# *HOST SPECIFICITY OF DNA PRODUCED BY ESCHERICHIA COLI, X. IN VITRO RESTRICTION OF PHAGE FD REPLICATIVE FORM*

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The functions involved in strain-specific modification and restriction of DNA produced by *Escherichia coli* are under the genetic control of the chromosome or of other genetic elements such as prophage and transfer factors.[1] They are active upon bacterial as well as many phage DNA's. The relatively small, biologically active phage DNA's which can be isolated in a homogeneous form provide a convenient system for studying the molecular mechanism of the functions. In this way it has been suggested for phage λ[2] and shown for phage fd[3] that modification is accompanied by the appearance of 6-methylamino purine at a limited number of sites within the DNA. The absence of this methylation might then allow an appropriate restriction activity to alter the DNA such that its biological activity is destroyed.

In order to study the restriction phenomenon *in vitro*, the replicative form (RF)[4] of phage fd appears to be particularly advantageous. This DNA is a small molecule of some 6000 base pairs with a closed, circular structure[5] which makes it resistant to exonucleases. Furthermore, fd strains with DNA containing a defined, small number of modification sites have been isolated.[6] Phage fd is restricted and modified by bacteria carrying the B- and P1-host specificities, but not by the K system,[7] presumably because fd DNA lacks the appropriate sites, nor by C strains,[8] which have not been observed to exhibit host-controlled modification. Hence, cell-free extracts were examined for activities which could reduce the infectious capacity of the RF and which were (1) specific to *E. coli* B or to K strains lysogenic for P1 and (2) inactive upon appropriately modified DNA.

Although no such activity was observed in crude extracts, a fractionation procedure, designed primarily to remove nonprotein material, yielded active fractions from both B and P1 lysogenic strains. *In vitro* complementation between extracts from certain nonrestricting bacterial mutants was also observed, suggesting that more than one gene function is required for the observed inactivation. Specific inactivation of infectious λ DNA by sonicates of restrictive bacteria carrying R factors[9] and by extracts from P1-lysogenic and K strains[10] have also been recently observed in other laboratories.

*Materials and Methods.—Materials:* Nucleoside triphosphates were purchased from Sigma Chemical Company. S-adenosylmethionine was purchased as the iodide salt from Calbiochem and purified by the method of Mudd.[11] It was stored frozen in 4 *N* acetic acid and neutralized with NaOH immediately before use. Soluble RNA from *E. coli* was purchased from Schwartz BioResearch, Inc. Streptomycin was purchased from Calbiochem and dissolved immediately before use. Methylated albumin, prepared as described by Ray, Bscheider, and Hofschneider,[5] was a generous gift of Dr. Rolf Benzinger. Thirty per cent bovine serum albumin "Povite" for spheroplast assays was a product of the Biotest Serum Institute, Frankfurt, Germany. Kieselguhr (J. M. Hyflo-Super-Cel) was purchased from Carl Roth, Karlsruhe, Germany.



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*Bacterial and phage strains:* The following strains of *E. coli* were employed: 519, an $F^+$ derivative of strain C;[12] 1101, an $F^+$ K strain deficient in endonuclease I;[13] 2276, a derivative of 1101 lysogenic for phage P1; B/41, an F'*lac* B strain deficient in endonuclease I;[13] 707, a λ-sensitive B strain;[14] 834, a nonrestricting and nonmodifying ($r_B^-$ $m_B^-$) spontaneous mutant of 707;[14] 837, a nonrestricting ($r_B^-m_B^+$) spontaneous mutant of 707;[14] 993, an $F^+r_K^+m_K^+$ strain;[7] 2027, an $F^+$ derivative of a K-B transduction hybrid $r_B^+m_B^+$ $thr_B^+leu_K^-met_K^-$.[7] Phage fd was obtained from Dr. H. Hoffmann-Berling.[15]

*Preparation of RF:* *E. coli* (strain 993 for RF·O, or strain 2027 for RF·B) was grown to a density of $3 \times 10^8$ cells/ml in 5 liters of Tryptone broth (1% Bactotryptone, 0.5% NaCl), then infected with fd phage at a multiplicity of infection of 5. After 90 min, cells were harvested, washed twice with 1 liter 0.1 *M* NaCl–0.01 *M* Tris-Cl, pH 8, then suspended in 500 ml 0.1 *M* NaCl–0.01 *M* Tris-Cl, pH 8–0.01 *M* EDTA. Isolation of intracellular DNA and separation of the RF on two successive MAK columns were performed as described by Ray *et al.*,[5] except that the volume of the first column was increased by tenfold. The final column fractions were concentrated by dialysis versus solid sucrose, then dialyzed versus 0.01 *M* Tris-Cl, pH 8, $10^{-3}$ *M* EDTA. As judged by electron microscopy, such preparations contained approximately 25% bacterial DNA.

*Assays of infectious DNA:* Lysozyme spheroplasts were prepared according to Ray *et al.*,[5] except that they were incubated for 30 min at 37° before cold storage. *E. coli* 993 spheroplasts were used to assay RF·O; strains 993 and 2027 yielded identical results with RF·B. To test for infectivity, 0.1 ml of DNA, appropriately diluted in 0.01 *M* Tris-Cl, pH 8–$10^{-3}$ *M* EDTA, was mixed with 0.9 ml assay mixture[16] and 0.3 ml spheroplast suspension, incubated 8–9 min at 37°, mixed with 3 ml sucrose-soft agar[16] (containing indicator bacteria of the same strain as the spheroplasts), and plated onto EMB agar,[17] containing 1% glycerol as a carbon source. Depending upon the particular preparation and age of the spheroplasts, $5 \times 10^5$ to $3 \times 10^6$ PFU per $OD_{260}$ of RF were obtained.[18] At least three simultaneous assays were done for each DNA sample being tested.

*Preparation of bacterial extracts:* All buffers contained $5 \times 10^{-3}$ *M* 2-mercaptoethanol and $5 \times 10^{-4}$ *M* EDTA, and all centrifugations were at 3,000 × *g* (low speed), or 20,000 × *g* for 10 min.

Bacteria were grown in 650 ml Tryptone broth to a density of 3–5 × $10^8$ per ml, harvested by low-speed centrifugation, and resuspended in 6 ml 0.05 *M* Tris-Cl, pH 8. The cells were disrupted by treatment for 3 min in an MSE ultrasonic disintegrator, and the debris were removed by low-speed centrifugation. The extract was made 0.035 *M* in $Mg^{++}$ by the addition for 1 *M* $MgCl_2$, and the turbid material was removed by centrifugation. A sufficient amount of a 5% streptomycin solution was then added to give a ratio of $A_{280}$:$A_{260}$ of 0.75 after centrifugation. The streptomycin supernatant fraction was made 60% saturated in ammonium sulfate by the addition of the dry salt, and after 20 min the precipitate was collected by centrifugation and suspended in 3 ml 0.05 *M* Tris-Cl, pH 8. The suspension was dialyzed twice versus 2 liters of 0.01 *M* potassium phosphate, pH 6.7, and then cleared of turbidity by centrifugation to yield the active "dialyzed ammonium sulfate fraction" used for the enzyme studies. For all strains of bacteria used, the procedure gave a fraction which had a ratio of $A_{280}$:$A_{260}$ of about 1.0, and which contained roughly 50% of the protein found in the crude sonicate and 10% of the 260 mμ absorbance found in the $Mg^{++}$ supernatant fraction.

*Detection of in vitro restriction:* Reaction mixtures (0.15 ml) contained 0.075 *M* Tris-Cl, pH 8; 7.5 mM $MgCl_2$; 1 mM 2-mercaptoethanol; 30 μM sRNA; 1.5 mM ATP; 10 μM SAM (and consequently, 0.02 *M* sodium acetate arising from the acetic acid used to store the compound); 0.04 density units (260 mμ) RF; and extract protein at a final concentration of 1.5 mg/ml. After incubation for 30 min at 37°, 15 μliters of 0.2 *M* EDTA was added and the mixtures were chilled and assayed for infectivity. Up to 10 μliters of this reaction mixture gives a linear response in the infectivity assay. Assays indicating greater than 99% inactivation are indicative only of an order of magnitude because of the small number of infective units surviving.

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*Other methods:* Protein was determined by the method of Lowry *et al.*,[19] ultraviolet absorption was determined with a Zeiss PMQ II spectrophotometer, and bacterial titers were determined by counting in a Petroff-Hausser bacteria counter.

*Results.—Specificity of the reaction:* An enzyme activity responsible for restriction of fd RF should (1) be identifiable only in bacterial strains which restrict phage fd, and (2) specifically inactivate nonmodified or inappropriately modified DNA. With regard to the first criterion, when fd RF·O is incubated with the dialyzed ammonium sulfate fractions from C, or K strains as described in *Materials and Methods*, there is relatively little inactivation of infectivity (Table 1). However, when the P1 prophage is introduced into the same

TABLE 1. *Effect of the dialyzed ammonium sulfate fractions from various strains of E. coli upon the biological activity of fd RF·O.*

| Source of extract | Per cent infectivity after incubation |
|---|---|
| Omit dialyzed fraction | = 100 |
| 519 = C | 67 |
| 1101 = K *endo*$^-$ | 76 |
| 2276 = K(P1) *endo*$^-$ | 4.4 |
| B/41 = B *endo*$^-$ | 0.4 |
| 707 = B $r^+m^+$ | 0.5 |
| 837 = B $r^-m^+$ | 76 |
| 834 = B $r^-m^-$ | 50 |

fd RF·O was incubated with the dialyzed ammonium sulfate fractions from various bacterial strains and assayed for infectivity as described in *Materials and Methods.*

K strain, fractions are now obtained from this P1-lysogenic strain, 2276, that give considerable inactivation. Likewise, fractions from the B strains, B/41 or 707, reduce infectivity below 1 per cent of control values. But mutants from B strain 707 which are unable to restrict phage fd *in vivo* yield cell-free fractions which also do not inactivate DNA. Hence the first criterion is fulfilled.

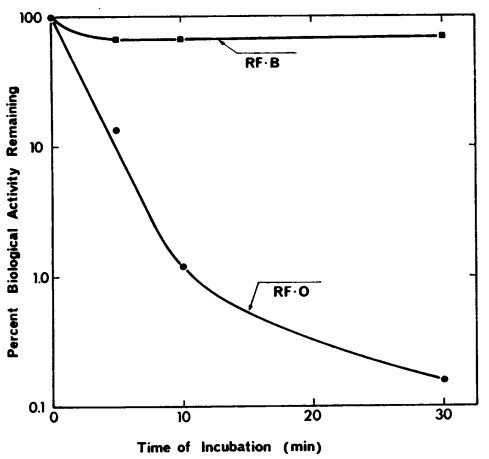


FIG. 1.—Effect of B/41 dialyzed ammonium sulfate fraction upon fd RF·O and fd RF·B. Reaction mixtures without protein were prepared as described in *Materials and Methods*, prewarmed, mixed with the dialyzed ammonium sulfate fraction from strain B/41, and incubated at 37° for the times indicated. After incubation they were chilled in ice and 15 $\mu$liters of 0.2 *M* EDTA was added. The zero time samples were mixed with EDTA before the addition of protein and showed no loss of activity compared to untreated RF.

With regard to the second criterion, when fd RF·B is used as substrate for the dialyzed ammonium sulfate fraction from strain B/41, little inactivation

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TABLE 2. *Effect of the dialyzed ammonium sulfate fractions from various strains of E. coli upon the biological activity of fd RF·O and fd RF·B.*

| Source of extract | Per Cent Infectivity after Incubation RF·O | RF·B |
|---|---|---|
| Omit dialyzed fraction | = 100 | = 100 |
| 1101 = K *endo*$^-$ | 87 | 95 |
| B/41 = B *endo*$^-$ | 1.6 | 97 |
| 2276 = K(P1) *endo*$^-$ | 4.4 | 6.1 |

The two types of RF were tested in parallel as to their sensitivity to the various ammonium sulfate fractions as described in *Materials and Methods*.

TABLE 3. *Conditions for restriction of fd RF·O by the dialyzed ammonium sulfate fraction from E. coli B/41.*

| Expt. | Reaction mixture | Per cent infectivity after incubation |
|---|---|---|
| I. | Omit dialyzed fraction | = 100 |
| | Complete system | 1.1 |
| | Omit $Mg^{++}$ | 76 |
| | Complete system plus 0.02 *M* EDTA | 94 |
| | Omit soluble RNA | 0.9 |
| | Omit 2-mercaptoethanol | 0.8 |
| | Omit ATP | 42 |
| II. | Omit dialyzed fraction | = 100 |
| | Complete system | 6 |
| | Omit S-adenosylmethionine | 89 |
| III. | Omit dialyzed fraction | = 100 |
| | Complete system (1.5 mg protein/ml) | 0.5 |
| | Complete, but 0.7 mg protein/ml | 23 |
| | Complete, but 0.3 mg protein/ml | 66 |
| | Complete, but protein heated 5 min 100°C | 108 |
| | Omit 37° incubation | 86 |
| IV. | Omit dialyzed fraction | = 100 |
| | Complete system | 1.6 |
| | Omit dialyzed fraction, add crude extract | 78 |
| | Complete system plus crude extract | 1.3 |

fd RF·O was exposed to the dialyzed ammonium sulfate fraction from *E. coli* B/41 as described in *Materials and Methods* with the modifications specified. Where indicated, S-adenosylmethionine was replaced by 0.02 *M* sodium acetate, or the crude extract was added at a concentration of 1.2 mg/ml. Fractions from the P1-lysogenic strain, 2276, have the same requirements.

occurs during a 30-minute period. Under these same conditions the infectivity of fd RF·O exhibits a time-dependent reduction to 0.15 per cent of its initial level (Fig. 1). However, whereas fd RF·B is resistant to the dialyzed ammonium sulfate fraction from strain B, it is not resistant to that from P1-lysogenic cells (Table 2). In summary, when assayed under these conditions, fractions from *E. coli* B inactivate fd RF·O, but not fd RF·B; fractions from *E. coli* K inactivate neither type of DNA, but lysogeny of this strain by phage P1 imparts the ability to inactivate both DNA's. These are the specificities expected from *in vivo* observations.

*Properties of the reaction:* The requirements for *in vitro* restriction are summarized in Table 3. The activity has a pH optimum of 8, requires $Mg^{++}$ (optimal concentration, 7 mM), and is inhibited by EDTA. It has an absolute requirement for SAM and ATP.[20, 21] Soluble RNA and 2-mercaptoethanol, although not affecting the rate of reaction, are added to overcome the effects of endonuclease I[22] and heavy metals, respectively, which have occasionally been observed. The inactivation requires incubation (37° is the optimum tempera-

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ture) and is dependent upon the amount of dialyzed ammonium sulfate fraction added. Neither heated dialyzed ammonium sulfate fraction nor crude sonicate inactivate the RF,[23] and, parenthetically, addition of crude extract does not affect the inactivation brought about by the dialyzed ammonium sulfate fraction.

Restriction of DNA *in vivo* ultimately results in extensive breakdown to acid-soluble products.[31] However, as judged by electron microscopy, RF·O is subject only to limited double-strand cleavage when treated as described above with fractions from *E. coli* B[8] suggesting that extensive degradation might be secondary to the restriction event. Experiments are in progress to determine whether the number of cleavage sites found is equal to that expected from the number of restriction sites known for the DNA of several strains of fd.

*Complementation between nonrestricting extracts:* Nonrestricting mutants of bacterial strains K and B as well as of phage P1 fall into two classes:[14, 24–26] those which still modify ($r^-m^+$) and those which neither restrict nor modify ($r^-m^-$). Since these two classes of mutants occur with approximately equal frequency, it is likely that they represent singular mutational events, one affecting both functions, the other only restriction. Although partially purified fractions from neither class of mutant cause *in vitro* restriction (Tables 1 and 4), it is nevertheless possible that each could supply the function lacking in the other, i.e., that a mixture of the two fractions would be active. Indeed, a mixture of the two dialyzed ammonium sulfate fractions exhibited an activity similar to that of wild-type fractions (Table 4), both in its extent and in its requirement for SAM

TABLE 4. *Complementation between dialyzed ammonium sulfate fractions from nonrestricting phenotypes.*

| Phenotype of bacteria used for extract | Per cent infectivity after incubation |
|---|---|
| Omit dialyzed fraction | = 100 |
| $r_B^-m_B^-$ | 71 |
| $r_B^-m_B^+$ | 71 |
| $r_B^-m_B^-$ plus $r_B^-m_B^+$ | 0.9 |
| Combination, omit ATP | 31 |
| Combination, omit SAM | 85 |

Incubations were carried out with 1.5 mg/ml of dialyzed ammonium sulfate fraction from strains 834 or 837 as described in *Materials and Methods*. In the "combination" samples each fraction was added at a final concentration of 1.2 mg/ml. Individual fractions at a concentration of 2.4 mg/ml give no more inactivation then when added at the lower concentration reported in this experiment.

and ATP.[21] These results suggest, therefore, that the restriction activity may be due to at least two separate functions. Fractions from the mutants noted above could then be used to detect individually either of these two functions. In this way it has been shown[8] that in the dialyzed ammonium sulfate fraction the activity specific only to restriction (i.e., that lost by the $r^-m^+$ mutants) is the rate-limiting step of the restriction process.

*Discussion.*—Studies with the various bacterial strains implicate the *in vitro* activity described here with *in vivo* restriction. Thus, extracts from K or C strains which do not restrict fd, or from B strains which have lost the ability to restrict, lack this activity. On the other hand, the introduction of the P1 restriction character into K strains via the P1 prophage is concomitant with the appearance of activity in extracts. Likewise, the introduction of the B modifica-

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tion character into the K strain, 993, to form the hybrid strain 2027, confers the ability to make RF which is inert to the *in vitro* activity from B, but not to that from P1-lysogenic K strains.

The requirement for S-adenosylmethionine parallels *in vivo* observations of the requirement of methionine for restriction.[27] The requirement for ATP does not seem to be involved in the generation of a methylating agent, since methionine has not been observed to stimulate the *in vitro* restriction activity.[8] Triphosphates, however, have been reported to stimulate several deoxyribonuclease activities.[28-30]

The complementation studies suggest that restriction activity is conferred by at least two gene functions, one of which is also required for modification. Whether these functions must act simultaneously or whether they can act on the DNA in sequence is not as yet known.

It is also of interest to know whether single-stranded phage DNA can be restricted. *In vitro* experiments have in fact shown that this activity proceeds at a much lower rate (less than one-tenth that found with the double-stranded RF), if at all.[8] More precise studies await further purification of the restriction enzyme(s) because of the presence in current fractions of an interfering activity which produces single-strand cleavage of the DNA.

*Summary.*—An activity has been found in fractionated extracts from *Escherichia coli* which reduces the infectivity of the replicative form of phage fd DNA. It is correlated with the *in vivo* restriction phenomenon by (1) its presence only in fractions from restricting strains of bacteria and (2) its specificity for nonmodified DNA. The inactivation requires S-adenosylmethionine, ATP, $Mg^{++}$, and the products of at least two gene functions; it seems to be accompanied by double-strand cleavage of the DNA.

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[4] The following abbreviations are used: RF, replicative form; fd RF·O and fd RF·B, replicative form isolated from nonmodifying or B strains of *E. coli*, respectively, infected with phage fd; *thr*, *leu*, *met*, *endo*, genetic markers for threonine, leucine, methionine, and endonuclease I biosynthesis, respectively; $r_i^-$ and $m_i^-$, mutants of strain *i* which are phenotypically nonrestricting, or nonmodifying, respectively; K, B, O, strains of *E. coli* with host-specificity phenotypes as carried by strains K12, B, and $r_i^-m_i^-$ mutants, respectively; EMB, eosin methylene blue; ATP, adenosine triphosphate; SAM, S-adenosylmethionine; EDTA, ethylenediaminetetraacetic acid; sRNA, soluble ribonucleic acid; MAK, methylated albumin Kieselguhr.

[5] As shown for the closely related phage M13 by Ray, D. S., H. P. Bscheider, and P. H. Hofschneider, *J. Mol. Biol.*, **21**, 473 (1966), and by Ray, D. S., A. Preuss and P. H. Hofschneider, *J. Mol. Biol.*, **21**, 485 (1966).

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[20] This observation confirms a finding made in another system by Yuan and Meselson[10] which had come to our attention during our experiments. The saturating concentration of SAM has not as yet been determined due to interference by the sodium acetate added concomitantly when high amounts are used. The ATP concentration, however, is saturating. UTP and CTP do not substitute for ATP, but variable activity has been noted for several GTP preparations.

[21] The lower infectivity levels observed upon omission of ATP do not appear to be related to the restriction activity, since similar reduction is noted with fractions from K, C, or $r_B^-$ strains.

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