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NOTES

In Vivo Methylation by *Escherichia coli* K-12 *mec*$^+$ Deoxyribonucleic Acid-Cytosine Methylase Protects Against In Vitro Cleavage by the RII Restriction Endonuclease (R·*Eco*RII)

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We have analyzed the susceptibility of the deoxyribonucleic acid (DNA) of phage fd replicative form (RF) and of *Escherichia coli* to in vitro cleavage by purified RII restriction endonuclease (R·*Eco*RII). The results are summarized as follows: (i) fd·*mec*$^-$ RFI, isolated from infected *E. coli* K-12 *mec*$^-$ bacteria (a mutant strain lacking DNA-cytosine methylase activity), is cleaved into at least two fragments, whereas fd·*mec*$^+$ RFI, isolated from the parental *mec*$^+$ strain, is not cleaved. (ii) *E. coli mec*$^-$ DNA is extensively degraded, whereas *E. coli mec*$^+$ DNA is resistant to cleavage. We conclude that the *E. coli mec*$^+$ DNA-cytosine methylase acts as an RII modification enzyme.

The N-3 and R15 drug-resistance-transfer (R) factors control a deoxyribonucleic acid (DNA) modification-restriction system (*hsp*II or RII) that acts on a variety of double-stranded DNA phages, e.g, λ, P22, P1, and φ80 (1, 3, 16, 17). The N-3 factor specifies a DNA-cytosine methylase (3, 5) that we demonstrated to be the RII modification enzyme (13). We also observed that phage λ was partially protected against restriction by N-3-containing cells if the phage had been previously grown in *mec*$^+$ hosts, but not after growth in *mec*$^-$ hosts (6). Results of further investigations led us to propose that the *Escherichia coli mec*$^+$ DNA-cytosine methylase has the same sequence specificity as the RII modification methylase (9, 10). It would follow that the *mec*$^+$ methylase should act as an RII modification enzyme; i.e., *mec*$^+$ methylation should protect DNA against degradation by the RII restriction endonuclease (R·*Eco*RII).

To investigate this question, fd RFI (covalently closed circular double-stranded DNA), isolated from infected *E. coli mec*$^-$ and *E. coli mec*$^+$ bacteria, was incubated with R·*Eco*RII and analyzed by agarose-gel electrophoresis. As can be seen in Fig. 1b, fd·*mec*$^+$ RFI is not subject to cleavage by R·*Eco*RII. In contrast, fd·*mec*$^-$ RFI is cleaved into at least two fragments (Fig. 1e). In the slab gel system employed, the larger fragment is poorly resolved from the unit-length, linear duplex (RFIII); however, under modifed conditions the large fragment (RII-A) is seen to migrate slightly faster than RFIII (Fig. 2). Recently, Vovis and co-workers, using the closely related phage f1 (15), have confirmed our observations and calculated that the two fragments (RII-A and RII-B) produced by R·*Eco*RII cleavage of f1·*mec*$^-$ RFI correspond to 85.5% and 14.5% of the length of RFIII, respectively. The fd fragments produced by R·*Eco*RII cleavage are identical in size to the f1 fragments (compare Fig. 1e and g).

fd·*mec*$^+$ RFI and f1·*mec*$^+$ RFI are both cleaved by R·*Hin*d at one site to produce RFIII (7, 11, 14). We observe that fd·*mec*$^-$ RFI is also cleaved once by R·*Hin*d (Fig. 1c and f). Thus, *mec*$^+$ methylation specifically protects fd RFI against degradation by R·*Eco*RII.

E. coli DNA methylated in vivo by the *mec*$^+$ DNA-cytosine methylase has almost the same 5-methylcytosine (MeC) content as *E. coli* DNA methylated by the RII modification methylase (6; unpublished data). Thus, it was of considerable interest to determine whether *E. coli mec*$^+$ DNA is also resistant to in vitro cleavage by R·*Eco*RII. *E. coli mec*$^+$ and *E. coli mec*$^-$ (N-3) DNAs are resistant to cleavage, whereas *E. coli mec*$^-$ DNA is extensively degraded by R·*Eco*RII (Fig. 3). That the *E. coli mec*$^+$ DNA

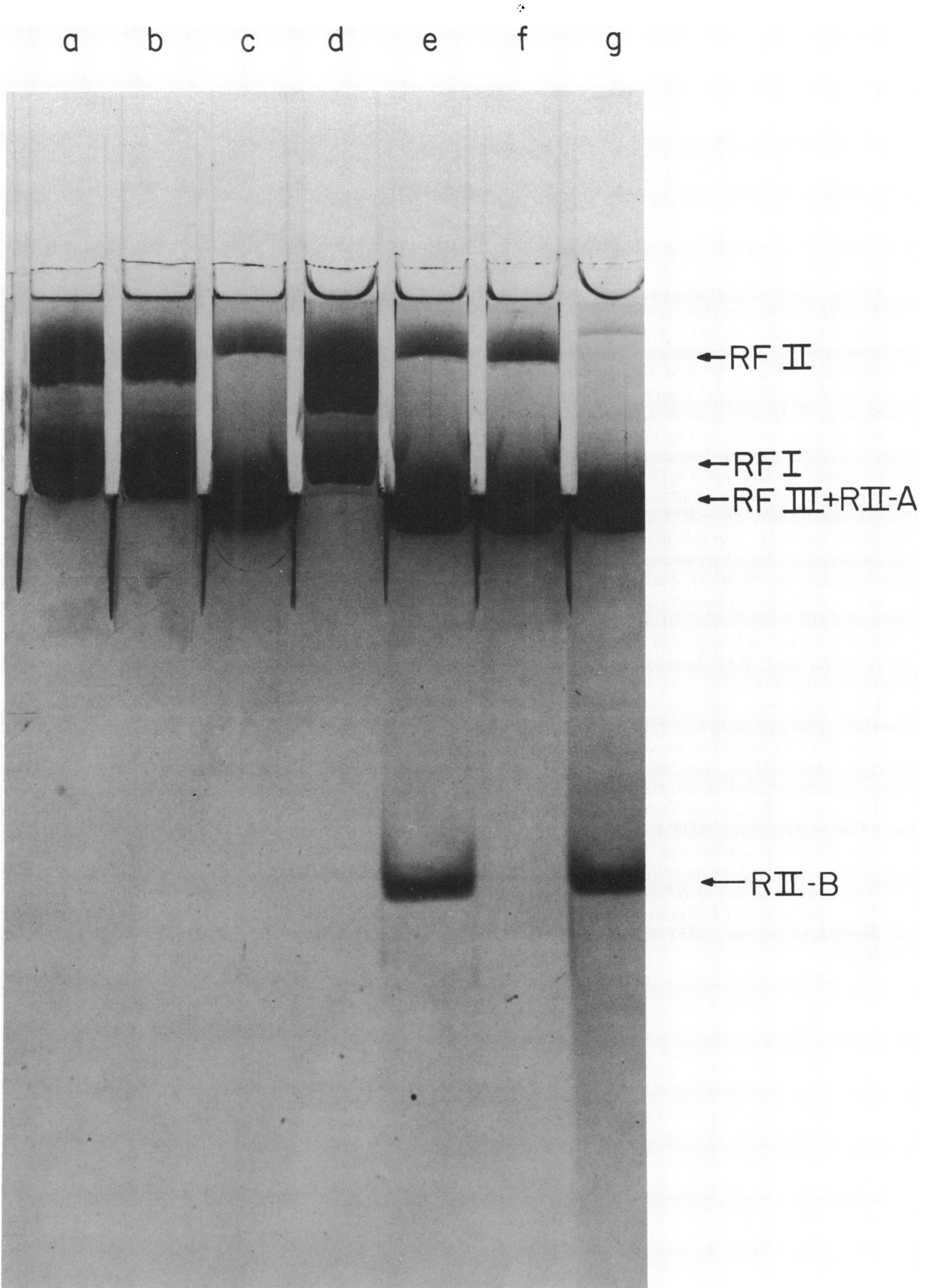
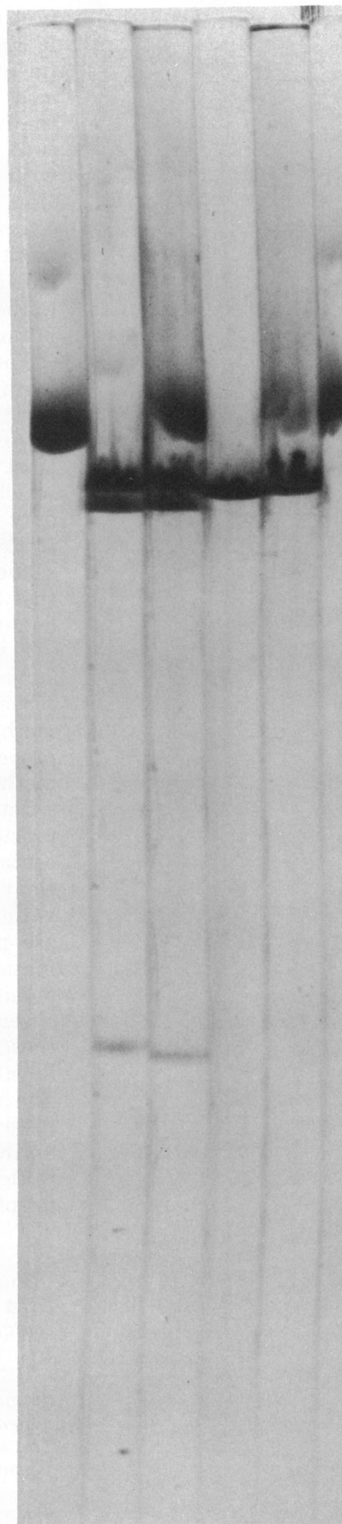


FIG. 1.

FIG. 1. *Agarose slab-gel electrophoresis of fd·RFI and f1·RFI treated with restriction endonucleases. The R·EcoRII reaction mixture contained: 4.5 μg of fd or f1 RFI; 5 μl of 0.9 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4 to 7.5; 5 μl of 50 mM $MgCl_2$; 1 μl of R·EcoRII; and water to bring the total volume to 50 μl. The samples were incubated at 37 C for 75 min. The R·Hind reaction mixture contained: 4.5 μg of fd RFI; 6 μl of 56 mM Tris-hydrochloride (pH 7.4 to 7.5); 6 μl of 56 mM $MgCl_2$; 1 μl of R·Hind; and water to bring the total volume to 50 μl. The enzyme preparation contained a mixture of two restriction nucleases, R·HindII and R·HindIII. The samples were incubated at 37 C for 75 min. Nuclease digestion reactions were terminated by the addition of 10 μl of 25% sucrose – 0.25 M disodium ethylenediaminetetraacetate (Na_2 EDTA), pH 8.0, and then 2 μl of 5% sodium dodecyl sulfate – 1% bromphenol blue. The samples were heated at 55 C for 2 min (to disrupt any possible association of "sticky ends"), chilled in ice, and then equilibrated for 1 min at 37 C. The entire sample was then layered on a 1.8% agarose slab gel. The running buffer consisted of 40 mM Tris-acetate (pH 7.4), 20 mM sodium acetate, 2 mM Na_2 EDTA, and 0.5 μg of ethidium bromide per ml. Electrophoresis was for 2 h at 80 V at 20 C. After electrophoresis the gel was illuminated under short-wavelength ultraviolet light and photographed. The DNA bands would normally appear white in the print; however, a copy-negative was first made, and the resulting print has the images reversed. The same photographic procedure was used in Fig. 2, 3 and 4. RFII is the open circular double-stranded DNA containing at least one discontinuity in one (or both) of the strands. (a) Untreated fd·mec^+ RFI; (b) fd·mec^+ RFI digested with R·EcoRII; (c) fd·mec^+ RFI digested with R·Hind; (d) untreated fd·mec^- RFI; (e) fd·mec^- RFI digested with R·EcoRII; (f) fd·mec^- RFI digested with R·Hind; (g) f1·mec^- RFI digested with R·EcoRII.*

FIG. 2. *Agarose tube-gel electrophoresis of fd·RF treated with restriction endonuclease. The reaction mixtures (see legend to Fig. 1) contained either 10 μl of R·EcoRII or 20 μl of R·Hind; after incubation for 120 min at 37 C the reactions were terminated by the addition of 20 μl of 25 mM disodium ethylenediaminetetraacetate (Na_2 EDTA), 20% sucrose, and 0.0125% bromophenol blue. After heating for 2 min at 65 C, the samples were chilled on ice, and approximately 0.6 μg of each RF sample was layered on cylindrical gels (0.45 by 13.5 cm) of 1% agarose (wt/vol) contained in tris-(hydroxymethyl)aminomethane (Tris)-borate buffer (10.8 g of Tris, 0.93 g of Na_2 EDTA; 5.5 g of boric acid; and 500 μg of ethidium bromide per liter); the gels had been subjected to electrophoresis at 20 C at 150 V until the bromophenol blue dye markers migrated at least 8 cm (ca. subjects to electrophoresis at 20 C at 150 V until the bromphenol blue dye markers migrated at least 8 cm (ca. 1.75 h). The presence of RF III in track (b) indicates that cleavage was incomplete. (a) Untreated fd·mec^- RFI; (b) fd·mec^- RFI digested with R·EcoRII; (c) untreated fd·mec^- RFI + fd·mec^- RFI digested with R·EcoRII; (d) fd·mec^- RFI digested with R·Hind; (e) untreated fd·mec^- RFI + fd·mec^- RFI digested with R·Hind.*



a
b
c
d
e
RF II
RF I
RF III
RII A
RII B

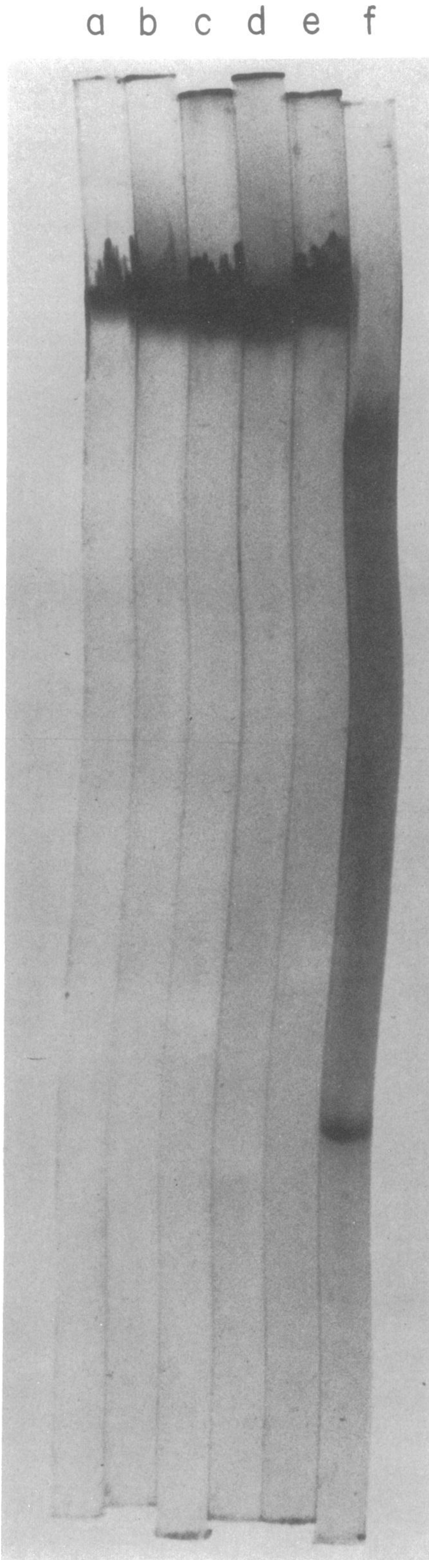
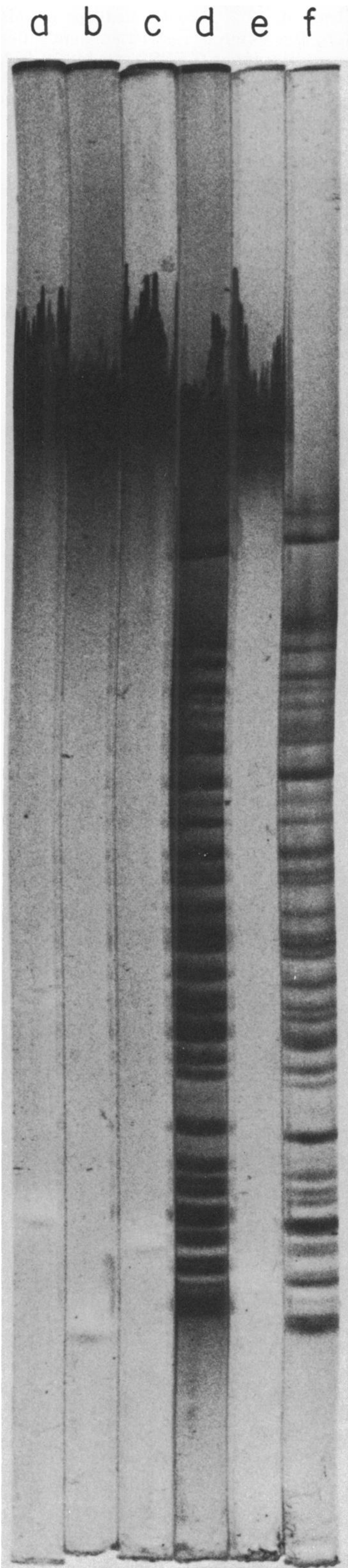


FIG. 3. *Agarose tube-gel electrophoresis of E. coli DNA treated with R·EcoRII. The reaction mixture contained: 1.0 to 1.8 μg of DNA; 5 μl of 0.95 M tris (hydroxymethyl) aminomethane-hydrochloride (pH 7.5)-0.05 M $MgCl_2$-0.1 mM disodium ethylenediaminetetraacetate; 20 μl of R·EcoRII (when added); and water to bring the total volume to 50 μl. The samples were incubated for 120 min at 37 C. Termination of the digestion and electrophoresis were as described in the legend to Fig. 2. (a,b) E. coli mec^- (N-3) DNA; (c,d) E. coli mec^+ DNA; (e,f) E. coli mec^- DNA; (a,c,e) untreated controls; (b,d,f) digested with R·EcoRII.*

preparation does not contain an inhibitor of R·*Eco*RII was demonstrated in a control experiment in which we treated a mixture of *E. coli* mec^+ DNA and λ·mec^- DNA with R·*Eco*RII; the fragment pattern produced with the mixture is a composite of the patterns obtained with the two DNA samples treated separately (Fig. 4). It should be noted that cleavage of *E. coli* mec^- DNA does not produce discrete fragment bands, as seen with phage λ DNA (compare Fig. 3f and 4f). We attribute this difference to the larger number and more complex distribution of RII sites in bacterial DNA.

The above results demonstrate that in vivo methylation of *E. coli* and fd RFI DNAs by the mec^+ methylase fully protects them against in vitro degradation by R·*Eco*RII. This is in contrast to the observation that λ·mec^+ DNA, which is incompletely methylated in vivo (9), is only partially protected against cleavage by R·EcoRII (8). Taken together with our earlier sequencing studies (9, 10), our findings suggest that the *E. coli* mec^+ DNA cytosine methylase behaves as an RII modification enzyme.

It should be noted that phage fd·mec^- is not subject to RII restriction in vivo, although it serves as a substrate for the RII modification methylase (4, 13). These observations led us to propose that the RII modification enzyme can methylate sequences not recognized by the restriction enzyme (4, 6). This possibility is now precluded by the fact that fd RFI lacking MeC contains at least two RII sites that can be cleaved in vitro; this correlates with the observation that in vivo the RII modification methylase produces two to three MeC residues per mature fd single-stranded DNA (4, 6) (it is possible that a third RII site exists that was not observed to be cleaved under the conditions of these experiments). It is not known why fd·mec^- DNA escapes RII restriction in vivo. The resistance to restriction may be due to a faster rate of modification versus cleavage or to an inhibitor of restriction produced after fd infection; the latter situation has been described for phage T7 and the B restriction system (2).



We are currently investigating these possibilities.

Finally, we have recently observed that DNA from several *mec*+ *Salmonella typhimurium* strains is also resistant to R·*Eco*RII (unpublished observations); thus, the resistance of a bacterial DNA to cleavage by R·*Eco*RII may serve as a diagnostic tool to determine whether the organism has a DNA-cytosine methylase with a specificity similar to that of the *E. coli* *mec*+ and RII modification enzymes.

We are most appreciative for the advice and assistance of the following people: P. Model, who gave us a procedure for purifying RFI prior to its publication (reference 12); G. F. Vovis, who collaborated in the initial experiment demonstrating that fd RFI and f1 RFI are sensitive to R·*Eco*RII, and who provided the f1-RFI for the experiment shown in Fig. 1; R. Roberts, who donated R·*Eco*RII used in the initial experiments; Lynn Goldstein, who helped us purify R·*Eco*RII and taught us the methodology of agarose (tube)-gel electrophoresis; and G. Wilson and K. Horiuchi, who donated R·*Hind* preparations.

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FIG. 4. *Agarose tube-gel electrophoresis of E. coli and λ DNAs treated with R·EcoRII. Conditions were as described in the legend to Fig. 2. (a,b) 1.5 μg of E. coli mec+ DNA; (c,d) 1.5 μg of E. coli mec+ DNA and 1.5 μg λ·mec− DNA; (e,f) 1.5 μg of λ·mec− DNA; (a,c,e) untreated controls; (b,d,f) digested with R·EcoRii.*

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