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Genetic Control of the Secondary Modification of Deoxyribonucleic Acid in *Escherichia coli*[1]

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The wild-type restriction and modification alleles of *Escherichia coli* K-12 and B were found to have no measurable effect on the patterns of methylated bases in the deoxyribonucleic acid (DNA) of these strains. The genetic region controlling the methylation of cytosine in *E. coli* K-12 was mapped close to *his*, and the presence or absence of this gene in *E. coli* B or *E. coli* K had no effect on the restriction and modification properties of these strains. Thus, only a few of the methylated bases in the DNA of these strains are involved in host modification, and the biological role of the remainder remains obscure.

The primary structure of nucleic acids can be altered after synthesis of the molecule. In the case of deoxyribonucleic acid (DNA) there are essentially two ways in which this can be done. First, the hydroxymethylcytosine residues of T-even phage DNA are glucosylated in characteristic patterns (16, 17, 20, 29) by phage directed enzymes. There is evidence that the biological role of glucosylated hydroxymethylcytosine is protection of the phage DNA from host cell nucleases (15, 26). Secondly, the DNA of different bacteria contains trace amounts (0.05 to 1.0 mole per cent) of 6-methylaminopurine (MAP) or 5-methylcytosine (MC), or both, in very characteristic patterns (9-12, 27). The methylation of adenine and cytosine in DNA takes place at the polymeric level with *S*-adenosyl-L-methionine serving as the methyl donor and involves at least two methylases (10, 12). The biological role of some of the methylated bases of DNA appears to be the protection of certain regions of the DNA from restriction endonucleases (1, 2, 18). However, as shown here and elsewhere (1, 13), the major portion of methylated bases of most DNA genomes still has no known biological role. We initiated the studies reported here as a possible approach for examining the function of methylated bases in DNA. We have taken advantage of the facts that *Escherichia coli* strains K-12 and B, although differing significantly in their pattern of methylated bases (11), have very similar genomes, and interstrain mating yields normal recombination frequencies and linkage data when the natural barrier of DNA restriction is eliminated (4).

We have found that the restriction and modification alleles of *E. coli* K-12 and B do not measurably contribute to the MAP or MC (or both) contents of these strains. We mapped the gene responsible for the methylation of cytosine in *E. coli* K-12 and constructed partial hybrid derivatives of *E. coli* B and K-12 with respect to the gene controlling the methylation of cytosine. No significant differences were found between the hybrid derivatives and the parental strains with respect to host-controlled modification and restriction of DNA.

MATERIALS AND METHODS

Bacterial strains. The organisms used here were derived from either *E. coli* B/r (obtained from E. Engelsberg) or *E. coli* K-12 (obtained from E. A. Adelberg). The nomenclature recommended by Demerec et al. (8) is used, and the pertinent strains are listed in Table 1. We will use *mec* to designate the gene controlling the methylation of cytosine in DNA and Mec^+ and Mec^- to designate the presence or absence of cytosine methylase activity.

Media. Unless otherwise designated, organisms were grown in L broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl, per liter of distilled water) and plated on nutrient agar plates (Difco). Minimal medium contained (per liter): $Na_2HPO_4 \cdot 7H_2O$, 8.2 g; KH_2PO_4, 2.7 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $Ca(NO_3)_2$, 5 mg; $FeSO_4 \cdot 7H_2O$, 0.125 mg; individual amino acids, about 300 to 700 mg; a carbon and energy source, 2 g; and streptomycin, 0.1 g.

Conjugation experiments. Conjugation experiments were carried out by mixing 0.1 ml of a log-phase cul-

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TABLE 1. *Escherichia strains*

Strain no.	Phenotype	Derivation
HB42	*E. coli* B/r	HfrB1 prototroph (*see* reference 5)
HB45	*E. coli* B/r	$F^-Thr^-Leu^-Pro^-Try^-His^-Met^-Arg^-Ara^-Lac^-Gal^-r_B^+m_B^+Sm^R$
HB50	*E. coli* B/r	$F^-Leu^-Pro^-Try^-His^-Met^-Arg^-Ara^-Lac^-Gal^-r_K^+m_K^+Sm^R$
HB67	*E. coli* K-12	$F^-Leu^-Pro^-Gal^-His^-Thi^-r_B^+m_B^+Sm^R$
HB78	*E. coli* K-12	$Hfr H Thi^-$
HB153	*E. coli* K-12	Hfr H ($r_B^+m_B^+$) (*see* reference 6)
HB200	*E. coli* B/r	recombinant (HB78 × HB50) carrying His^+Mec^+
HB201	*E. coli* K-12	recombinant (HB42 × HB67) His^+Mec^-
HB202	*E. coli* B/r	recombinant (HB153 × HB45) His^+Mec^+
HB203	*E. coli* B/r	recombinant (HB78 × HB50) His^+Mec^-
HB204	*E. coli* K-12	recombinant (HB42 × HB67) His^+Mec^+

ture of the male strain (5×10^8 cells/ml), 1.5 ml of a log-phase culture of the female strain (5×10^8 cells/ml), and 0.5 ml of L broth and incubating the mixture at 37 C for 90 min. The appropriate dilutions of the mating were plated on minimal media selecting for various classes of recombinants. Stremptomycin was used for counter selection of the male strains. The male and female cultures were plated separately on the selective media as controls.

Extracts. Cultures (250 ml of L broth) of purified recombinants, grown to a density of about 10^9 cells/ml and supplemented with L-methionine (0.025 g/liter), were harvested by centrifugation at 7,500 × *g* for 10 min. The pellets were washed twice with triethanolamine buffer [0.05 M triethanolamine, 0.005 M 2-mercaptoethanol, 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.01 M $MgCl_2$, *p*H 8.8] and resuspended in 4 ml of buffer. The suspensions were sonically treated for three 30-sec intervals with intermittent cooling on a model LS75 Branson sonic oscillator. The extracts were cleared of debris by centrifugation at 12,000 × *g* for 15 min. The extracts were treated with ribonuclease (Calbiochem, heated at 80 C for 10 min) at a final concentration of 0.5 mg/ml for 30 min at 37 C.

Protein assays. The protein concentrations of the extracts were determined by the method of Lowry et al. (21).

DNA extraction. *E. coli* DNA used as a substrate for in vitro methylases was prepared by the method of Marmur (22).

Methylase assay. The methylase activities of the extracts were assayed in a total volume of 0.25 ml containing the following: tris(hydroxymethyl)aminomethane (Tris), 20 μmoles, *p*H 8.3; 2-mercaptoethanol, 4 μmoles; EDTA, 40 μmoles; *S*-adenosyl-L-methionine ($^{14}CH_3$-SAM, 51 mCi/nmole); extract, 0.5 to 1 mg of protein; "B" DNA, 130 nmoles.

The reactions were incubated for 30 min at 37 C and terminated by chilling and then adding 4 ml of cold 2 N HCl. After 10 min on ice, the precipitate was collected on glass-fiber filters (Reeve-Angel, N.J.) prewetted with cold 2 N HCl. The precipitates were washed on the filters with 20 ml of cold 2 N HCl, dried, and placed in 10 ml of toluene-liquifluor (NEN), and the radioactivity was determined in a Beckman LS200 liquid scintillation counter.

In vivo methylation. Cultures (10 ml) were grown in minimal glucose medium supplemented with 18.6 mg of L-methionine per ml at a concentration of 8×10^8 cells/ml. The cells were collected by centrifugation, washed with 30 ml of minimal glucose medium without L-methionine, and resuspended in 100 ml of minimal glucose medium (2×10^7 cells/ml). After 10 min, ^{3}H-L-methionine (Nuclear-Chicago Corp.) was added (12.5 μCi/ml). The cultures were incubated at 37 C until the cell density reached saturation (about 2×10^9 cells/ml), harvested by low-speed centrifugation, and washed twice in 100 ml of Tris-EDTA buffer (0.01 M Tris, 0.05 M EDTA, *p*H 8.0). The DNA was extracted by the method of Lark (19).

Chromatography of methylated bases. Purified DNA was incubated at 30 C in 0.1 N NaOH for 24 hr to hydrolyze any contaminating ribonucleic acid (RNA). After alkaline hydrolysis, the DNA was neutralized with 0.1 N acetic acid. The neutralized DNA was applied to a G-200 Sephadex column (38 by 1 cm) and eluted with 0.1 SSC (0.015 M NaCl, 0.0015 M trisodium citrate, *p*H 7.0). The concentration of DNA present in the peak tubes was determined by the method of Burton (7). The DNA was dried in vacuo over KOH and P_2O_5. The dry residue was sealed in a tube with 0.1 ml of 90% formic acid for every 15 μg of DNA and hydrolyzed at 175 C for 30 min. The hydrolysate was dried, redissolved in 0.1 ml of 90% formic acid, and applied to Whatman no. 1 paper with 25 μg each of 5-methylcytosine and 6-methylamino purine as carrier. The paper was subjected to two-dimensional chromatography by the method of Fujimoto et al. (10). The MAP and MC spots were detected by ultraviolet light and excised, and the tritium decay was counted directly in 10 ml of scintillation mix [4 g of 2,5-diphenyloxazole and 50 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene].

RESULTS

Methylation of DNA by restriction and modification alleles. *E. coli* strains K-12 and B have unique patterns of methylated bases in their DNA. Kühnlein, Linn, and Arber (18) presented evidence that the basis of host-controlled modification of DNA is the methylation (via SAM) of bases in DNA. However, other investigations in-

dicate that only a small percentage of the total methylated bases in a genome is involved in host-controlled modification. We examined the pattern of methylated bases in (i) an *E. coli* K-12 strain (HB67) carrying the r_B^+ m_B^+ alleles and (ii) an *E. coli* B strain (HB50) carrying the r_K^+ m_K^+ alleles and found no significant change in the ratios of MAP to MC (Table 3) when compared to published values (11). These data independently confirm the suggestion that the majority of methylated bases in the bacterial DNA of *E. coli* is not involved in host-controlled modification of DNA.

Mapping the cytosine methylase gene. The only detectable methylated base in *E. coli* B DNA is MAP; MAP and MC, however, are found in *E. coli* K-12 DNA (10, 11). *E. coli* B DNA can be used as a substrate for the K-12 cytosine methylase, because *E. coli* B apparently does not have cytosine methylase activity but does have substrate sites in its DNA for the K-12 cytosine methylase (11). Therefore, extracts of recombinants generated from a K-12 male and a B female which have received the K-12 gene controlling the methylation of cytosine should methylate *E. coli* B DNA.

Several classes of recombinants were selected from a cross between a K-12 male (HB78) and a B female (HB50). The normal restriction barrier between these strains was eliminated by using a B female with the K-12 restriction and modification genes. Extracts of purified recombinant clones were tested for their ability to methylate *E. coli* B DNA. Extracts of His^+ recombinants (34/39) usually had methylase activity typical of the K-12 strain (Table 2).

A second cross was carried out with a B male (HB42) and a K-12 female (HB67) which has the *E. coli* B restriction and modification alleles, and selection was made for His^+ Str^R recombinants. Most of the K-12 hybrid recombinants tested (16/19) had no in vitro methylase activity with the B DNA substrate.

TABLE 2. *Linkage of cytosine methylase gene to his locus*[a]

Selected marker	No. of recombinants tested	No. of recombinants with 5-methylcytosine methylase activity
Pro^+	20	0
Gal^+	20	0
Try^+	20	0
His^+	39	34
Met^+	9	2

[a] Extracts of recombinants were tested for methylase activity as described in Materials and Methods. Extracts with 5-methylcytosine methylase activity methylated B DNA to the extent of about 0.1 pmole of methyl group per reaction assay. Extracts without methylase activity methylated B DNA less than 0.01 pmole of methyl group per reaction assay.

In vivo methylation of cytosine by hybrid recombinants. The in vivo methylation of cytosine by hybrid recombinant derivatives was used to verify the in vitro analysis of the hybrids. The content of MAP and MC found in the DNA of two of the hybrid recombinants (HB201 and HB200) was determined (*see* above). Although *E. coli* K-12 and B have similar amounts of MAP (0.134 and 0.185 mole per cent, respectively), they differ considerably in MC content, 0.08 and ~0.0005 mole per cent, respectively (*see* Table 3). The *E. coli* B strain (HB200) with the *his* region of the K-12 genome and which had in vitro cytosine methylase activity had a MAP and MC content similar to the paternal K-12 strain. The *E. coli* K-12 strain (HB201) with the *his* region of the B genome having no in vitro cytosine methylase activity had a MAP and MC content similar to that of the paternal B strain. We consider the variations in the MAP content to be within experimental error. The actual counts found in the MC spots in the case of *E. coli* B (HB50) and the *E. coli* K-12 hybrid recombinant (HB201) were barely above background and therefore the mole content of MC in those strains is very approximate. However, consideration of the ratio of MAP to MC in these strains clearly shows the differences between the maternal and recombinant strains with respect to MC content and agrees with the in vitro methylation obtained with extracts of these strains.

Characteristics of recombinants. Recombinants from the crosses outlined above were used for most of the subsequent experiments. Recombinants HB200 and HB203 were derived from

TABLE 3. *DNA methylation pattern of Escherichia coli B, K-12, and hybrid recombinants*

Strain	Mole per cent		Ratio of 6-methylaminopurine to 5-methylcytosine
	6-Methylaminopurine	5-Methylcytosine	
(HB67) *E. coli* K-12	.134	.08	1.7
(HB50) *E. coli* B	.115	.005	232
(HB200) *E. coli* B (K-12-*his*)	.185	.105	1.8
(HB201) *E. coli* K-12 (B-*his*)	.091	.0015	61

the HB78 (K-12 ♂) × HB50 (B ♀) cross and differ in their ability to methylate cytosine. Likewise, HB201 and HB204 were derived from the HB42 (B ♂) × HB67 (K-12 ♀) cross and differ in their ability to methylate cytosine. Other than the difference in cytosine methylase and His^+, these recombinants had none of the other detectable paternal markers.

The presence or absence of MC does not affect the growth rate of these strains in broth or minimal glucose or minimal succinate media (Table 4). These strains were also examined for their sensitivity to ultraviolet irradiation, and the HB200 and HB203 strains were inactivated at the same rate (average lethal dose, 0.75 min of exposure) and HB201 and HB204 strains were inactivated at the same rate (average lethal dose, 1.25 min of exposure).

The chemical basis of host modification of phage DNA involves the methylation of bases in DNA (1, 2, 18). Therefore, we examined the HB201 and HB204 strains for their ability to modify and restrict phage λ. Although both of these strains are primarily *E. coli* K-12, they have the restriction and modification alleles of *E. coli* B in lieu of their normal alleles, and their phenotype is $r_B^+ m_B^+$. These strains modify and restrict phage λ in an identical fashion (Table 5). Therefore, the presence or absence of the *mec* gene does not affect the restriction and modification phenotype of this strain.

Recombination frequencies and marker linkage are sensitive indices of the restriction of bacterial genomes (4, 24). Therefore, we examined the recombination frequencies and marker linkage of a cross involving an Mec^+ hybrid B recombinant which carried the $r_B^+ m_B^+$ alleles (HB202) as the recipient and an Hfr B (HB33) donor. The assumption here is that any restriction activity associated with the cytosine methylase activity would be controlled by a gene closely linked to the *mec* locus. No anomalous data were obtained from this particular conjugation experiment (Table 6). We did not examine the behavior of K-12 Mec^- hybrids as recipients in conjugation experiments because they were not adequately marked.

TABLE 4. *Growth of hybrid recombinants of Escherichia*[a]

Growth medium	Doublings per hour			
	HB200 (*E. coli* B) His^+ Mec^+	HB203 (*E. coli* B)	HB201 (*E. coli* K-12)	HB204 (*E. coli* K-12)
L broth	2.0	1.8	1.6	1.6
Minimal glucose	1.1	1.2	0.9	0.9
Minimal succinate	0.4	0.3	0.4	0.5

[a] Doublings per hour were calculated from optical density increments and viable count increments over a 1.5-hr period of logarithmic growth. See Table 1 for a more detailed description of the recombinants. The parenthetical designation refers to the maternal origin of the hybrid recombinant.

DISCUSSION

The biological role of the methylated bases in DNA is interesting because of the widespread occurrence of these bases in the DNA of organisms and the species specific patterns of methylated bases in DNA (9–12, 27). The hypothetical functions proposed for methylated bases in DNA have ranged from regulation and carcinogenesis

TABLE 5. *Modification and restriction of phage λ by hybrid recombinant*

Phage lysate[a]	HB204	HB201	*E. coli* B	*E. coli* K
λ·B	1.0	1.0	1.0	10^{-4}
λ·C	10^{-4}	10^{-4}	10^{-4}	2×10^{-4}
λ·K	10^{-4}	10^{-4}	10^{-4}	1.0
λ·204	1.0	1.0	1.0	3×10^{-4}
λ·201	1.0	1.0	1.0	10^{-4}

[a] The symbols λ·B, λ·C, etc., designate the host strains on which the phage stocks were prepared. All phage stocks were standardized with *Escherichia coli* C which does not restrict or modify phage λ. See Table 1 for details of strain phenotypes.

TABLE 6. *Recombinant frequencies and gene linkage*

Cross		Recombinants/0.1 ml		
♀	♂	Try^+	Pro^+	Gal^+
HB202	HB33	2.8×10^6	1.7×10^5	2.4×10^5
HB45	HB33	3.2×10^6	1.8×10^5	8.0×10^5

Cross	Selected Marker	Unselected Marker		
		Try^+	Gal^+	Pro^+
		%	%	%
HB202 × HB33	Try^+		1.5	<0.5
	Gal^+	53		13
	Pro^+	50	55	
HB45 × HB33	Try^+		2.5	0.5
	Gal^+	45		10
	Pro^+	47	45	

to the biochemical basis of memory (3, 14). At the present time, only the host modification of DNA which protects regions of the genome against specific endonuclease attack has been substantially correlated with methylated bases in DNA (1, 2, 18). As we have shown here, the genes controlling the r^+m^+ phenotypes of *E. coli* K-12 and B do not measurably affect the pattern of methylated bases in the DNA of these strains. In consideration of this and other findings, we must conclude that only a small fraction of the total methylated bases in a modified genome are concerned with host-controlled modification. As a corollary to this, we mapped the gene controlling the methylation of cytosine close to the *his* region of *E. coli* K-12. This region of K-12 can be introduced to *E. coli* B, and the hybrid recombinant methylates DNA similarly to *E. coli* K-12. The restriction and modification property of such a strain was not altered when assessed by conjugation. When the *his* region of *E. coli* B was introduced to *E. coli* K-12, the hybrid recombinant strain no longer methylated cytosine, but the restriction and modification of phage λ by this strain were not changed.

The *E. coli* K-12 and B strains with altered patterns of methylated cytosine were not measurably different from the maternal parent strains with respect to other parameters examined. For example, if the methylation of cytosine had a role in the regulation of gene expression or repair of ultraviolet-damaged DNA, one might expect some differences between these strains. Thus, the biological role of most methylated bases in DNA still remains obscure, but the specificity and the widespread occurrence of such a mechanism insures a significant role for it.

We would like to speculate briefly in general terms about the function of the methylated bases not involved in host-controlled modification of DNA. This speculation is based on conclusions made about the role of methylated bases in host-controlled modification of DNA. In the latter case, the methylated base prevents a restriction endonuclease from introducing phosphodiester bond cleavages in certain regions of DNA (23, 25) because it prevents the enzyme from binding to the DNA (28; Boyer, *unpublished data*). Thus, the other methylated bases in DNA may serve a similar function such as keeping certain regions of DNA free of nonspecific protein, or protecting these regions of DNA from enzymatic attack, or both.

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