Recombinant Derivatives of *Bacillus subtilis* Phage Z Containing the DNA Methyltransferase Genes of Related Methylation-proficient Phages

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The DNA methyltransferase (Mtase) genes of temperate *Bacillus subtilis* phages SPR, ϕ 3T, SP β and ρ 11 can be transferred by transfection and recombination to the genome of the related non-modifying phage Z. Integration of the Mtase genes occurs in phage Z DNA at a unique location which is homologous with the flanking regions of the Mtase genes of the related phages. In lysogenic cells carrying recombinant phages, expression of the Mtase genes is repressed, irrespective of whether the Mtase genes were derived from phage donors which were homo- or heteroimmune to phage Z.

INTRODUCTION

The temperate *Bacillus subtilis* phages SPR, SP β , ϕ 3T and ρ 11 code for DNA methyltransferases (Mtases) with multiple sequence specificity (Günther & Trautner, 1984). In non-modifying phage Z (Hemphill *et al.*, 1980), which also belongs to this group of related phages, the Mtase gene is deleted (Tran-Betcke *et al.*, 1986). DNA fragments of SPR, ϕ 3T, SP β , and ρ 11 containing the Mtase gene as well as a DNA fragment of phage Z, which cross-hybridized to such fragments, have been cloned (Kiss & Baldauf, 1983; Noyer-Weidner *et al.*, 1985). Sequence analysis (Tran-Betcke *et al.*, 1986) revealed that all Mtase genes are flanked at their 3' ends by a highly conserved region, while sequences at their 5' ends are variable. Regions homologous to the 3' flank of all Mtase genes and to the 5' flank of the SPR Mtase gene are also present in the genome of phage Z, where they form a contiguous DNA segment. We have shown that the Mtase genes of modification-proficient phages can be introduced by recombination into the phage Z genome (Noyer-Weidner *et al.*, 1983). In this study we investigate whether the integration of the Mtase genes occurs at random positions in the phage Z genome or specifically at the region which is homologous to sequences flanking the Mtase genes. Mtase-proficient phage Z derivatives, containing the Mtase genes of phages heteroimmune to Z, have also been analysed for expression of the Mtase genes in the lysogenic state.

METHODS

Bacteriophages, strains and plasmids. Phages SPR, ϕ 3T, SP β , ρ 11 and Z as well as recombinant phages (nomenclature see below) were purified and the DNA extracted as described by Noyer-Weidner *et al.* (1983). The nonlysogenic r^-m^- *B. subtilis* strain SB1207 (Trautner *et al.*, 1980) served as host for these phages. The Z-lysogenic derivative of strain CU1065 (Zahler *et al.*, 1977) obtained by H. E. Hemphill was used as recipient strain in transfection experiments; the r^+m^+ strain TB804 (Trautner *et al.*, 1980) was used to select *BsuR*-resistant phages. Recombinant plasmids pBKU1 (Tran-Betcke *et al.*, 1986), pSPR5 (=pSU2; Kiss & Baldauf, 1983) and pBN16 (Noyer-Weidner *et al.*, 1985) were used in DNA/DNA hybridization experiments. The cloned phage DNA fragments are shown in Fig. 1.

Recombination experiments. Competent cells of strain CU1065 lysogenic for non-modifying phage Z were

Abbreviations: AdoMet, S-adenosyl-L-methionine; MC, mitomycin C; Mtase, DNA methyltransferase; r/m , *BsuR* restriction/modification genotype.

Figure 1 shows schematic diagrams of DNA constructs. (a) Linear maps of pBK U1, pSPR5, and pBN16. pBK U1 is 4.85 kb. pSPR5 is 2.5 kb with an EcoRI site (E) at the start. pBN16 is 1.8 (1.6) kb with a BamHI site (B) at the start. (b) Recombinant constructs R/Z-E1-E2-B3, R/Z-B4-B5, and R/Z-P6-P7. R/Z-E1-E2-B3 is 2.5 kb. R/Z-B4-B5 is 4.0 kb. R/Z-P6-P7 is 2.9 kb. (c) Recombinant constructs ϕ/Z , β/Z , ρ/Z , and β/Z -P3. ϕ/Z , β/Z , and ρ/Z are 4.0 kb. β/Z -P3 is 1.0 kb.

Fig. 1. Schematic representation of parental and recombinant genomes in the Mtase coding region. The Mtase genes and their direction of transcription are marked by a solid (SPR) or an open (ϕ 3T = SP β , ρ 11) arrow. Homologous regions outside the Mtase genes are marked identically. Non-homologous regions are left blank. No information for the region 5' of the *Eco*RI site of SPR DNA is available. Unless otherwise indicated, vertical arrows represent *Hind*III sites; E, *Eco*RI sites. The numbers give the size (kb) of DNA fragments. (a) Genomes of recipient (Z) and donor (SPR, ϕ 3T, SP β , ρ 11) phages. The extent of phage DNA contained in plasmid probes pBKU1, pSPR5 and pBN16 is marked with heavy double arrows. (b) Recombinants produced with SPR DNA. R/Z-B3 and R/Z-B4 do not contain the most leftward *Eco*RI site from SPR. (c) Recombinants produced with ϕ 3T, SP β and ρ 11 DNAs. The configuration of DNA of β /Z-P3 to the right of the 1 kb *Hind*III fragment could not be assessed with the DNA probes available.

transfected with either uncleaved or *in vitro*-restricted DNA of heterologous Mtase-proficient phage. Transfected cells were plated on the indicator strain, TB804.

General techniques. Restriction enzymes were purchased from Boehringer and New England Biolabs and used according to the recommendations of the suppliers. For DNA/DNA hybridization the probes were labelled by nick-translation following the procedure of Rigby *et al.* (1977). DNA polymerase I was purchased from Boehringer, and [α - 32 P]GTP (3000 Ci mmol $^{-1}$, 110 TBq mmol $^{-1}$) from Amersham. DNA fragments were separated in 1% (w/v) agarose gels (Maniatis *et al.*, 1982) and blotted on Genescreen membranes (New England Nuclear) according to the method of Southern (1975). Hybridization and washing of Genescreen membranes was done under stringent conditions (60 °C) as described by the suppliers.

Preparation of crude extracts. Lysogenic *B. subtilis* cells were propagated in TY medium at 37 °C. Two cultures of each strain to be analysed were grown in parallel. At a cell titre of about 2×10^8 ml $^{-1}$ one of the cultures was induced with mitomycin C (MC) (0.25 μ g ml $^{-1}$). After further incubation for 60 min the cells were harvested and disrupted in a French press cell (13000 lbf in $^{-2}$; 90 MPa). The crude extracts were clarified by centrifugation at 50000 g for 40 min followed by dialysis against storage buffer containing 50% (v/v) glycerol (Günther *et al.*, 1981). The parallel culture was processed similarly without MC induction.

In vitro methylation assay. DNA methylation activity in crude extracts was determined as described previously (Günther *et al.*, 1981). *Micrococcus luteus* DNA (Serva) was used as substrate, and S-adenosyl[3 H]methionine ([3 H]AdoMet; 15 Ci mmol $^{-1}$, 550 GBq mmol $^{-1}$; Amersham) as donor of methyl group. The Mtase unit is defined as the amount of enzyme that incorporates 1 pmol of methyl groups from AdoMet into DNA in 1 h at 37 °C. Protein concentration in crude extracts were determined by the method of Lowry.

Nomenclature. In the designation of recombinant bacteriophages the first entry indicates the source of donor DNA (R, β , ϕ or ρ), the second entry separated by a slash shows the name of the recipient phage Z. If recombinants are derived from *in vitro*-restricted DNA a symbol representing the enzyme used for restriction (B = *Bam*HI, E = *Eco*RI, P = *Pst*I) follows the designation of the recombinant phage. Independently isolated phages derived from a single recombinant experiment are numbered sequentially. Example: R/Z-E2 is isolate number 2 of a methylation-proficient Z derivative obtained by recombination with *Eco*RI-restricted SPR DNA.

RESULTS

Characterization of modification-proficient phage Z derivatives

Competent cells of the Z-lysogenic *B. subtilis* strain CU1065 were transfected with either uncleaved or *in vitro*-restricted DNAs of phages SPR, ϕ 3T, SP β and ρ 11 as previously described (Noyer-Weidner *et al.*, 1983). For reasons unknown, such DNAs are biologically inactive in the transfection of non-lysogenic cells (Williams & Young, 1977; Noyer-Weidner *et al.*, 1983). To select for methylation-proficient phages, the transfected cells were plated on the *Bsu*R r $^+$ m $^+$ strain TB804. Table 1 shows that compared to uncleaved DNA, partial or total *in vitro* restriction of the donor DNA reduced or abolished its potential to generate *Bsu*R-resistant phages, although none of the enzymes used cleaves within the Mtase genes (Noyer-Weidner *et al.*, 1983, and authors' unpublished observations). This finding can be rationalized on the basis of the size of the Mtase-gene-carrying fragments (Table 1) and, in the case of ϕ 3T and SPR, where mapping and/or sequencing data are available (Noyer-Weidner *et al.*, 1985; Tran-Betcke *et al.*, 1986), the location of the Mtase genes within these fragments. Phages obtained from single-plaque isolates proved to be recombinants since their resistance to *Bsu*R restriction was stably acquired.

The phages used in this study constitute two immunity groups, represented by phages SPR, SP β , Z and phages ϕ 3T and ρ 11 (for a review see Zahler, 1982). While SPR, SP β and Z form turbid plaques, ϕ 3T and ρ 11 yield clear plaques, presumably due to a higher spontaneous phage induction rate (Noyer-Weidner *et al.*, 1981). Transfection of CU1065(Z) with ϕ 3T and ρ 11 DNAs yielded recombinant phages forming either turbid or clear plaques, which were homo- or heteroimmune respectively to Z. Immunity defined by this plaque morphology was verified by plating samples of phage lysates on Z or ϕ 3T lysogenic strains.

The restriction patterns of DNAs from various independently isolated recombinants indicated that their DNA is either resistant to *Hae*III and *Hpa*II (donor phage SPR) or *Hae*III and *Fnu*4HI cleavage (donor phages ϕ 3T, SP β , ρ 11) (Terschüren, 1985). Hence the recombinant phages express the complete modification potential of the donor phage. Further, the *Hind*III restriction patterns of Mtase-proficient SPR/Z recombinants (Fig. 2) differ markedly from the

Fig. 2. Southern blot of representative recombinants. All phage and plasmid DNAs analysed were digested with *Hind*III. The digests were separated by gel electrophoresis (a), transferred to Genescreen membranes and probed with nick-translated pBKU1 (b) and pSPR5 (c) DNAs. The tracks represent DNAs of: (1) molecular mass standard (*Eco*RI-degraded SPP1 DNA: Ratcliff *et al.*, 1979); (2) SPR; (3) Z; (4)–(10) recombinants R/Z-E1, -E2, -B3, -B4, -B5, -P6, -P7, respectively; (11) pBKU1; (12) pSPR5. In contrast to the 1.1 kb DNA fragment of SPR, a 0.46 kb DNA fragment of pSPR5 carries the homology region shared between sequences upstream of the Mtase gene and pBKU1. This is due to the presence of a *Hind*III site in the vector (pBR322) moiety located 30 bp upstream of the leftmost *Eco*RI site confining the pSPR5 insert shown in Fig. 1(a).

Table 1. *Generation of methylation-proficient phage Z derivatives*

Uncleaved (0.75 µg) or *in vitro*-restricted (4.5 µg) DNA was used for transfection. Recombination experiments (see Methods) were repeated once and the results resembled those presented. The sizes of the Mtase-carrying DNA fragments were previously determined by Kiss & Baldauf (1983), Noyer-Weidner *et al.* (1983) and M. Noyer-Weidner (unpublished).

Phage DNA used for transfection of CU1065(Z)	Restriction endonuclease used for phage DNA cleavage	Approx. size of the Mtase-carrying DNA fragment in cleaved DNA (kb)	No. of p.f.u. obtained on TB804 (<i>Bsu</i> R ⁺ m ⁺)
SPR	None	–	2.4 × 10 ⁴
	<i>Eco</i> RI*	4.3	2.7 × 10 ²
	<i>Bam</i> HI	≥ 30	9.3 × 10 ²
	<i>Pst</i> I*	ND	4.5 × 10 ¹
SPβ	None	–	3.9 × 10 ³
	<i>Eco</i> RI	12	9.8 × 10 ²
	<i>Pst</i> I	15	5.0 × 10 ²
φ3T	None	–	2.0 × 10 ⁴
	<i>Eco</i> RI	12	1.5 × 10 ³
	<i>Bgl</i> II	4.5	< 10
	<i>Pst</i> I	14	5.8 × 10 ²
ρ11	None	–	1.2 × 10 ⁴
	<i>Eco</i> RI	12	3.8 × 10 ²

ND, Not determined.

* Partially digested DNA was used for transfection. Complete digests yielded no p.f.u. on TB804.

donor phage DNA patterns but resemble the pattern of phage Z DNA. Therefore only a limited amount of donor DNA was introduced into the Z genome in the generation of these phages. Restriction patterns of recombinant clones also differ from each other. Hence, different recombination events must have taken place in the generation of the various SPR/Z recombinants. Analogous results were obtained by analysis of the *Hind*III restriction pattern of SPβ/Z and φ3T/Z or ρ11/Z phages forming turbid plaques (data not shown).

Fig. 3. *Hind*III restriction pattern of ϕ 3T- and ρ 11/Z Mtase-proficient phages with ϕ 3T/ ρ 11 immunity. The tracks represent DNAs of: (1) molecular mass standard; (2) ϕ 3T; (3) ρ 11; (4) Z; (5)–(10) recombinants ϕ /Z-5, -6, -7, -8, ρ /Z-3, -4, respectively.

The restriction patterns of Mtase-proficient ϕ 3T/Z and ρ 11/Z phages forming clear plaques differ significantly from the restriction patterns of the donor and the recipient phage DNAs (Fig. 3) and among each other. Phages of this type must have been generated by multiple recombinational events between the donor and recipient DNAs, which also included genes affecting immunity.

Localization of the Mtase genes within the recombinant phage genomes

To localize the Mtase genes within the recombinant phage genomes, DNA/DNA hybridization experiments were done with two types of plasmid DNAs as probes.

(1) pBKU1 carries a 4.85kb fragment of phage Z DNA comprising the sequences homologous to the flanks of the Mtase genes of the related phages (Fig. 1). Hybridization to pBKU1 reveals whether this fragment is conserved in the recombinant phages or has been affected by recombination. The latter would indicate specific integration of the Mtase genes into the homologous region of the phage Z genome.

(2) pSPR5 and pBN16 contain Mtase-coding DNA fragments of phages SPR and ϕ 3T respectively. Hybridization to these plasmids proves the presence of the Mtase genes in the recombinant phages and reveals whether their immediate environment was involved in the recombination events. pBN16 was also used as a probe against SP β /Z and ρ 11/Z DNAs since the Mtase-coding fragments of ϕ 3T, SP β and ρ 11 are extensively homologous (Noyer-Weidner *et al.*, 1985).

Analysis of the results of the DNA/DNA hybridization experiments (Fig. 2), indicates that the unique restriction fragment of Z DNA hybridizing with pBKU1 is absent from all SPR/Z recombinant DNAs. Instead, two new fragments give a hybridization signal. These fragments

Table 2. *Mtase activity in crude extracts of lysogenic SB1207 strains ($r^- m^-$)*

Mtase activity and protein concentration were measured as described in Methods. Duplicate values were taken at two different concentrations of crude extracts; they varied by 10% or less. Mean results are presented.

Prophage	Specific Mtase activity [units (mg protein) ⁻¹]	
	Uninduced	MC-induced
Z	69	65
ϕ 3T	708	933
R/Z-E2	81	223
R/Z-B3	49	238
β /Z-E1	64	172
β /Z-P3	78	144
ϕ /Z-E1	52	239
ϕ /Z-P3	65	366
ρ /Z-E1	78	252

are among those which also hybridize against the SPR Mtase-coding DNA fragment contained in pSPR5. This hybridization pattern shows that the Z fragment containing the flanks of the SPR Mtase gene has become disrupted and associated with DNA of the Mtase genes. The Mtase genes of all SPR/Z recombinants are thus contained in a unique genomic location, which is defined by DNA homologous to the region of the SPR Mtase gene.

Within this general scheme, different hybridization patterns of individual recombinant clones were observed (Fig. 2). Differences in the DNA structure defining the flanks of the SPR Mtase gene or the corresponding fragment of Z DNA (Fig. 1) have permitted us to describe three types of recombination events affecting the 3' flank of the SPR Mtase gene. These are represented by phages R/Z-E1, -E2, -B3; R/Z-B4, -B5; and R/Z-P6, -P7 (Fig. 1). Recombination at the 5' end must have occurred in phages R/Z-B3, -B4 between the beginning of the gene and the 5'-proximal *Eco*RI site and in the other R/Z phages at a location upstream of this site. This distinction was made by hybridization of pSPR5 against *Hind*III/*Eco*RI double digests of recombinant DNAs (not shown).

Analogous hybridization against *Hind*III-cleaved ϕ 3T-, SP β - and ρ 11/Z DNAs (Terschüren, 1985, and unpublished) led to similar conclusions to those for the SPR/Z recombinants. In all the recombinant DNAs the Z DNA fragment contained in pBKU1 was absent, irrespective of whether they were derived from turbid or clear plaque-forming phages. This indicates that during generation of these recombinants the Mtase genes were integrated at the region of the Z genome characterized by homology with their flanks.

Further analysis of the hybridization patterns of the DNA of turbid-plaque-forming recombinants (Terschüren, 1985) revealed that they are more homogeneous than the patterns of SPR/Z phages. In nine of the 10 recombinant DNAs analysed (four, three and two derived from recombinations between Z and DNA of ϕ 3T, SP β and ρ 11 respectively), recombination at the 3' end must have occurred within the homologous sequences shared by Z and the regions immediately downstream of the Mtase gene (ϕ /Z, β /Z and ρ /Z in Fig. 1c). The tenth recombinant, β /Z-P3, was generated by recombination at a location outside the probed DNA fragments (Fig. 1c). All ϕ 3T-, SP β - and ρ 11/Z recombinants forming turbid plaques were generated by recombination events involving structures at the 5' flank of the Mtase genes, which are located outside the region corresponding to the probed DNA fragments. This reflects the absence of homology between phage Z DNA and structures directly upstream of the ϕ 3T, SP β , ρ 11 genes.

Regulation of the Mtase gene expression in recombinant phage Z derivatives

The expression of the Mtase genes in Mtase-proficient phages is repressed in the lysogenic state (Noyer-Weidner *et al.*, 1981; Jentsch, 1982). To investigate whether the same type of control operates in recombinant phages whose Mtase genes are derived from homoimmune or

heteroimmune phages, we determined the Mtase activity in crude extracts prepared from either uninduced or MC-induced lysogenic strains (Table 2). The specific Mtase activities of extracts prepared from uninduced strains lysogenic for recombinant phages have background levels as defined by induced or non-induced Z lysogens. The high level of Mtase activity found in extracts of uninduced ϕ 3T lysogens is presumably caused by a rather high spontaneous induction rate of phage ϕ 3T (Noyer-Weidner *et al.*, 1981). In extracts prepared from MC-treated lysogens a significant increase of Mtase activity (2- to 6-fold in recombinant phage lysogens; 1.3-fold in ϕ 3T lysogens) was found. These results demonstrate that in the recombinant phages heteroimmune to the donor phage the expression of the Mtase gene is repressed in the lysogenic state.

DISCUSSION

The results presented here show that in all Mtase-proficient phage Z derivatives, the Mtase genes were integrated into a single region of the phage Z genome, which carries sequences homologous to the flanks of the Mtase genes. The responsible double recombination event could involve various alternative homologous DNA sequences shared between Z and regions immediately 3' to the Mtase genes. While at least some of the SPR/Z recombinants were generated by recombination events affecting the immediate 5' flank of the SPR Mtase gene, no recombination between Z DNA and the immediate 5' environment of the ϕ 3T, SP β and ρ 11 Mtase genes was detected. This reflects the differences in the relatedness of sequences preceding the various phage Mtase genes to phage Z DNA (Fig. 1).

Since DNA with the size of the Mtase genes (about 1.4 kb) can be integrated into a specific region of the Z genome without interfering with phage viability, this offers the possibility of constructing a *B. subtilis* cloning system based on phage Z. In a way similar to that described for temperate *B. subtilis* phage ϕ 105 (Savva & Mandelstam, 1984), foreign genetic material inserted into pBKU1 or a derivative of this plasmid could be directed by recombination into the non-essential region of phage Z defined by our experiments.

The Mtase genes of recombinant phages having Z immunity are repressed in the lysogenic state, irrespective of whether they are derived from a phage homoimmune (SP β , SPR) or heteroimmune (ϕ 3T, ρ 11) to phage Z. The repressor defining phage immunity is therefore not directly involved in the repression of the Mtase genes. Most likely the Mtase gene forms part of a larger, phage-repressor-controlled, transcriptional unit that is directly repressed in the prophage state. It remains to be elucidated whether additional DNA structures involved in the control of Mtase gene expression are located on the DNA segments derived from the modification-proficient donor phages or are provided by DNA sequences of the recipient phage Z. Such an understanding requires an analysis of *in vivo* transcription.

Beyond our primary objective to localize the Mtase genes from various donor DNAs in the genome of recipient phage Z, we have recognized extensive recombination between donor and recipient DNA also in other areas of the Z genome (Figs 2 and 3). Such recombination became genetically manifest in the exchange of genetic material determining the immunity of phages. In addition to this, we have observed an exchange of genes for antigenic determinants in transfections with DNA of phage SPR. Phage SPR represents a serotype distinct from that of the other phages studied here (Noyer-Weidner *et al.*, 1983). Obviously extensive sequence homology exists between the various phage genomes in spite of significant polymorphism in restriction patterns. Analogous observations within the SPP1 family of *B. subtilis* phages have recently been reported (Santos *et al.*, 1986).

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