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Restriction and Modification in *Bacillus subtilis*: DNA Methylation Potential of the Related Bacteriophages Z, SPR, SPβ, φ3T, and ρ11

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The DNA methylation capacity and some other properties of the related temperate *Bacillus subtilis* phages Z, SPR, SPβ, φ3T, and ρ11 are compared. With phage mutants affected in their methylation potential, we show that phage-coded methyltransferase genes are interchangeable among the phages studied. DNA/DNA hybridization experiments indicate that phage methyltransferase genes are structurally related, whereas no such relationship is observed to a bacterial gene, specifying a methyltransferase with the same specificity.

Several restriction/modification systems have been identified in *Bacillus subtilis* (11, 25), including the *Bsu*R restriction/modification system with the target sequence GGCC (2). In view of the anticipated frequency of this tetranucleotide sequence in phage DNA, surprisingly few of the known *B. subtilis* phages are affected by the *Bsu*R restriction/modification system. In some bacteriophages, this resistance is a consequence of the absence or paucity of the *Bsu*R target sequence in their DNA (12, 19). In contrast, it could be demonstrated for one group of large temperate phages of *B. subtilis* that resistance of phage DNA to *Bsu*R-specific restriction and modification is caused by "self-modification" (4, 16, 26). Genomes of these phages contain genes for *Bsu*R-specific DNA methyltransferases which are expressed during phage growth and which modify the target sequences contained in the DNA of such phages. The genes for these DNA methyltransferases are present in the absence of genes for the matching restriction enzymes.

In this communication, we expand our previous studies on phage-borne DNA methyltransferases and focus on comparative aspects of the DNA-methylation potential of phages Z, SPR, SPβ, φ3T, and ρ11. Some data reported here have appeared in preliminary publications (13, 14).

MATERIALS AND METHODS

Abbreviations. MC, mitomycin C; 5mC, 5-methylcytosine; MOI, multiplicity of infection; Mtase, methyltransferase; NMNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; r/m, restriction/modification; WT, wild type.

Media. Modified M medium (17) was used for growth of bacteria, preparation of phage lysates, and in plating.

Bacteriophages and bacteria. Phages and bacteria used in this study are listed in Tables 1 and 2. All bacteria used, with the exception of CU1065, were derivatives of the nonlysogenic, nonrestricting, nonmodifying strain SB1207. Phage stocks were obtained either by induction of lysogenic cells with MC as described in reference 29 or (in the case of clear plaque mutants) by infection of growing cells of SB1207 (MOI, 0.1 to 1.0). Phages and phage DNA were purified as described for SPP1 (25).

Phage mutagenesis with NMNG was described in reference 26.

Phage crosses were performed either by superinfection of MC-induced lysogenic cells as described previously (26) or by mixed infection of strain SB1207. *Bsu*R-resistant phages were identified by plating the lysate on the restricting strain TB804.

Marker rescue experiments. Competent *B. subtilis* cells lysogenized with *Bsu*R-sensitive mutant phages were prepared as described previously (21). After transfection with either degraded or undegraded DNA, the transfected cells were plated on the restricting strain TB804 with top agar.

Phage antiserum. SPR and SPβ phages were purified by isopycnic centrifugation and had titers of 5×10^{11} PFU/ml. Three portions of 2 ml each of these phage stocks were injected into rabbits together with Freund complete adjuvant (Difco Laboratories) over a 2-month period. The antisera had *K* values of 250 per min (SPR) and 340 per min (SPβ).

5mC determinations. 5mC determinations in [6-^{3}H]uridine-labeled phage DNA were performed by thin-layer chromatography of hydrolyzed DNA as described by Günthert et al. (9).

DNA restriction. Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.) and Boehringer (Mannheim, Germany) and were used as described by the manufacturers. Analytical agarose gel electrophoresis was performed as described previously (1). Individual bands of DNA were

TABLE 1. Bacterial strains used and their properties

Bacterial strain	Chromosomal markers	Prophage	Comments and origin
SB1207	*su*$^{3+}$ *leu metB5 thr r*$^{-}$ *m*$^{-}$	Nonlysogenic	Derivative of strain CU1050 (30) obtained from I. Stroynowski
TB804	*su*$^{3+}$ *leu r*$^{+}$*m*$^{+}$	Nonlysogenic	Restricting/modifying derivative of SB1207 (26)
CU1065 (Z)	*trpC2*	Z	Obtained from E. Hemphill
TB201	Lysogenic derivatives of SB1207	Z	
TB106	Lysogenic derivatives of SB1207	SPR	
TB104	Lysogenic derivatives of SB1207	SPR19	
TB102	Lysogenic derivatives of SB1207	SPR26	
TB101	Lysogenic derivatives of SB1207	SPβ	
TB300	Lysogenic derivatives of SB1207	φ3T	
TB311	Lysogenic derivatives of SB1207	φ3T11	
TB312	Lysogenic derivatives of SB1207	φ3T12	

isolated from the gel by the method of Vogelstein and Gillespie (28).

Hybridization techniques. DNA of agarose gels was blotted on nitrocellulose filters according to Southern (22) and hybridized against a nick-translated DNA probe (20). Deoxycytidine 5′-[α-^{32}P]triphosphate was purchased from Amersham Corp. (3,000 Ci/mmol).

RESULTS

Biological properties of phages analyzed. Bacteriophages Z (10), SPβ (29), φ3T (27), and ρ11 (5) were previously described. Bacteriophage SPR was isolated in our laboratory from the restricting/modifying strain *B. subtilis* R (25) and was originally described as SPβ because of their morphological similarity and homoimmunity. After Fink et al. (6) published restriction data on SPβ DNA, we realized that the phage isolated in our laboratory, now termed SPR, was not identical with SPβ. We point out that the experiments of preceding publications from our laboratory (7, 8, 13, 14, 16, 26) were performed with SPR rather than with SPβ, as described therein.

All phages described here have very similar morphologies. Their DNAs have molecular weights of about 80 megadaltons. Distinguishing properties of these phages, which were established in our own and other laboratories, are summarized in Fig. 1 and Tables 2 and 3. We specifically want to draw attention to the following points. (i) Each phage DNA can be identified by a characteristic *Eco*RI restriction pattern (Fig. 1a). No instability of these restriction patterns has been observed. (ii) The phages fall into two unrelated serological groups represented by SPR and Z, SPβ, φ3T, and ρ11. (iii) All phages

TABLE 2. Phages used and their properties

Phage	Capacity to methylate the target sequence of: *Bsu*R (GGCC)	*Hpa*II/*Msp*I (CCGG)	*Fnu*4HI (GCNGC)	5mC content[a] $\left(\% \frac{5mC}{C+5mC}\right)$	Comments and origin
Z	–	–	–	0.13	Isolated from strain CU1065 (Z)
SPR WT	+	+	–	1.37[b]	(26)
SPR19	–`	+	–	0.99[b]	(14)
SPR26	–	–	–	0.31[b]	(26)
SPβ	+	–	+	1.76	(29)
φ3T WT	+	–	+	1.81	(16)
φ3T12	–	–	+	2.03	Isolated in this laboratory after NMNG mutagenesis
φ3T11	–	–	–	0.67	(16)
ρ11	+	–	+	1.65	(5)
SPP1 WT					(25)

[a] The values represent the average of three to four independent determinations of the 5mC content (detection limit: 0.01 to 0.02% methylated base per main base). The 5mC content of *B. subtilis* SB1207 DNA was 0.11%.

[b] Data taken from reference 14.

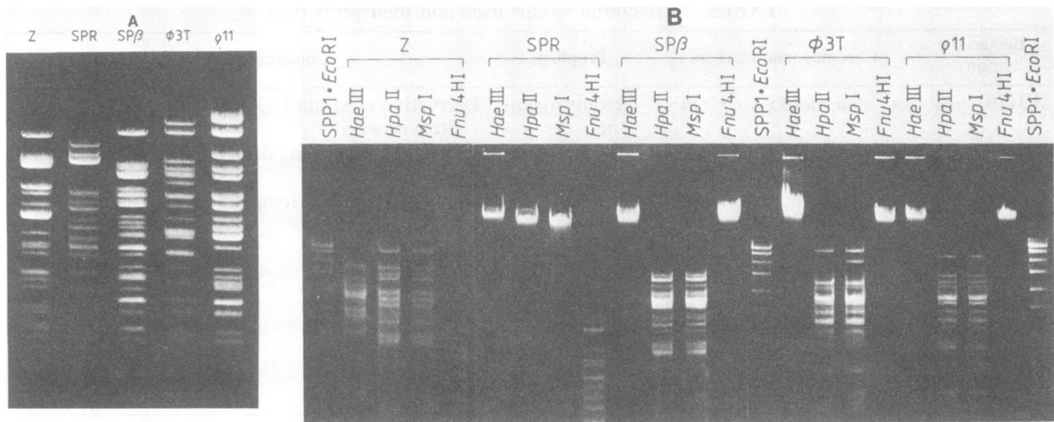

FIG. 1. (A) *Eco*RI degradation patterns of DNAs of phage Z, SPR, SPβ, φ3T, and ρ11. (B) Methylation potential of phage Z, SPR, SPβ, φ3T, and ρ11; gel electrophoresis of phage DNAs after restriction with *Hae*III, *Hpa*II, *Msp*I, and *Fnu*4HI. *Eco*RI-restricted DNA of phage SPP1 served as size reference (18).

with the exception of Z are resistant to *Bsu*R restriction in vivo. This resistance pattern is paralleled by differential sensitivity of the phage DNAs to *Hae*III (isoschizomer of *Bsu*R) degradation (Fig. 1b). (iv) Among the *Bsu*R-resistant phages, SPR and SPβ/φ3T/ρ11 can be distinguished on the basis of their potential to methylate target sequences in addition to GGCC. Thus, SPR DNA is also resistant to *Msp*I and *Hpa*II degradation, whereas the DNAs of the other three phages carry modifications rendering them *Fnu*4HI resistant (Fig. 1b).

Phage mutants affected in their methylation potential. We have previously reported on the isolation of mutants of SPR (14, 26) and φ3T (16) deficient in *Bsu*R Mtase activity. Such phage mutants were identifiable through their sensitivity to *Bsu*R restriction. In an analysis of six of these independent *Bsu*R-sensitive mutants from each of SPR and φ3T, we identified two types of mutants. In both phages, five mutants had lost both the capacity for *Bsu*R methylation and the capacity to express the accompanying non-*Bsu*R-specific methyltransferase, i.e., their DNAs were also sensitive to *Hpa*II-*Msp*I (SPR) or to *Fnu*4HI (φ3T) degradation. Only one mutant each of SPR and φ3T had maintained the *Hpa*II–*Msp*I- or *Fnu*4HI-specific modification

TABLE 3. Biological properties of *B. subtilis* phage Z, SPR, SPβ, φ3T, and ρ11

Phage	Property					
	Cross-reaction[a] against:		Homoimmunity group[c]	Production of bacteriocin by lysogens[d]	Potential for specialized transduction of the thymidylate synthetase gene[e]	Sensitivity to *Bsu*R restriction[f]
	Anti-SPR serum	Anti-SPβ serum[b]				
Z	−	+	I	+	+	+
SPR	+	−	I	+	+	−
SPβ	−	+	I	+	−	−
φ3T	−	+	II	−	+	−
ρ11	−	+	II	−	+	−

[a] Purified phage were exposed to antisera, which were in a 1:100 dilution from the stock, for 8 min at 37°C. +, Reduction of PFU to $<10^{-4}$; −, no reduction of PFU.
[b] See also reference 10.
[c] See also references 5 and 10. The efficiencies of plating of SPR on TB201 and TB101 and of Z and SPβ on TB106 were $<10^{-8}$ relative to those on the nonlysogenic strain SB1207.
[d] Reference 10; S. A. Zahler, personal communication.
[e] References 5, 23, 24, 27; S. A. Zahler, personal communication.
[f] See also references 4, 16, and 26. +, Reduction of the plating efficiency to $<10^{-6}$ in strain TB804 compared with SB1207; −, no reduction.

TABLE 4. Homologous recombination among Mtase mutants[a]

Competent cells	PFU after recombination with:					
	SPR WT	SPR19	SPR26	φ3T WT	φ3T11	φ3T12
TB104 (SPR19)	1.8×10^5	<10	1.1×10^5	—	—	—
TB102 (SPR26)	1.1×10^5	5.1×10^4	<10	—	—	—
TB311 (φ3T11)	—	—	—	1.2×10^5	<10	4.0×10^4

[a] Competent cells were transfected with 0.5 μg of the DNAs indicated. Infective centers were plated on TB804. Cells of TB312, lysogenic for phage φ3T12, could not be made competent for reasons not clarified. —, Recombination values of these heterologous crosses were not determined in this experiment.

potential of the corresponding WT phage (Table 2).

Table 2 also summarizes determinations of the 5mC content of the DNAs of WT phages and their Mtase-deficient mutants. In general, the methylation potential of a phage is correlated with the 5mC content of its DNA. Among the WT phages, 5mC values are high in SPR, SPβ, φ3T, and ρ11 and low in the restrictable phage Z. The same correlation holds for SPR WT and its two mutants. In contrast, in φ3T the 5mC content of the WT DNA is similar to that of the φ3T mutant 12, whereas the DNA of mutant 11 contains significantly less 5mC. The meaning of this observation remains to be elucidated. The residual methylation level found in the DNAs of the doubly deficient Mtase mutants is, however, still higher than that of the DNAs of phage Z or strain 1207. We cannot yet assign this methylation to any defined base sequence. No methyladenine was detectable in any of the phages.

Genetic analysis of phage Mtase genes. To determine whether the mutation sites causing single and double deficiency in Mtase activity were overlapping or separable, we performed crosses with such mutants. Cells lysogenic for Mtase-deficient mutants of SPR and φ3T were transfected with the DNAs of Mtase-deficient phages and as a control of WT phages. The transfected cells were plated on the restricting/modifying strain TB804 (Table 4). Plaque formation on the selective host was not a consequence of complementation, since resistance to restriction of phages isolated from single plaques proved to be genetically stable after a passage through the nonrestricting/nonmodifying strain SB1207. This result indicates that in both phages, the more frequent mutation leading to the loss of both Mtase activities has occurred at a site different from that causing loss of only the *Bsu*R methylation potential. A model which accomodates this observation will be discussed later.

In SPR, we have neither by mutagenesis nor by recombination been able to separate determinants for the *Hpa*II and *Msp*I activities. Also in enzyme purification (U. Günthert et al., unpublished data) the two activities could not be separated from each other.

To investigate the correlation among the various Mtase genes, we determined whether Mtase-proficient phages would be generated in heterologous combinations of WT and mutant phages. Cells lysogenic for the nonmodifying mutant 11 of φ3T or 26 of SPR and for phage Z were transfected with either *Bam*HI-, *Bgl*II-, or *Eco*RI-degraded DNAs of the various WT phages. Such DNAs are biologically inactive in the transfection of nonlysogenic cells (fewer than 10 plaques produced per 50 μg of DNA). Degradation of these DNAs by restriction enzymes did not affect their capacity to contribute the WT allele of the Mtase gene to progeny when tested in homologous combinations. As in homologous crosses involving Mtase-deficient mutants, Mtase-proficient recombinants can be selectively identified by plating transfected cells on a nonlysogenic r^+ m^+ strain. From the results of several such experiments (Table 5), we conclude the following.

(i) Mtase genes are interchangeable among phages SPR, SPβ, φ3T, and ρ11.

(ii) Provided the Mtase gene is not destroyed by a restriction cut, the efficiency of donating the Mtase gene to a recipient genome depends on the endonuclease used to generate the active donor DNA digest.

(iii) In spite of the inability of phage Z DNA digests to donate Mtase proficiency, Z-lysogenic cells will produce Mtase-proficient phages after transfection with DNA from heterologous Mtase-proficient phage.

Some hybrid phages produced in these experiments have been analyzed. Uniformly, these phages have the methylation potential of the donor DNA (see Fig. 4), and they fall into the immunity group of the recipient prophage. They have either the serotype of the recipient or a new serotype characterized by sensitivity to both anti-SPR and anti-SPβ serum (Table 6). These results demonstrate integration of the donor Mtase gene and in some cases also of genes determining serotype into the prophage genome. Further experiments will determine whether this

TABLE 5. Marker rescue experiments

Phage DNA	Restriction endonuclease used for DNA degradation	PFU obtained with 0.5 μg of degraded phage DNA after transfection of lysogens		
		CU1065 (Z)	TB102 (SPR26)	TB311 (φ3T11)
Z	*Eco*RI	<10	<10	<10
SPR	*Eco*RI	<10	1.2×10^2	<10
	*Bam*HI	1.5×10^4	1.1×10^3	7.4×10^4
SPβ	*Eco*RI	2.1×10^3	3.2×10^2	2.5×10^5
φ3T	*Eco*RI	6.3×10^2	1.4×10^2	1.9×10^5
	*Bgl*II	8.0×10^1	<10	1.0×10^3
ρ11	*Eco*RI	5.8×10^2	1.7×10^2	2.1×10^5
SPP1[a]		8.0×10^4	7.7×10^2	2.7×10^3

[a] Undegraded SPP1 DNA (0.5 μg) served to determine the degree of competence of the different lysogens.

integration reflects the substitution of recipient Mtase alleles by donor DNA or the addition of donor DNA to the prophage.

Structural relationships among Mtase genes. To obtain information on whether the phage Mtase genes are physically related with each other and with corresponding bacterial genes, we performed DNA/DNA hybridization experiments. For this purpose, the DNAs of phages Z, SPR, SPβ, φ3T, and ρ11 and of bacterial strains SB1207, TB804, and TB106 were degraded with *Eco*RI and fractionated on agarose gels. The DNA was then transferred to nitrocellulose paper and hybridized with ^{32}P-labeled SPP1 DNA into which we had cloned a 0.98-megadalton fragment of SPR DNA, 0.7 megadalton of which represented the SPR Mtase gene (the construction of this phage will be reported separately). Since SPP1 DNA alone has no homology with any of the DNAs probed (data not shown), any hybridization observed must have been due to the cloned SPR DNA fragment. As expected, we found strong hybridization of the probe to a restriction fragment of SPR DNA (Fig. 2). However, homology to SPR DNA is also observed with one DNA fragment each of SPβ, φ3T, and ρ11 DNAs. It is remarkable that there is also homology with one fragment of phage Z DNA, although this phage is lacking DNA Mtase activity.

To establish that the heterologous hybridizations observed are not accidental but reflect correspondence among Mtase genes, we isolated from agarose gels of *Eco*RI-degraded SPβ, φ3T, and ρ11 DNAs those DNA bands which had the electrophoretic mobility of the hybridizing material. This DNA was then tested by transfection for its ability to donate Mtase proficiency to Mtase-deficient mutants (see above). In all cases, a positive response was observed. The plaque numbers obtained under these conditions were three orders of magnitude above the marker rescue activity observed with control fragments.

With respect to bacterial DNAs, homology is apparent with the DNA of strain TB106 which is SPR lysogenic. Homology is absent in DNA of strain SB1207, the nonlysogenic parent strain of TB106. Also, no homology is found with TB804 DNA, which is a nonlysogenic derivative of SB1207, containing the bacterial gene for *Bsu*R Mtase (Fig. 2).

We conclude that the Mtase genes of all phages studied are structurally related. These genes are physically unrelated to the bacterial Mtase gene. To interpret the homology between

TABLE 6. Methylation potential, homoimmunity, and serological properties of recombinant phage

Recombinant phage[a]	Property			
	Methylation potential	Homoimmunity group[b]	% Survival of phage after treatment with[c]: Anti-SPR serum	Anti-SPβ serum
φ3T WT-SPR26	*Hae*III, *Fnu*4HI	I	4.1	3.0
SPR WT-φ3T11	*Hae*III, *Hpa*II, *Msp*I	II	100	2.0
SPR WT-Z	*Hae*III, *Hpa*II, *Msp*I	I	33.0	13.0

[a] The first entry of each pair is the genotype of the donor DNA; the second is the genotype of the prophage of the recipient cell.
[b] See Table 3.
[c] Antiserum treatment was for 5 min at 37°C with an antiserum diluted 1:500.

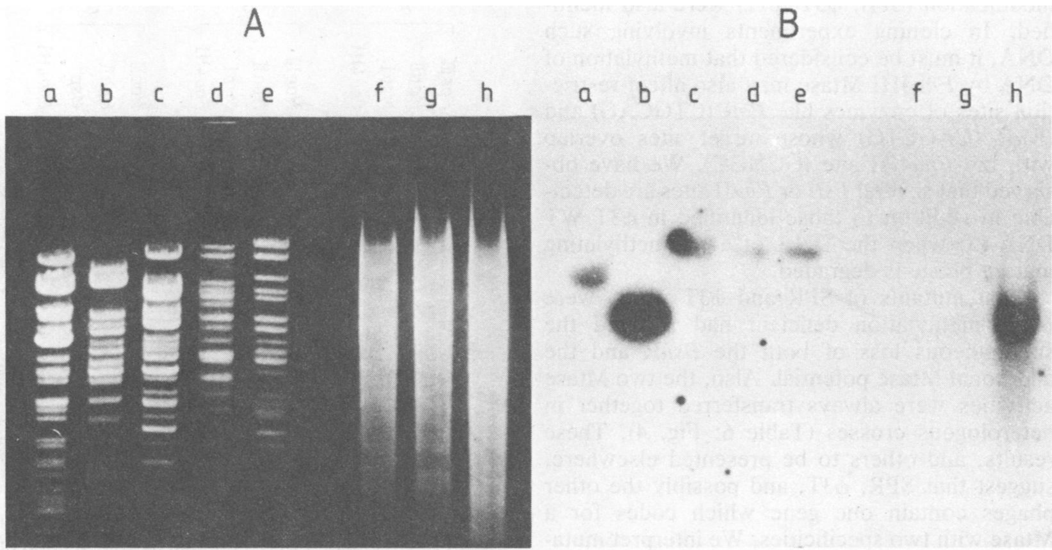

FIG. 2. Agarose gel electrophoresis (A) and autoradiography (B) after hybridization with [^{32}P]DNA of the cloned Mtase gene of SPR. Lanes a to e, *Eco*RI digests of Z, SPR, SPβ, φ3T, and ρ11 DNAs. Lanes f to h, *Eco*RI digests of DNAs of bacterial strains SB1207, TB804, and TB106.

the SPR DNA probe and a fragment of Z DNA, one might assume that also phage Z at some time carried such a gene which had been mutated during evolution.

Localization of the Mtase genes in the restriction maps of φ3T and ρ11. To obtain a tentative localization of the Mtase genes of φ3T and ρ11, DNA of these phages was degraded with restriction enzymes used in the establishment of the published restriction maps (3, 15). After gel electrophoresis, the degraded DNAs were transferred to nitrocellulose filters and hybridized against a ^{32}P-labeled probe of the SPR Mtase gene in phage SPP1 (Fig. 3a). Hybridization occurred against *Pst*I fragment C and *Bgl*II fragment J of φ3T DNA and against *Bam*HI fragment A, *Sal*I fragment A and *Bgl*II fragment A of ρ11 DNA (Fig. 3a). Figure 3b shows parts of the restriction maps of φ3T and ρ11 DNA (3, 15), with the hybridizing fragments indicated by crosshatching. In both cases, the Mtase genes are localized at similar positions within the central region of the phage genomes.

DISCUSSION

The temperate phages SPR, SPβ, φ3T, and ρ11 owe their resistance to *Bsu*R restriction to their capacity to modify *Bsu*R restriction sites in their DNA through phage-determined DNA Mtases. Additional Mtase activities, providing *Hpa*II-*Msp*I modification (SPR) and *Fnu*4HI

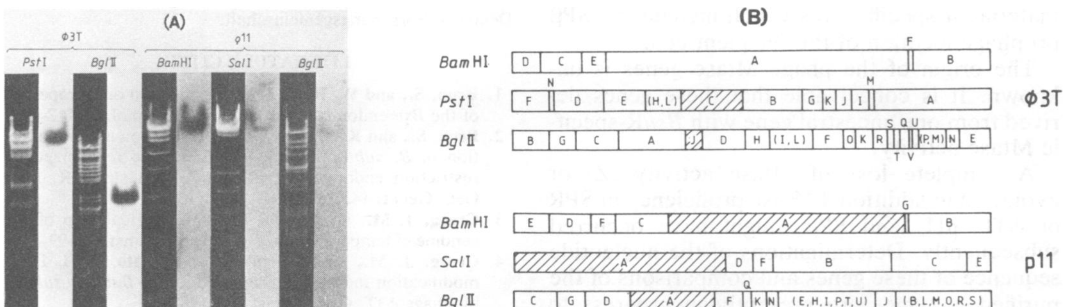

FIG. 3. (A) Gel electrophoresis of φ3T and ρ11 DNA digests and autoradiography after hybridization against a cloned fragment carrying the SPR Mtase gene. (B) Position of the Mtase genes within the published maps of φ3T and ρ11 (3, 15).

modification (SPβ, φ3T, ρ11) were also identified. In cloning experiments involving such DNA, it must be considered that methylation of DNA by *Fnu*4HI Mtase may also affect restriction sites of enzymes like *Pst*I (CTGCAG) and *Pvu*II (CAGCTG) whose target sites overlap with the *Fnu*4HI site (GCNGC). We have observed that several *Pst*I or *Pvu*II sites are detectable in addition to those identified in φ3T WT DNA (3) when the DNA of a nonmethylating mutant phage is degraded.

Most mutants of SPR and φ3T which were *Bsu*R methylation deficient had suffered the simultaneous loss of both the *Bsu*R and the additional Mtase potential. Also, the two Mtase activities were always transferred together in heterologous crosses (Table 6; Fig. 4). These results, and others to be presented elsewhere, suggest that SPR, φ3T, and possibly the other phages contain one gene which codes for a Mtase with two specificities. We interpret mutations affecting both enzymatic activities to fall into a domain of the enzyme responsible for a general step in the methylation reaction, whereas mutations causing only the loss of *Bsu*R modification activity might alter a different domain responsible for specificity. This interpretation also accommodates our observation of the generation of WT recombinants in crosses between the two types of mutants. An alternative to this interpretation would be that the two Mtase activities are associated with two distinct proteins whose genetic determinants would be closely linked. Mutants affecting both activities could represent regulatory mutations.

DNA/DNA hybridization experiments (Fig. 2) and also heterologous marker rescue experiments (Table 5) suggest homologies between phage Mtase genes and indicate the presence of a pseudogene in the DNA of nonmodifying phage Z. These homologies are apparently not the only ones existing in this group of phages. Stroynowski (23, 24) has shown that the integration of the *thyP3* gene of φ3T into Thy⁻ cells of *B. subtilis* involves insertion of this genetic material at specific sites which include the SPβ prophage location of the recipient cell.

The origin of the phage Mtase genes is unknown. It is conceivable that these genes derived from one ancestral gene with *Bsu*R-specific Mtase activity.

A complete loss of Mtase activity (Z) or evolution to additional Mtase proficiency in SPR or φ3T, ρ11, and SPβ, might have occurred subsequently. Determinations of the nucleotide sequence of these genes and comparisons of the purified enzymes are presently in progress to obtain precise information on the relatedness of these genes. Such studies involving the mutants found will also serve to define the postulated domains of these enzymes. The phage Mtase genes are not related to the bacterial gene encoding *Bsu*R methylation. Hence, the situation of the Mtase genes contained in these phages is different from that of the *thyP3* gene which is present in phages SPR, Z (H. E. Hemphill and S. A. Zahler, personal communication), φ3T (27), and ρ11 (5). This gene of φ3T has been demonstrated to be identical to the *thyA* gene of *B. subtilis* (23, 24).

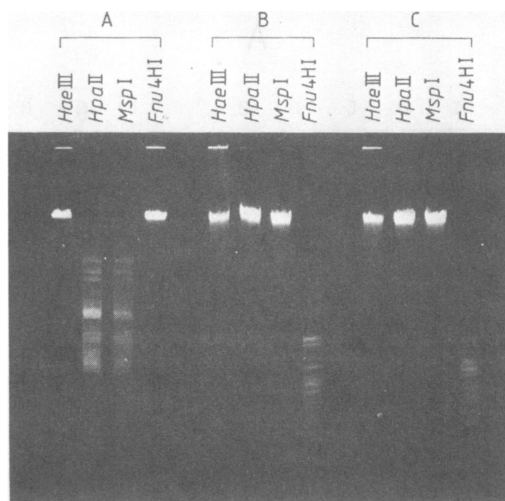

FIG. 4. Modification potential of recombinant phage. Recombinants were obtained by transfection of (A) TB102 (SPR26) with *Eco*RI-degraded φ3T WT DNA, (B) TB311 (φ3T11) with *Bam*HI-degraded SPR WT DNA, and (C) TB201 (Z) with *Bam*HI-degraded SPR WT DNA. Recombinant DNAs were degraded to completion with the enzymes indicated and subjected to agarose gel electrophoresis. For the serotypes of these phage, see Table 6.

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