
	MT1100	+	2,520 (15)	1,640 (7)
	MT1101	—	1,990 (23)	1,600 (11)
Exonuclease I	<i>E. coli</i> B	+	194	268
	ER39	—	251	355
	ER22	+	275	293
	AC2519	+	272	235
	MT1111	—	268	219
	MT1100	+	300	244
	MT1101	—	293	237
Exonuclease II ^c	<i>E. coli</i> B	+	27	15
	ER39	—	42	20
	ER22	+	31	15
	AC2519	+	27 [12]	12 [7]
	MT1111	—	28 [13]	10 [5]
	MT1100	+	37 [18]	10 [4]
	MT1101	—	28 [12]	10 [5]
Exonuclease III ^c	<i>E. coli</i> B	+	52	22
	ER39	—	67	26
	ER22	+	44	16
	AC2519	+	37 [2]	18 [2]
	MT1111	—	38 [2]	15 [1]
	MT1100	+	39 [4]	14 [4]
	MT1101	—	47 [1]	13 [4]

^a sRNA (25 μ g) was present in all assays except endonuclease I.

^b Figures in parentheses are endonuclease I blank values. See Table 2.

^c Figures in brackets were obtained in the presence of 100 μ M ZnCl₂ per assay.

DNA is the preferred substrate in all cases, and, when endonuclease I does not interfere, the T*2 DNA substrate is hydrolyzed about twice as rapidly as T2 DNA.

As a check on the specificity of the assays, ZnCl_2 was added to a final concentration of 3.3×10^{-4} M for several of the strains examined (Table 5). Exonuclease III activities were 70 to 100% inhibited, whereas exonuclease II activities fell by 40 to 60%. These results are in accord with the known inhibition of purified exonuclease III by ZnCl_2 (17) and the fact that exonuclease III has substantial activity under the conditions of the exonuclease II assay (19).

DISCUSSION

Several kinds of indirect evidence (for reviews, see 13 and 15) had suggested that a deoxyribonuclease, possibly endonuclease I, might play a major role in the restriction of T* phages. This specific suggestion concerning endonuclease I could predict one or more of the following: (i) it should show in vitro a marked preference for T* DNA relative to T DNA, (ii) nonrestricting strains should lack it, and (iii) *E. coli* mutants selected as *dnsA*⁻ should also prove to be *rst*⁻. Our results fail to support any of these predictions. *E. coli* endonuclease I was found to hydrolyze T*2 DNA only about twice as fast as T2 DNA, and the *Rst*⁻ host, *S. sonnei*, had a high level of a deoxyribonuclease activity which resembled endonuclease I of *E. coli* in that it (i) was sRNA inhibitable, (ii) was released by osmotic shock, and (iii) showed an almost identical pattern of substrate preference. In addition, strain ER39, the mutant of *E. coli* B selected as *rst*⁻, remained *dnsA*⁺, and *dnsA*⁻ *E. coli* B and K-12 strains remained *rst*⁺.

The above evidence, although showing that the mere presence or absence of endonuclease I activity cannot explain restriction, is essentially negative, and it may be premature to eliminate the endonuclease I protein entirely in considering the phenomenon of restriction. The same protein, for example, might have a second active site not expressed or detected under our assay conditions. It may be recalled that the residual endonuclease I activity of ER22 extracts showed a 5-fold preference in one case (Table 2) and a 20-fold preference in another experiment (Table 5) for T* DNA over T DNA, a result which perhaps hints at a second enzyme or activity detectable by the standard in vitro assay. Also, while endonuclease I appears to be localized between cell membrane and wall (14), its locus with respect to the phage injection sites is unknown, and this intracellular location might differ in *rst*⁺ and *rst*⁻ hosts (see also 13 and 15).

The evidence concerning the three exonucleases of *E. coli* is less complete because mutants lacking these activities were not available. However, it appears that these enzymes are present in both *rst*⁺ and *rst*⁻ strains at comparable levels and have the same patterns of substrate preference.

Revel's study (15) of the enzymology of T-even restriction closely paralleled the approach reported here and led to similar results. She found that sonic extracts of *rst*⁺ and *rst*⁻ strains contained comparable levels of endonuclease I, exonuclease III, and the DNA-phosphatase associated with exonuclease III, and that the *dnsA*⁻ strain K-12-1100 restricts T* phage, as was also reported by Takano et al. (20). Comparable levels of endonuclease I in spheroplast supernatant fractions of *rst*⁺ and *rst*⁻ strains are reported in the accompanying paper (13). In addition, spheroplasts of *rst*⁺ hosts retain restriction activity in spite of the loss of the bulk of their endonuclease I (15; B. Molholt, Ph.D. Thesis, Indiana Univ., Bloomington, 1967). Richardson (16) has studied the four highly purified *E. coli* deoxyribonucleases in their action on T* DNA and T DNA. The results indicated that endonuclease I and exonuclease I had no marked preference for T* DNA and were therefore similar to the data on crude extracts presented here, and by Revel for endonuclease I. However, purified exonucleases II and III showed a significant (20-fold or more) preference for T* DNA, both in terms of rates and extents of hydrolysis. In the cell extracts, only a twofold preference for T* DNA was found for these enzymes, and exonuclease III in Revel's sonic extracts also preferred T* DNA by a factor of two. The significance of this discrepancy between the results of the assays with purified enzymes and cell extracts is difficult to assess, especially since Richardson found that limited endonuclease I digestion sensitized T DNA to hydrolysis by exonuclease III, and Shortman and Lehman (19) noticed that exonuclease II and III activities could vary by factors of up to five when assayed with different preparations of the same type of DNA substrate.

The properties of the *rst*⁻ mutant ER39 indicate that this strain differs from the *rst*⁻ mutants of *E. coli* B isolated by Revel (15) and Molholt (Ph.D. Thesis, Indiana Univ., Bloomington, 1967). Revel obtained several strains that are *thi*⁻ and *Rst*⁻ for T*2, T*4, and T*6 (*Rst* 2,4,6⁻), the thiamine requirement being inseparable from the *Rst*⁻ property. Molholt's strains are also *Rst* 2,4,6⁻ but are *thi*⁺. ER39 is *thi*⁻ but *Rst* 2,6⁻, and Revel obtained a temperature-sensitive mutant which is *thi*⁺ and *Rst* 6⁻. There thus appear to be distinct classes of *Rst*⁻

mutants obtained by single-step mutations from *E. coli* B. In contrast, *E. coli* K-12 appears to mutate first to Rst 6⁻, and only by a second step to Rst 2,4,6⁻ (15).

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LITERATURE CITED

1. ADELBERG, E. A., M. MANDEL, AND G. C. C. CHEN. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K12. *Biochem. Biophys. Res. Commun.* **18**:788-795.
2. ALBERTS, B. M. 1967. Fractionation of nucleic acids by dextran-polyethylene glycol two-phase systems, p. 566-581. In L. Grossman and K. Moldave [ed.], *Methods in enzymology*, vol. 12A. Academic Press, Inc., New York.
3. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315-322.
4. CUMMINGS, D. J., AND L. MONDALE. 1966. Density-gradient banding of denatured deoxyribonucleic acid in cesium sulfate. *Biochim. Biophys. Acta* **120**:448-453.
5. EIGNER, J., AND P. DOTY. 1965. The native, denatured and renatured states of deoxyribonucleic acid. *J. Mol. Biol.* **12**:549-580.
6. ERIKSON, R. L., AND W. SZYBALSKI. 1964. The Cs₂SO₄ equilibrium density gradient and its application for the study of T-even phage DNA: glucosylation and replication. *Virology* **22**:111-124.
7. HATTMAN, S. 1964. The functioning of T-even phages with unglucosylated DNA in restricting *Escherichia coli* host cells. *Virology* **24**:333-348.
8. LEHMAN, I. R. 1960. The deoxyribonucleases of *Escherichia coli*. I. Purification and properties of a phosphodiesterase. *J. Biol. Chem.* **235**:1479-1487.
9. LEHMAN, I. R., AND C. C. RICHARDSON. 1964. The deoxyribonucleases of *Escherichia coli*. IV. An exonuclease activity present in purified preparations of deoxyribonucleic acid polymerase. *J. Biol. Chem.* **239**:233-241.
10. LEHMAN, I. R., G. G. ROUSSOS, AND E. C. PRATT. 1962. The deoxyribonucleases of *Escherichia coli*. II. Purification and properties of a ribonucleic acid-inhibitable endonuclease. *J. Biol. Chem.* **237**:819-828.
11. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
12. MASSIE, H. R., AND B. H. ZIMM. 1965. The use of hot phenol in preparing DNA. *Proc. Natl. Acad. Sci. U.S.A.* **54**:1641-1643.
13. MOLHOLT, B., AND D. FRASER. 1968. Host-controlled restriction of T-even bacteriophages: relation of endonuclease I and T-even-induced nucleases to restriction. *J. Virol.* **2**:313-319.
14. NOSSAL, N. G., AND L. A. HEPPEL. 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *J. Biol. Chem.* **241**:3055-3062.
15. REVEL, H. R. 1967. Restriction of nonglucosylated T-even bacteriophage: properties of permissive mutants of *Escherichia coli* B and K12. *Virology* **31**:688-701.
16. RICHARDSON, C. C. 1966. Influence of glucosylation of deoxyribonucleic acid on hydrolysis by deoxyribonucleases of *Escherichia coli*. *J. Biol. Chem.* **241**:2084-2092.
17. RICHARDSON, C. C., I. R. LEHMAN, AND A. KORNBERG. 1964. A deoxyribonucleic acid phosphatase-exonuclease from *Escherichia coli*. II. Characterization of the exonuclease activity. *J. Biol. Chem.* **239**:251-258.
18. SAITO, H., AND K. I. MIURA. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta* **72**:619-629.
19. SHORTMAN, K., AND I. R. LEHMAN. 1964. The deoxyribonucleases of *Escherichia coli*. VI. Changes in enzyme level in response to alterations in physiological state. *J. Biol. Chem.* **239**:2964-2974.
20. TAKANO, T., T. WATANABE, AND T. FUKASAWA. 1966. Specific inactivation of infectious λ DNA by sonicates of restrictive bacteria with R factors. *Biochem. Biophys. Res. Commun.* **25**:192-198.
21. THOMAS, C. A., JR., AND J. ABELSON. 1966. The isolation and characterization of DNA from bacteriophage, p. 553-568. In G. L. Cantoni and D. R. Davies [ed.], *Procedures in nucleic acid research*. Harper and Row, Publishers, New York.
22. THOMAS, C. A., JR., K. I. BERNIS, AND T. J. KELLY, JR. 1966. Isolation of high molecular weight DNA from bacteria and cell nuclei, p. 535-540. In G. L. Cantoni and D. R. Davies [ed.], *Procedures in nucleic acid research*. Harper and Row, Publishers, New York.