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Mutants of the N-3 R-Factor Conditionally Defective in *hsp*II Modification and Deoxyribonucleic Acid-Cytosine Methylase Activity

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The N-3 drug resistance (R) factor specifies a deoxyribonucleic acid (DNA)-cytosine methylase and a DNA restriction-modification (*hsp*II) system. We have isolated three independent mutants that are conditionally defective in their ability to modify bacteriophage λ and to methylate DNA-cytosine residues. The ratio of 5-methylcytosine to N^6-methyladenine in bacterial DNA and in the DNA of phages λ and fd was determined after labeling with [*methyl*-^{3}H]methionine at various growth temperatures. Although the ability of the wild-type N-3 factor to modify phage λ and to methylate DNA-cytosine residues was unaffected with increasing temperature, two of the mutants exhibited a parallel loss in modification and cytosine methylation ability. The ability of the third mutant to carry out these functions was dependent on the presence or absence of an amber suppressor mutation in the host genome. These results offer further support for the notion that *hsp*II modification is mediated by a DNA-cytosine methylase. Evidence is also presented that the modification methylase is responsible for the in vivo methylation of phage fd DNA (which is not subject to *hsp*II restriction in vivo).

The genetic control of deoxyribonucleic acid (DNA) restriction-modification (or host specificity) may be exerted either by the host genome (5, 10–12, 16, 39) or by extrachromosomal factors; e.g., the host specificity system, *hsp*II (3, 4) is controlled by the drug resistance (R) factors N-3 and R 15 (or RTF-2) (37, 38). This system affects a variety of double-stranded DNA-containing *Escherichia coli* phages (2–4, 24, 37, 38, 40), as well as *Salmonella* phage P22 (26, 38), but it does not affect the male-specific single-stranded DNA phages fd and M13 (1, 25, 27).

Previous reports from this laboratory have shown that after propagation in hosts containing the N-3 plasmid a specific increase in the content of 5-methylcytosine (MeC) is observed in the DNA of phage λ (24), P22 (26), fd, and M13 (25, 27); no effect on the level of the methylated base N^6-methyladenine (MeAde) is observed. Furthermore, *E. coli* B DNA, which normally lacks MeC (13–15, 22, 26, 30), was observed to contain this base when the N-3 plasmid was present (26). All other host specificity systems thus far studied have been found to be based on the methylation of specific adenine residues in the DNA (8, 29, 31, 33, 34, 36; for a recent summary see reference 35). Our data suggested that the methylation of specific cytosine residues may be involved in the *hsp*II modification process. This notion was supported further by our observation that an *E. coli* K-12 mutant which lost the ability to produce MeC also lost the ability to confer partial protection to λ DNA against restriction by N-3-containing cells (27).

The present communication describes the isolation and characterization of conditional modification-defective mutants of the N-3 factor; the ability to methylate DNA-cytosine residues by these strains parallels their ability to modify λ DNA. These results support the direct involvement of cytosine methylation in the *hsp*II modification. Recently, Boyer and his co-workers (6) have shown that the R 15 (*hsp*II) modification enzyme is a DNA-cytosine methylase.

MATERIALS AND METHODS

Phage and bacterial strains. Phage λcI_{857}*ind*$^-$ was obtained from B. Dottin; phages λ*vir* and λc were from S. E. Luria. Phage fd was from D. Marvin. *E. coli* B 834 *met*$^-$*gal*$^-$ r_B^- m_B^- *sup*$^-$*mec*$^-$ (hereafter referred to as r_B^- m_B^-) and *E. coli* 1100 *sup*$^+$*mec*$^+$*rgl*$^-$*endo* I$^-$ B_1^- r_K^- m_K^+ were obtained from H. Revel. These strains were made lysogenic for λcI_{857} and/or a recipient of an F factor. *E. coli* F$^+$1100

mec$^-$ was derived in this laboratory from the parental strain above (27); *mec*$^+$ and *mec*$^-$ refer to the ability or inability to methylate DNA-cytosine residues.

Media and chemicals. Minimal medium containing 0.1 or 0.2% Casamino Acids (wt/vol) was used in all labeling experiments (23). Phosphate buffer contained (per liter): KH_2PO_4, 3 g; Na_2HPO_4, 7 g; NaCl, 0.5 g; and NH_4Cl, 1.0 g. LB broth contained (per liter): tryptone (Difco), 10 g; yeast extract (Difco), 5 g; and NaCl, 5 g (adjusted to pH 7.0 with 1 N NaOH). L-[*Methyl*-^{3}H]methionine (Amersham/Searle and New England Nuclear Corp.), 5-methylcytosine (Schwarz/Mann), N^6-methyladenine (Calbiochem), tetracycline hydrochloride (Squibb), streptomycin sulfate (Eli Lilly), ethyl methane sulfonate (Eastman Kodak), and Triton X-100 (Rohm-Haas) were obtained from the firms indicated in parentheses.

Determination of the MeC/MeAde ratio. Cultures containing the N-3 factor were grown overnight in the presence of 20 μg of tetracycline per ml or 40 μg of streptomycin per ml; log-phase cells used for the labeling experiments were grown after 50- to 100-fold dilution of the overnight culture into a drug-free medium.

Growth and purification of labeled phage DNA was essentially as described previously (24, 25). Hydrolysis of DNA in 70% perchloric acid and paper chromatographic analysis were as before (26) with the modifications described previously (27).

Determination of the MeC/MeAde ratio in bacterial DNA was performed as described previously (26) except that cells were labeled for only 60 min and alkaline hydrolysis was for 2 h at 42 C in 1 N NaOH.

Isolation of the $r_{N3}^-m_{N3}^+$ plasmid. An overnight culture of *E. coli* $r_B^-m_B^-$ (N-3) was diluted 50-fold into 10 ml of broth and grown to a density of 5 × 10^8 cells per ml at 37 C. The cells were then harvested and suspended in 1.5 ml of phosphate buffer containing 0.3 M ethyl methane sulfonate at 0 C. After incubation at 37 C for 40 min with no aeration, the culture was serially diluted in phosphate buffer. Isolation of restrictionless mutants was carried out by a modification of Revel's technique (32). Samples (0.1 ml) from several dilutions were spread on LB plates containing 10^5 to 10^6 unmodified λc·$r_B^-m_B^-$ phage. After incubation at 37 C for 12 to 20 h, "nibbled" colonies (presumptive restrictionless mutants) were picked and streaked onto LB plates containing tetracycline (to ensure that these cells still contained the N-3 plasmid). One colony from each streak was picked and grown to log phase in broth. Each culture was then tested for its ability to restrict unmodified λ. One of the isolates obtained was a restrictionless mutant that still retained its ability to modify λ; this mutant was designated $r_{N3}^-m_{N3}^+$. The plasmid was transferred into several different unmutagenized host strains where the $r_{N3}^-m_{N3}^+$ and drug resistance phenotypes were still observed; this ruled out the possibility that the original mutation induced by ethyl methane sulfate was on the host chromosome.

Isolation of modification-defective mutants. Ethyl methane sulfate mutagenesis and isolation of modification-defective plasmid mutants were essentially as described for obtaining the *mec*$^-$ mutant of *E. coli* F$^+$ 1100 (27). The *ts*-1 and *am*-1 plasmids were isolated after mutagenesis of *E. coli* r_B^- m_B^- (r_{N3}^- m_{N3}^+) (λcI_{857}); the *ts*-2 plasmid was isolated after mutagenesis of *E. coli* F$^+$1100 *mec*$^-$ (r_{N3}^- m_{N3}^+) (λcI_{857}). All three mutants still retained their ability to confer drug resistance and to be transferred. These plasmids were still found to be modification defective after transfer to unmutagenized cells, indicating that the mutation was on the plasmid and not on the host chromosome. Plasmids transferred to unmutagenized cells were used in all the studies presented in this communication.

RESULTS

Modification-defective mutants of the N-3 drug resistance factor. The approach we used to study N-3-controlled modification (*hsp*II) was to isolate and characterize mutants defective in carrying out this function. First, a restrictionless mutant was isolated (designated r_{N3}^- m_{N3}^+) that retained its ability to modify λ DNA. Then, three independent modification-defective mutants were isolated after a second ethyl methane sulfonate mutagenesis. These mutants still retained their ability to be transferred and to confer drug resistance.

The defect in modification ability was demonstrated by serially propagating phage λ in host strains containing a mutant or wild-type plasmid and then assaying the relative plating ability on restricting versus permissive cells. For this study the restricting cells contained a wild-type N-3 factor. Phage λ *vir* propagated at different temperatures exhibited different plating abilities on permissive versus r_{N3}^+ plasmid-containing hosts (Table 1). For example, λ grown in the presence of the r_{N3}^- m_{N3}^+ plasmid plated with nearly equal efficiency on both indicator strains. Thus, the r_{N3}^- m_{N3}^+ plasmid confers full modification to λ at both 32 and 37 C (Table 1). In contrast, the ability of the *ts*-1 and *ts*-2 mutants to modify λ against N-3 restriction is temperature sensitive; e.g., at 37 C *ts*-1 gave partial modification of λ and *ts*-2 did not modify, whereas at 32 C *ts*-1 gave almost full modification, but *ts*-2 gave only partial modification.

There are two plausible explanations for the decrease in modification ability of these mutants at high temperature; viz., the plasmid is lost or the activity of the modification enzyme is destroyed at high temperature. To distinguish between these possibilities, cells were grown at 40 to 42 C for more than 6 h (at least eight generations) in the absence of drugs. Appropriately diluted samples were spread on plates with or without tetracycline and streptomycin. No significant difference was observed in the

TABLE 1. *Effect of growth temperature on the relative plating efficiency of λvir propagated in various E. coli strains*[a]

Phage	Relative plating efficiency of λvir[b] propagated at:	
	32 C	37 C
λ·r_B^- m_B^-	3.4×10^{-5}	3.5×10^{-5}
λ·r_B^- m_B^- (r_{N3}^- m_{N3}^+)	0.51-1.0	0.69-0.84
λ·r_B^- m_B^- (r_{N3}^- m_{N3} *ts*-1)	0.17-0.36	$1.6\text{-}7.8 \times 10^{-3}$
λ·r_B^- m_B^- (r_{N3}^- m_{N3} *ts*-2)	3.4×10^{-3}	3.0×10^{-5}
λ·r_B^- m_B^- (r_{N3}^- m_{N3} *am*-1)	5.2×10^{-5}	3.0×10^{-5}

[a] Various *E. coli* r_B^- m_B^- derivatives were grown to log phase in broth at 32 or 37 C and infected with approximately 1 λvir phage per 50 cells. After several hours of incubation, lysis was completed by the addition of $CHCl_3$, and the cell debris removed by low-speed centrifugation.

[b] Relative plating efficiency = titer on r_B^- m_B^- (r_{N3}^+ m_{N3}^+)/titer on r_B^- m_B^-. The range of values for several independent assays is given in some cases.

number of colonies formed on the two sets of plates incubated at 42 C. These results show that the mutant plasmids are not lost during growth at high temperature (and that the drug resistance phenotype is not temperature sensitive).

A third mutant factor, designated *am*-1, did not even partially modify phage λ at 32 C when harbored by non-amber-suppressing hosts (*sup*$^-$) (Table 1); however, it fully modified λ when harbored in amber-suppressing strains (*sup*$^+$) (Table 3). The plasmid is transferrable, confers drug resistance, and is not lost after continued growth in a *sup*$^-$ host. It is still not clear whether the loss of modification ability is due to an amber mutation within the structural gene for the modification enzyme or to a polarity effect of the amber mutation in a neighboring gene; similarly, it is not yet ruled out that the temperature-sensitive mutations occur in some regulatory or specificity gene.

Bacterial DNA methylation in cells harboring various N-3 modification mutants. Since we had originally proposed that the N-3 modification enzyme is a DNA-cytosine methylase (24, 26, 27), it was of interest to determine the effect of temperature on the enzyme activity exhibited by the various plasmid mutants. To this end, we measured the ratio of 5-methylcytosine to N^6-methyladenine (MeC/MeAde) in bacterial DNA after growth in a medium containing [*methyl*-^{3}H]methionine. Since *S*-adenosylmethionine is the methyl donor for all DNA methylases (18-21), the MeC and MeAde produced become radioactively labeled. We showed previously that the presence of the N-3 plasmid has no effect on the level of MeAde in bacterial DNA (27); thus, a decrease in the MeC/MeAde ratio reflects a decrease in the MeC content. In essence, this constitutes an in vivo assay for DNA-cytosine methylase activity.

In agreement with others (13-15, 22, 30), we found that *E. coli* B DNA is devoid of MeC at all the growth temperatures employed (Table 2). *E. coli* B harboring the r_{N3}^- m_{N3}^+ plasmid contains MeC, and the MeC/MeAde ratio was virtually the same at 32, 37, and 42 C (Table 2); the ability to confer N-3 modification also remained complete at these temperatures. In contrast, the MeC/MeAde ratio in cells harboring the *ts*-1 and *ts*-2 plasmids were affected strongly by growth temperature; i.e., both plasmids exhibited decreasing activities of cytosine methylase with increasing growth temperature (Table 2). These data correlate well with the observed loss in N-3 modification ability with increasing growth temperature. It is also interesting to note that the *ts*-1 and *ts*-2 plasmids exhibited different temperature sensitivities; for example, at 37 C the *ts*-2 plasmid lacked cytosine methylase activity and the ability to modify λ DNA, whereas *ts*-1 exhibited reduced cytosine methylase activity and partially modified λ DNA (Tables 1 and 2).

Methylation of phage λ DNA in strains harboring various N-3 mutants. The results presented above demonstrate a parallel loss in the ability of various N-3 plasmid mutants to confer modification to phage λ and to methylate cytosine residues in host cell DNA. These studies were extended to measurements on phage λ

TABLE 2. *Effect of growth temperature on the content of methylated bases in bacterial DNA*[a]

DNA from strain	MeC/MeAde ratio[b] observed after labeling at:		
	32 C	37 C	42 C
r_B^- m_B^-	≤0.03	<0.01	<0.001
r_B^- m_B^- (r_{N3}^- m_{N3}^+)	0.60	0.55	0.57
r_B^- m_B^- (r_{N3}^- m_{N3} *ts*-1)	0.58	0.34	0.013
r_B^- m_B^- (r_{N3}^- m_{N3} *ts*-2)	0.53	0.03	0.005

[a] Various *E. coli* r_B^- m_B^- derivatives were grown to log phase (2×10^8 to 3×10^8 cells per ml) in glucose-minimal medium at the temperatures indicated. [*Methyl*-^{3}H]-methionine was added and incubation continued for 1 h. The cells were harvested and the DNA was extracted. See references 26 and 27 for details on the labeling, DNA extraction, and MeC/MeAde determination.

[b] The figures listed here are the mean values obtained from analysis of at least two independent preparations; in most cases each preparation was subjected to duplicate chromatographic analysis. The variation was generally ±10%. This applies to all subsequent tables.

DNA. For this purpose we prepared strains lysogenic for the λcI_{857} prophage, which produces a temperature-labile repressor. After growth at 32 C, the prophage was heat-induced at 42 C and then incubated at 37 C in the presence of [*methyl*-^{3}H]methionine until lysis occurred. The phage was purified, and the MeC/MeAde ratio of the labeled DNA was determined. Phage λ grown in the *ts*-1-containing host had a significantly lower MeC/MeAde ratio and reduced plating ability on N-3-containing cells than did λ grown on the m_{N3}^+ host (Table 3). In addition, λ grown in *ts*-2-containing host had no MeC; these results are consistent with the observation that *ts*-2 is more temperature sensitive than *ts*-1 (Tables 1 and 2). Finally, λ grown in *mec*$^-$ *sup*$^-$ cells containing the *am*-1 plasmid was devoid of MeC and N-3 modification; in contrast, when the *am*-1 plasmid was contained in a *mec*$^-$ *sup*$^+$ host, the λ progeny had the same MeC content and N-3 modification as λ grown in the presence of the m_{N3}^+ factor. These findings further support the direct involvement of DNA-cytosine methylation in the modification process.

Methylation of phage fd DNA in strains harboring various N-3 mutants. The single-stranded DNA-containing *E. coli* phages fd and M13 are not subject to *hsp*II restriction (1, 25, 27); however, they showed a specific increase in MeC content after propagation in hosts containing the m_{N3}^+ plasmid (25, 27). The question was raised whether this methylation is mediated by the N-3 modification enzyme or by a second DNA-cytosine methylase also controlled by the plasmid. To answer this, fd was propagated on various plasmid-containing hosts, and the MeC/MeAde ratio was analyzed as a function of growth temperature. When fd was grown on *ts*-2 at 37 C, it was devoid of MeC (Table 4); at this temperature *ts*-2 also completely lost its ability to modify λ*vir* (Table 1) and to methylate host DNA (Table 2). At 30 C *ts*-2 exhibited reduced methylation of fd and incompletely modified λ*vir*. A similar parallel in the ability of the *ts*-1 plasmid to methylate cytosine residues on fd (Table 4) and to modify λ*vir* (Table 1) was also seen. In contrast, the m_{N3}^+ plasmid retained both full modification and DNA-cytosine methylation capacity up to 39 C. Thus, if fd methylation by the m_{N3}^+ plasmid were due to a second cytosine methylase activity, we should not have observed a loss in fd methylation capacity by the *ts*-2 or *ts*-1 plasmids; this was not the case. Therefore, we concluded that the N-3 modification enzyme is responsible for the in vivo methylation of fd DNA.

TABLE 3. *Analysis of methylated bases and plating ability of* λ *produced in various E. coli strains*[a]

Phage	Relative plating[b] efficiency	MeC/MeAde
λcI *mec*$^-$*sup*$^-$	4×10^{-5}	<0.01
λcI *mec*$^-$*sup*$^-$ (r_{N3}^- m_{N3}^+)	1.0	1.12
λcI *mec*$^-$*sup*$^-$ (r_{N3}^- m_{N3} *ts*-1)	$0.9\text{–}1.5 \times 10^{-3}$	0.033
λcI *mec*$^-$*sup*$^-$ (r_{N3}^- m_{N3} *ts*-2)	3.2×10^{-3}	<0.01
λcI *mec*$^-$*sup*$^-$ (r_{N3}^- m_{N3} *am*-1)	$7\text{–}10 \times 10^{-5}$	<0.01
λcI *mec*$^-$*sup*$^+$	7.8×10^{-5}	<0.01
λcI *mec*$^-$*sup*$^+$ (r_{N3}^- m_{N3}^+)	0.85–1.0	0.70
λcI *mec*$^-$*sup*$^+$ (r_{N3}^- m_{N3} *ts*-1)	1.8×10^{-3}	0.07[c]
λcI *mec*$^-$*sup*$^+$ (r_{N3}^- m_{N3} *am*-1)	0.92–1.0	0.70

[a] Cells lysogenic for λcI_{857} were grown to log phase (about 3 × 10^8 cells per ml) at 32 C in glucose-minimal medium, heat-induced at 42 C for 18 min, and incubated at 37 C in the presence of [*methyl*-^{3}H]methionine. See references 23, 24, and 26 for details on the labeling, phage purification, and MeC/MeAde determination. The *mec*$^-$*sup*$^-$ host is *E. coli* r_B^- m_B^-; the *mec*$^-$*sup*$^+$ host is from F$^+$1100.

[b] Relative plating efficiency = titer on r_B^- m_B^- (r_{N3}^+ m_{N3}^+)/titer on r_B^- m_B^-.

[c] The range in values was 0.10 to 0.047.

DISCUSSION

Modification in all *hsp* systems previously studied has been found to be based on methylation of adenine residues contained within specific sequences on double-stranded DNA (8, 29, 31, 33, 34, 36). We presented evidence suggesting that the *hsp*II modification-restriction system involved DNA-cytosine methylation (24, 26, 27). In this communication we report the isolation and properties of three conditionally defective modification mutants of the N-3 plasmid; two of them exhibit a temperature-sensi-

TABLE 4. *Effect of growth temperature on the MeC/MeAde in fd grown in various strains of E. coli*[a]

Phage	MeC/MeAde in fd phage DNA grown at:		
	30 C	37 C	39 C
fd·*mec*$^-$	<0.01	<0.01	<0.01[b]
fd·*mec*$^-$ (r_{N3}^- m_{N3}^+)	0.64	0.59	0.59[b]
fd·*mec*$^-$ (r_{N3}^- m_{N3} *ts*-1)	0.62	ND	<0.01[b]
fd·*mec*$^-$ (r_{N3}^- m_{N3} *ts*-2)	0.11[c]	<0.01	ND

[a] Various derivatives of *E. coli* F$^+$ 1100 *mec*$^-$*sup*$^+$ were grown to 10^8 to 2 × 10^8 cells per ml in glucose-minimal medium at the indicated temperatures and infected at a ratio of 50 to 100 fd per cell. One hour after infection, [*methyl*-^{3}H]methionine was added, and incubation continued for 4 h. See references 25 and 26 for details on the labeling, phage purification, and MeC/MeAde determination. ND, Not done.

[b] Based on a single determination.

[c] Assuming an average of 4.0 MeAde per fd DNA (25, 27), this would correspond to approximately 0.4 MeC per fd DNA.

tive phenotype and one appears to be an amber mutant. In each instance, we observed a direct correlation between the plasmid's ability to modify phage λ (protect it against restriction by an r_{N3}^+ containing host) and to methylate DNA-cytosine residues. The loss of modification-methylation ability is attributed to the loss of methylase function; it is clearly not due to loss of the plasmid during prolonged growth at high temperature or in the absence of antibiotics. These results further support the view that the N-3 (*hsp*II) modification requires DNA-cytosine methylase activity. We still don't know whether the three modification mutants were produced by mutational events in the structural gene for the DNA-cytosine methylase or in a regulatory gene affecting its activity (or specificity). While this work was in progress, Boyer and his co-workers reported that the R15 plasmid (*hsp*II) modification enzyme is a DNA-cytosine methylase (6).

Although the single-stranded DNA-containing phages M13 and fd are not subject to restriction by N-3- or R15-containing cells (1, 25, 27), they serve as in vivo substrates for the DNA-cytosine methylase (Table 4; 25, 27). The results presented here indicate that this methylation is mediated by the modification enzyme and not by a second DNA-cytosine methylase also controlled by the N-3 factor. This situation raises the following question. Is fd phage modified in vivo before the restriction nuclease can act, or is the N-3 specific methylation occurring at sites not involved in the protection against *hsp*II restriction? The latter would imply that the modification enzyme can methylate a sequence(s) not recognized by the restriction enzyme; this situation could arise if the restriction nuclease were to possess a much more stringent sequence specificity than the modification methylase. The presence or absence of *hsp*II restriction sites can be experimentally tested by determining whether the double-stranded replicative form from fd·*mec*⁻ (devoid of MeC) is cleaved in vitro by the *hsp*II restriction nuclease. These experiments are in progress.

It is interesting to note that at 32 C *ts*-2 gives almost the normal MeC level on host DNA, whereas at 30 C its ability to produce MeC on fd DNA is significantly reduced (Tables 2 and 4). This apparent discrepancy may actually be a reflection of the differences in the DNA substrates. For example, bacterial DNA is probably always available as a substrate for methylation, whereas phage DNA might be packaged into mature phage particles before all potential sites can be methylated. Experiments are currently in progress to test this hypothesis.

It is also interesting to note that the λcI_{857} progeny released after heat induction is not as strongly restricted as λ*vir* serially propagated in the *ts*-2-containing host (Tables 1 and 3). We attribute this difference to the methods of preparing the phage; viz. λ*vir* was propagated through several lytic cycles at 37 C, and λcI_{857} was released after heat induction and a single growth cycle at 37 C. Thus, the progeny released after heat induction probably include particles containing the original prophage DNA (this may actually be in a hybrid containing one "old" prophage strand plus one "new" de novo synthesized strand). Such particles would be expected to be resistant to N-3 restriction since the prophage DNA should have been modified during cell growth at the permissive temperature. In contrast, λcI_{857} progeny released from the *am*-1-containing *sup*⁻ host exhibits the same low plating efficiency as the λ*vir* propagated serially. This is expected because the *am*-1 plasmid cannot modify the prophage DNA even during growth at low temperature.

Finally, we should point out that modification-defective mutants have been isolated in a variety of other *hsp* systems (7, 9–11, 16, 17, 28, 39). Many of these mutants were intermediate between the fully defective and wild-type phenotypes; hence, they were designated $m^{\pm}$. The *ts*-1 mutant, in fact, exhibits this phenotype at 37 C. Therefore, it is likely that many of the $m^{\pm}$ mutants in existence may be temperature-sensitive mutants.

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