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Determination of DNA Sequences Containing Methylcytosine in *Bacillus subtilis* Marburg

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The methylcytosine-containing sequences in the DNA of *Bacillus subtilis* 168 Marburg (restriction-modification type *Bsu*M) were determined by three different methods: (i) examination of in vivo-methylated DNA by restriction enzyme digestion and, whenever possible, analysis for methylcytosine at the 5′ end; (ii) methylation in vitro of unmethylated DNA with *B. subtilis* DNA methyltransferase and determination of the methylated sites; and (iii) the methylatability of unmethylated DNA by *B. subtilis* methyltransferase after potential sites have been destroyed by digestion with restriction endonucleases. The results obtained by these methods, taken together, show that methylcytosine was present only within the sequence 5′-TCGA-3′. The presence of methylcytosine at the 5′ end of the DNA fragments generated by restriction endonuclease *Asu*II digestion and the fact that in vivo-methylated DNA could not be digested by the enzyme *Xho*I showed that the recognition sequences of these two enzymes contained methylcytosine. As these two enzymes recognized a similar sequence containing a 5′ pyrimidine (Py) and a 3′ purine (Pu), 5′-PyTCGAPu-3′, the possibility that methylcytosine is present in the complementary sequences 5′-TTCGAG-3′ and 5′-CTCGAA-3′ was postulated. This was verified by the methylation in vitro, with *B. subtilis* enzyme, of a 2.6-kilobase fragment of lambda DNA containing two such sites and devoid of *Asu*II or *Xho*I recognition sequences. By analyzing the methylatable sites, it was found that in one of the two PyTCGAPu sequences, cytosine was methylated in vitro in both DNA strands. It is concluded that the sequence 5′-PyTCGAPu-3′ is methylated by the DNA methyltransferase (of cytosine) of *B. subtilis* Marburg.

Procaryotic DNA contains 5-methylcytosine and *N*-6-methyladenine as modified bases. These two methylated bases are present in specific base sequences which vary among different bacteria, and the sequence specificity may even vary within strains of the same species (40). A great diversity of methylated-base-containing sequences was observed in different species of the genus *Bacillis* (20, 26, 38, 41). DNA methylation in bacteria is generally associated with restriction-modification (R-M) phenomena, although other roles are also attributed to DNA methylation (3, 14, 19, 31).

The presence of R-M systems in different strains of *Bacillus subtilis* and the chromosomal loci of the genes controlling these systems have been reported (23, 33, 35, 38). Of the six different strain-specific R-M systems identified so far, only the restriction endonuclease of strain R has been isolated and characterized (7). Different investigators have determined the methylcytosine-containing sequences in different R-M systems present in *B. subtilis* (24, 25). Although in *B. subtilis* Marburg (identified as *Bsu*M in the R-M system) the presence of methylcytosine as the predominant methylated base has been known for some time, the sequences that contain the methylcytosine remain unknown. A hint that one of the bases in the sequence recognized by the restriction endonuclease *Xho*I may be methylated came from the observation that *B. subtilis* 168 Marburg chromosomal DNA remains unrestricted by this enzyme (8). As *Xho*I is known to be sensitive to both cytosine and adenine methylation (29), it seemed reasonable to assume that in this strain methylation in vivo occurs at *Xho*I sites. It has also been reported that in strain Marburg, the only methylcytosine-containing site is the sequence recognized by *Xho*I (25).

Based on methylation experiments in vivo and in vitro, I found that in *B. subtilis* 168M the *Xho*I recognition site is not the only methylated sequence and that cytosine methylation occurs in 5′-PyTCGAPu-3′ sequences.

MATERIALS AND METHODS

Bacteria and plasmids. The bacterial strains used are listed in Table 1. Recombinant plasmid pGsOB2 (6) and the bifunctional vector plasmid pHV33 (30), which can replicate in both *Escherichia coli* and *B. subtilis*, were used for this work. Transformation of competent bacteria with plasmids was carried out by standard methods for *E. coli* and *B. subtilis* (6). For the preparation of plasmid and chromosomal DNA, cells were cultured in LB medium (10).

Culture of phage lambda. *E. coli dam-3 dcm-6* grown in LB medium containing 5 mM $CaCl_2$ was infected with bacteriophage lambda *c*I857 *S*am7 (2) at a multiplicity of infection of 0.1. After induction, cells were grown overnight at 37°C and lysed with chloroform, and phage were precipitated with polyethylene glycol 6000 (final concentration, 10%). The precipitate was dissolved in 10 mM Tris-hydrochloride (pH 7.4)–10 mM $MgSO_4$ and purified further by CsCl banding (2).

DNA preparations. (i) Plasmid and chromosomal DNA. Plasmid DNA was prepared by the method of Birnboim and Doly (4) and purified on cesium chloride gradients. DNA from *B. subtilis* and methyltransferase-deficient *E. coli* was isolated from lysozyme lysates (digested with RNase and proteinase K) by centrifugation in cesium chloride gradients containing 1% Sarkosyl (10).

(ii) Lambda DNA. Lambda DNA was isolated by formamide extraction (10) and dialyzed exhaustively against 10 mM Tris (pH 7.4)–1 mM EDTA.

Unmethylated chromosomal, phage, or plasmid DNA was from an *E. coli* methyltransferase-deficient strain (*dam-3*

TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
B. subtilis		
168M	*trpC2*	C. Anagnostopoulos
JH648	*trpC2 phe-1, spo0B136*	J. Hoch (21)
RM125	*leuA8 argA15 SP90*(s) *hsdR168 hsdM168*	*Bacillus* Genetic Stock Center (39)
E. coli		
C600	F⁻ *leu thr supE gal thi*	1
GM48	*dam-3 dcm-6* derivative of C600	27

dcm-6). The absence of methylation in these DNA preparations was checked by *Mbo*I and *Eco*RII digestion.

5′-Terminal labeling of DNA restriction fragments and chromatography of ^{32}P-labeled mononucleotides. Chromosomal or plasmid DNA (10 μg) was usually digested overnight with 20 to 40 U of the appropriate restriction enzyme and dephosphorylated with bacterial alkaline phosphatase (0.05 U/μg of DNA) for 3 h at 60°C. The restriction fragments were purified by phenol extraction and concentrated by alcohol precipitation. The dephosphorylated DNA fragments (0.5 to 1.0 μg) were labeled at the 5′ end by incubation with [γ-^{32}P]ATP and 10 U of polynucleotide kinase. After incubation for 45 min at 37°C, the reaction was stopped by adding ammonium acetate solution, and the DNA was extracted by ethanol precipitation (28). The labeled DNA, in the presence of 10 μg of carrier calf thymus DNA (total volume, 20 μl), was digested overnight at 37°C with 4 μg of snake venom phosphodiesterase. From 2 to 5 μl of the digest was applied to the corner of polyethyleneimine thin-layer plates (10 by 10 cm) which had been spotted with 2 μl of a mixture of methyl-dCMP and dCMP (3 mg/ml each). The plates were washed with methanol and dried before chromatography.

Chromatography in the first dimension (from bottom to top) was carried out in 1 M acetic acid adjusted to pH 3.4 with concentrated ammonia (5). Chromatography in the second dimension (from left to right), after the plates were washed with methanol, was done in saturated ammonium sulfate solution–1 M acetic acid–isopropanol (80:18:2) (16). The chromatograms were then washed with methanol, dried, and exposed overnight at −70°C to Kodak X-Omat S film with intensifier screens (Du Pont). Methyl-dCMP and dCMP from the radioactive spots were counted in a toluene-based scintillation mixture. The percentage of methylcytosine was calculated from the ratio of methylcytosine to total cytosine (methylated and unmethylated).

Assay and purification of DNA methyltransferase. The assay mixture used for DNA methyltransferase described previously (17) was modified to contain, in a total volume of 100 μl, 50 mM Tris-hydrochloride (pH 8.0), 50 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol, 200 μg of bovine serum albumin per ml, and 2.5 μM of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]SAM) (1 μCi of [^{3}H]SAM per assay unless mentioned otherwise). The amount of unmethylated DNA was usually 5 to 10 μg per assay. In standard assays, incubation was at 37°C for 15 min. The reaction was stopped by adding 100 μl of 0.5 mM unlabeled SAM, followed by 200 μl of 20% trichloroacetic acid. After 20 min in ice, the precipitate was pelleted by centrifugation and washed three times with 1 ml of 10% trichloroacetic acid containing 5 μM SAM. The DNA was degraded by heating the pellet in 100 μl of 10% trichloroacetic acid at 90°C for 20 min. After centrifugation, the solution was removed, mixed with 1 ml of water, and counted in Aquasol (New England Nuclear Corp.).

DNA methyltransferase was purified from *B. subtilis* 168M. Crude extract was prepared as described previously (17). For most in vitro methylation assays, the enzyme was purified further. Briefly, the homogenate was centrifuged at 30,000 × *g*, and the supernatant was precipitated with 0.6% Polymin P. The precipitate was extracted in a buffer containing 1 M NaCl. The enzyme in the supernatant was then precipitated with ammonium sulfate. The fraction precipitating between 38 and 66% ammonium sulfate saturation was collected, dissolved in buffer, and purified through a DEAE-cellulose column with a gradient of NaCl. Active fractions were pooled and concentrated by ammonium sulfate precipitation. This preparation, termed the DEAE eluate, was used for some experiments. Base analysis (17) of DNA methylated in vitro by DEAE eluate has shown that only cytosine is methylated. For other experiments the DEAE eluate was further purified on a heparin-agarose column. The eluate was then concentrated by centrifugation in Centricon-10 (Amicon) and, after dialysis against buffer containing 50% glycerol, was stored at −20°C. The purification procedure and the properties of the enzyme will be described elsewhere.

Isolation of 2.6-kb restriction fragment of lambda DNA and its methylation in vitro. Lambda DNA (100 μg) was digested overnight at 37°C with 200 U of *Cla*I, and the fragments were separated by electrophoresis on a preparative gel. The band corresponding to the 2.6-kilobase (kb) DNA fragment was removed and electroeluted in an ISCO electroelution apparatus. The eluate was brought to 0.3 M in sodium acetate, and the DNA was precipitated with 3 volumes of 95% alcohol at −20°C overnight. The precipitate, collected by centrifugation, was dissolved in 0.5 ml of 10 mM Tris (pH 7.4)–1 mM EDTA–300 mM NaCl. The solution was centrifuged, and the supernatant was applied to a NACS Prepac column (Bethesda Research Laboratories). The column was washed with at least 5 ml of adsorption buffer, and the DNA was eluted with the same buffer containing 1 M NaCl. Fractions (100 μl) were collected and checked by electrophoresis on agarose gels for the presence of DNA fragments. Fractions containing DNA were pooled, concentrated by two ethanol precipitations, and dissolved in 10 mM Tris (pH 7.4)–1 mM EDTA. It was found that agarose strongly inhibited DNA methyltransferase activity and therefore it was necessary to remove traces of agarose for methylation to occur.

The 2.6-kb DNA was methylated for 3 h with 2 μg of heparin-agarose-purified methyltransferase in the presence of 10 μCi of [^{3}H]SAM (specific activity, 81 Ci/mmol) and precipitated with alcohol after phenol extraction and the addition of 10 μg of yeast tRNA as carrier. *Taq*I digestion of the in vitro-methylated fragment was carried out in 25 μl of solution, and separation was done on 6% acrylamide gels (28).

Destruction of potential methylatable sites. To destroy potential sites for methylation, chromosomal and lambda DNAs were exhaustively digested with *Xho*I, *Asu*II, or *Taq*I. The restriction fragments were purified by phenol extraction and concentrated by ethanol precipitation. These fragments were used as the substrate for methyltransferase assays. The cohesive ends were destroyed by heating at 70°C for 5 min before in vitro methylation.

Enzymes and isotopes. Restriction endonucleases were

obtained from New England Biolabs and Boehringer Mannheim and were used as specified by the suppliers. *Eco*RI methylase was purchased from New England Biolabs. *Eco*RII was purchased from Miles Laboratories. *Asu*II (11) was from A. De Waard, Sylvius Laboratories, Leiden, The Netherlands. Marker nucleotides were from P-L Biochemicals GmbH. DNase, snake venom phosphodiesterase, and polynucleotide kinase were from Boehringer Mannheim. Bacterial alkaline phosphatase was from Amersham International. [γ-^{32}P]ATP (2,900 Ci/mmol) was purchased from New England Nuclear Corp., and [^{3}H]SAM was from Amersham International.

Specificity of *Asu*II restriction. The specificity of *Asu*II restriction was determined by digesting lambda DNA either with *Asu*II alone or with *Asu*II followed by *Xho*I. It was found that digesting lambda DNA with *Asu*II gave rise to eight fragments (seven sites) of the sizes predicted by the sequence coordinates (9). A 3.9-kb *Asu*II fragment which contained the *Xho*I site remained undegraded. This fragment disappeared after further digestion with *Xho*I, giving rise to two fragments of 3.1 and 0.8 kb.

DNA sequence coordinates. The DNA sequences in the text are written from left to right, from 5′ end to 3′ end. Lambda DNA restriction site coordinates were obtained from the computed data (9), and the data for other sequences were from the complete nucleotide sequence of the phage as published previously (34).

RESULTS

Methylation in vivo. Bifunctional plasmids are useful tools for comparative analysis of base methylation in the DNA of *E. coli* and gram-positive bacteria (12). To determine the sequences containing methylated bases, I used a bifunctional plasmid, pGsOB2, carrying an early sporulation gene of *B. subtilis* (6), which replicates in both *B. subtilis* and *E. coli* (methyltransferase-positive and methyltransferase-negative strains). During replication in *E. coli* C600, this plasmid was found to acquire the host-specific methylation pattern. On the other hand, the same plasmid derived from a methyltransferase-deficient strain (*E. coli dam-3 dcm-6*) was restricted by the endonucleases *Mbo*I and *Eco*RII, indicating an absence of *E. coli*-specific methylation (19). This finding suggested that the plasmid, when replicated in *B. subtilis*, will acquire a host-specific methylation pattern. First it was determined that the plasmid obtained from the three different sources mentioned above showed the same restriction profile when digested with restriction endonucleases that are insensitive to the presence of methylated bases.

The comparative analysis for methylation in specific sequences was carried out by digesting the plasmid with restriction isoschizomers, one of which was sensitive to the presence of a methylated base in the recognition sequence and the other of which was insensitive to such methylation. When such a pair of isoschizomers was not available, the *B. subtilis* JH648-derived plasmid was digested with a methylated-base-sensitive enzyme, and the restriction profile was compared with that of the unmethylated plasmid. In the *B. subtilis*-derived plasmid, the following sequences were devoid of methylated cytosine (the restriction enzymes used are indicated in parentheses): CCGG (*Hpa*II and *Msp*I), GGCC (*Hae*III), GCGC (*Hha*I), and GATC (*Sau*3AI; see reference 13). Plasmid pGsOB2, as determined on the unmethylated form, did not contain any *Xho*I or *Xba*I sites.

Further searching for sites methylated in vivo was done with the chromosomal DNA of *B. subtilis* 168M. Chromosomal DNA was digested with several restriction enzymes

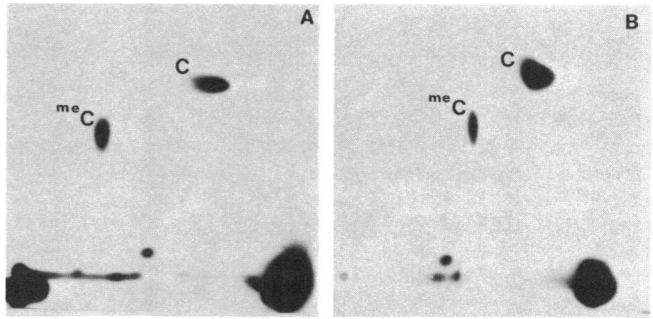

FIG. 1. Thin-layer chromatography of ^{32}P-labeled 5′-terminal nucleotides of the restriction fragments of *B. subtilis* chromosomal DNA digested with (A) *Taq*I or (B) *Asu*II. Fragments from the *Taq*I (0.5 μg) and *Asu*II (1.0 μg) digests were labeled with ^{32}P and digested with snake venom phosphodiesterase in a total volume of 20 μl. The *Taq*I and *Asu*II digests (2 and 5 μl, respectively) were applied to polyethyleneimine plates and chromatographed in two dimensions. After chromatography, spots corresponding to methyl-dCMP (^{me}C) and dCMP (C) were cut out and counted for radioactivity. The percentage of methylcytosine (average for two plates) was 39.2% with *Taq*I and 12.1% with *Asu*II.

which recognize four-base sequences and expose a cytosine at the 5′ end. This cytosine was labeled with ^{32}P as described above and analyzed by two-dimensional chromatography for the presence of methylcytosine. Only the fragments generated by *Taq*I (recognition sequence, 5′-TCGA-3′) digestion showed the presence of 5-methylcytosine at the 5′ end. In a DNA preparation from post-exponential-phase cells, almost 40% of the cytosine at the *Taq*I sites was in methylated form (Fig. 1A).

Since the *Xho*I recognition sequence is a subsequence of *Taq*I cleavage sites, it seemed plausible that the partial methylation observed in *Taq*I sites was due to the methylcytosine present in the *Xho*I recognition sequence. In fact, by a different method of analysis, Jentsch (25) observed the occurrence of methylcytosine in *Taq*I sites and concluded that this is due to the methylation of *Xho*I sites alone. However, the method used by Jentsch (25) does not allow methylcytosine to be quantified, and it was not clear whether the amount of methylcytosine present in the *Taq*I recognition sequence could really account for methylated bases being present only in *Xho*I sites. Moreover, Jentsch (25) did not try to determine whether any other DNA sequence containing TCGA was also methylated. Several six-base-pair-recognizing enzymes have the core sequence TCGA in their recognition sites (cleavage sites are shown by arrows; the endonuclease is given in parentheses): AT$^{\downarrow}$CGAT (*Cla*I); G$^{\downarrow}$TCGAC (*Sal*I); C$^{\downarrow}$TCGAG (*Xho*I); TT$^{\downarrow}$CGAA (*Asu*II).

I have tried to analyze the restriction sites of these enzymes for the presence of methylcytosine by the ^{32}P end-labeling method. The restriction sites of *Sal*I and *Xho*I could not be analyzed by this method for two reasons. One is that these two enzymes are affected in their restriction capacity by cytosine methylation in their recognition sequences (29). Another reason, evident from the examples above, is that the cleavage products of these enzymes have a protruding thymine instead of a cytosine at their 5′ end. However, it was observed that the unmethylated recombinant plasmid pGsOB2 had a single *Sal*I site and that this plasmid, when replicated in *B. subtilis*, still showed total linearization afer *Sal*I digestion, indicating that there was no cytosine methylation at this site. The absence of restriction

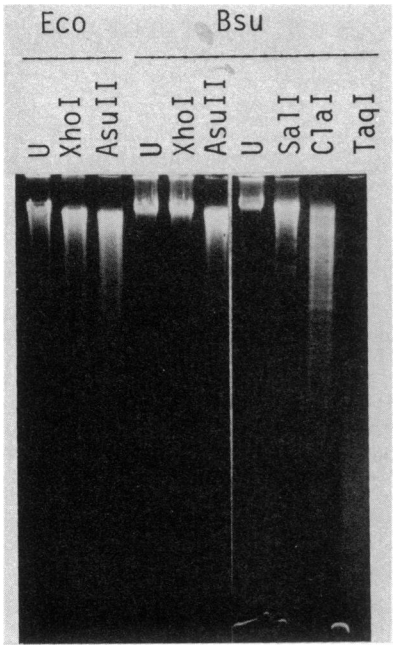

FIG. 2. Restriction patterns of *E. coli dam-3 dcm-6* (Eco) and *B. subtilis* 168M (Bsu) DNAs with different endonucleases (ethidium bromide staining). U, Undigested chromosomal DNA. Gel electrophoresis was done with 0.8 and 1% agarose for the first six and last four lanes, respectively.

of *B. subtilis* DNA by *Xho*I (Fig. 2) would also suggest total methylation of this site. Moreover, Jentsch (25) has shown that DNA from phage φ105C propagated in an R-M-deficient strain can be restricted with *Xho*I. The *Cla*I site, analyzed for methylcytosine, totally lacked the modified base (data not shown). When chromosomal DNA from *B. subtilis* was restricted with *Asu*II, it was found that about 12% of the total cytosine at this site was methylated (Fig. 1B). The same proportion of methylcytosine in *Asu*II sites was observed in both exponential-phase and post-exponential-phase *B. subtilis* DNA. This finding also shows that the restriction capacity of *Asu*II was unimpaired by cytosine methylation.

Methylation in vivo of recombinant plasmid pGsOB2. Since no methylated site could be identified by comparative restriction pattern analysis of plasmid pGsOB2, there was some reason to doubt whether the plasmid is methylated in vivo by the host enzyme. As the chromosomal *Asu*II sites were found to contain methylcytosine, I digested the plasmid overnight with *Asu*II (4 U of enzyme per μg of DNA), which allowed its complete linearization. The 5′ end was analyzed for methylcytosine, and it was found that 33% of the total cytosine was methylcytosine. Since each plasmid has only one *Asu*II site, it is evident that one in three plasmids was methylated in vivo.

Methylation in vitro. The information obtained by analyzing in vivo-methylated chromosomal and plasmid DNA appeared to be incomplete. It was then necessary to obtain further information by methylating different DNAs in vitro.

(i) Plasmid pGsOB2. In an initial experiment, plasmid pGsOB2 and its vector plasmid pHV33 were methylated in vitro with a methyltransferase preparation. Each assay tube contained 5 μg of purified plasmid DNA, 5 μCi of [^{3}H]SAM (specific activity, 73 Ci/mmol), and 52.4 μg of crude extract as a source of methyltransferase, and the tubes were incubated for 1 h at 37°C. pGsOB2 incorporated 4,225 cpm of [^{3}H]SAM, whereas pHV33 incorporated none (for comparison, 1 pmol of [^{3}H]SAM was 15,870 cpm). It is interesting that these two plasmids did not contain any *Xho*I site. One *Asu*II site was present in pGsOB2 within the sporulation gene insert (6). This experiment showed that the *B. subtilis* enzyme can methylate a DNA sequence even in the absence of an *Xho*I recognition sequence.

To determine whether the *Asu*II site of pGsOB2 was totally methylatable, the plasmid derived from the methyltransferase-deficient strain of *E. coli* was methylated for 3 h in the presence of heparin-agarose-purified methyltransferase. Half of the in vitro-methylated plasmid was digested with *Asu*II, and the other half was digested with *Taq*I. The completeness of digestion was checked by agarose gel electrophoresis. A portion of each digest was analyzed for methylcytosine by ^{32}P end labeling. About 63% of the cytosine in the *Asu*II sites was methylated in vitro; in the *Taq*I digest, 23% of the cytosine was methylated.

(ii) *E. coli dam-3 dcm-6* chromosomal DNA. Further evidence of methylation in *Asu*II sites came from in vitro-methylated chromosomal *E. coli* DNA. When the unmethylated DNA was methylated in vitro with DEAE eluate and analyzed for methylcytosine as for the in vitro-methylated plasmid DNA, it was found that 37% of the cytosine in *Asu*II sites and 15% of the cytosine in *Taq*I sites was methylated. In addition, it was observed that in vitro-methylated chromosomal DNA digested with *Xho*I showed less restriction than unmethylated DNA did.

(iii) 2.6-kb fragment of lambda DNA. The recognition sequences of *Asu*II and *Xho*I are similar in that both sequences contain, apart from the common TCGA, a pyrimidine (Py) at the 5′ end and a purine (Pu) at the 3′ end. The question arose whether the other two possible PyTCGAPu sequences, which are complementary in double-stranded DNA, could also be methylated by *B. subtilis* DNA methyltransferase. This was determined by using lambda DNA, which contains two such sequences about 2 kb apart within a 2.6-kb fragment (from nucleotide 43825) generated by *Cla*I digestion. This fragment lacked *Asu*II and *Xho*I sites but contained one *Eco*RI site. Each assay tube contained 0.5 μg of the 2.6-kb fragment, 5 μCi of [^{3}H]SAM (specific activity, 73 μCi/mmol), and 28.9 μg of DEAE eluate from *B. subtilis* or 1 U of *Eco*RI methylase, and the tubes were incubated for 1 h at 37°C. Blank counts (without DNA) were deducted. The 2.6-kb fragment was methylated in vitro by the *B. subtilis* enzyme (4,237 cpm incorporated), indicating that sites other than those of *Asu*II or *Xho*I can also be methylated by the DNA methyltransferase of *B. subtilis* Marburg. With the *Eco*RI methylase, the 2.6-kb lambda DNA fragment incorporated 7,232 cpm.

To identify the methylated-base-containing sequences, the ^{3}H-methylated 2.6-kb DNA was digested to completion with *Taq*I, which cleaves within the PyTCGAPu sequences, allowing determination of whether the cytosines in the sequences TTCGAG and CTCGAA are methylated differently. In the PyGCGAPu sequence starting from nucleotide 43891, after digestion with *Taq*I the methylatable cytosine in the sequence CTCGAA was segregated in a 0.41-kb fragment, whereas the methylatable cytosine of the TTCGAG sequence was in the contiguous 0.068-kb fragment. Similarly, in the PyTCGAPu sequence starting from nucleotide 45894, the methylcytosine in the two complementary sequences was segregated into 0.054- and 0.26-kb fragments, respectively. The digest was electrophoresed on an acrylamide gel and autoradiographed. Among the 13 nonoverlapping *Taq*I fragments, only the bands correspond-

ing to the 0.26- and 0.054-kb fragments contained radioactivity (Fig. 3). This indicates that only the PyTCGAPu sequence starting from nucleotide 45894 is methylated in both strands, whereas the PyTCGAPu sequence starting from nucleotide 43891 is totally unmethylated.

Destruction of potential methylatable sites and methyl-accepting capacity of DNA. It is known that the potentially methylatable sites in DNA can be destroyed by digestion with specific restriction endonucleases (36). To identify such sites, unmethylated DNA was digested overnight at 37°C with *Xho*I, *Asu*II, or *Taq*I. The resulting restriction fragments were used as the substrate for subsequent methylation. Undigested DNA served as a control. Digestion with *Xho*I or *Asu*II caused only a partial loss of methyl-accepting capacity (Table 2), indicating that the recognition sequence for either enzyme is not the exclusive site for methylation. It was also evident (Table 2) that digesting either chromosomal or lambda DNA with *Taq*I caused a total loss of methyl-accepting capacity, indicating that all methylatable cytosines are located within the *Taq*I site.

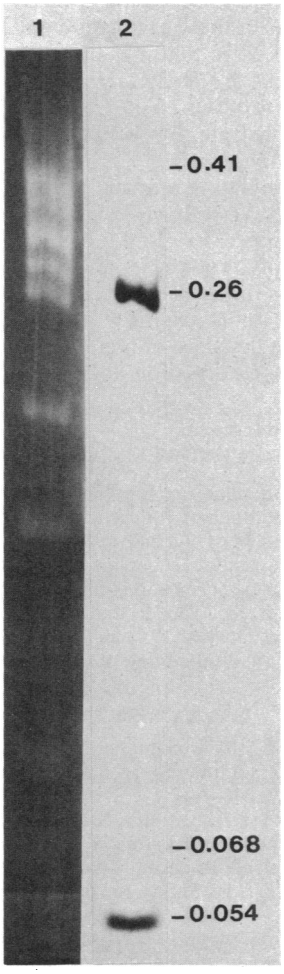

FIG. 3. Autoradiography of the ^{3}H-labeled, *Taq*I-digested fragments of in vitro-methylated 2.6-kb lambda DNA. Electrophoresis was carried out on a 16.5-cm-long, 1-mm-thick 6% acrylamide gel for 15 h at 50 V in buffer containing 45 mM Tris, 45 mM boric acid, and 1.125 mM EDTA (pH 8.3). The gel was stained with ethidium bromide (1 μg/ml) (lane 1), photographed, dried, and exposed to Kodak X-Omat S film for 12 days (lane 2). Fragment sizes (in kilobases) are indicated. The radioactive bands at 0.26 and 0.054 kb correspond to the complementary sequences TTCGAG and CTCGAA, respectively (from nucleotide 45894). The second PyTCGAPu site at nucleotide 43891 was totally unmethylated. The fragment sizes were determined from the lambda sequence data and also from an *Hpa*II digest of plasmid pBR322 (not shown).

TABLE 2. Effect of restricting DNA on in vitro methylation[a]

DNA and digesting enzyme	[^{3}H]SAM incorporated (μmol/mg of protein per 15 min)	% Activity remaining
E. coli dam-3 dcm-6		
None	2.04	100
*Xho*I	1.15	56
*Asu*II	1.50	73
*Taq*I	0	0
Lambda		
None	0.48	100
*Xho*I	0.32	66
*Taq*I	0	0

[a] Different DNA preparations were digested overnight at 37°C with 4 U of restriction enzyme per μg of DNA. Undigested DNA was sonicated twice for 1 min each at 100 W in a Branson sonifier. Digested DNAs were extracted with phenol and concentrated by ethanol precipitation. Each assay tube contained 3.4 μg of DNA (0.068 A_{260} unit) and 2.4 μg of heparin-agarose-purified DNA methyltransferase from *B. subtilis*. Incubation was at 37°C for 15 min. A separate experiment showed that in *Asu*II-digested lambda DNA only 37% of the activity remained compared with that in the undigested control. *Sal*I digestion caused practically no loss of activity.

DISCUSSION

To identify the methylated sites in the DNA of a particular bacterial strain, the cleavage site revealed by the homologous restriction enzyme is often determined first. The eventual protection from cleavage by methylation with a homologous methylating enzyme permits the localization of methylated-base-containing sequences (36). Although in *B. subtilis* Marburg the methyltransferase of cytosine is considered part of the R-M system, no restriction enzyme has so far been detected in this strain. Previous attempts (23), including those in my laboratory, to detect any restriction activity in this strain have been unsuccessful. However, several findings on a restriction-deficient (of phage ϕ105C) mutant of strain Marburg, strain RM125 (39), suggest that this methyltransferase may be a part of the R-M system. The DNA of this mutant is restricted by *Xho*I (25) and can serve as a methyl acceptor with the *Bsu*M DNA methyltransferase (unpublished data). Moreover, preliminary experiments with a crude extract of mutant strain RM125 showed that this mutant is also deficient in DNA methyltransferase activity. Experimental details will be published later.

Several phage-induced DNA methyltransferases in *B. subtilis* were reported and reviewed by Gunthert and Trautner (18). The sequences methylated by these enzymes were analyzed by restriction endonuclease isoschizomers; none of them, however, methylated the cytosine in the sequence TCGA. Moreover, the persistence of DNA methylation in a strain of *B. subtilis* 168 Marburg excised of its resident phage SPβ (42) indicates that this methylation enzyme is of chromosomal origin (25).

The facts that chromosomal DNA in strain Marburg was not restricted by *Xho*I and that the DNA of an R-M-deficient strain was susceptible to *Xho*I restriction clearly indicate the presence of a methylated base in the *Xho*I recognition sequence (CTCGAG). It is not known whether the adenine or the cytosine in this sequence is methylated or, in the latter case, whether it is the internal or external cytosine. Jentsch

(25) found that the internal cytosine of the *Xho*I recognition sequence is methylated, and he concluded that methylation occurs only in the *Xho*I site.

Several findings, however, suggest that the *Xho*I recognition sequence is not the only methylatable site in strain Marburg (*Bsu*M). In initial experiments I found that plasmid pGsOB2 can be methylated in vitro by the *B. subtilis* enzyme even though it does not contain any *Xho*I site. Moreover, digesting unmethylated lambda DNA with *Xho*I resulted in only a 33% loss of methylatability. If the *Xho*I recognition sequence were the sole site of methylation by the *B. subtilis* enzyme, digestion would have caused a total loss of methylatability. These results together indicated that methylatable sites other than those of the *Xho*I recognition sequence also exist in strain Marburg. Moreover, Jentsch (25) did not try to determine whether the sequences containing TCGA recognized by other six-base-pair-recognizing enzymes (see above) are also methylated. The findings reported here show clearly that, in fact, another such site recognized by *Asu*II (TTCGAA) is also methylated, both in vivo and in vitro. Since the possibility of methylation in *Cla*I and *Sal*I sites is excluded by the results reported herein, my attention was drawn to the fact that *Asu*II and *Xho*I recognition sequences bear some similarity: a 5′ pyrimidine, a common TCGA, and a 3′ purine. The question I posed was whether the other two complementary PyTCGAPu (CTCGAA and TTCGAG) sequences are also methylatable. Since no restriction enzyme identified to date can recognize these two sequences, analysis by restriction was not possible. A 2.6-kb *Cla*I fragment of lambda DNA without *Asu*II or *Xho*I sites carries two such sequences. As this DNA fragment was found to be methylatable by the *B. subtilis* enzyme, it was analyzed further to detect the methylated sequences. *Taq*I digestion was chosen because this enzyme, which is insensitive to cytosine methylation, produces a cut within the PyTCGAPu sequences and segregates the methylatable cytosine of each DNA strand into a separate contiguous fragment. It is thus possible to determine whether the cytosines in the two nonpalindromic but complementary sequences TTCGAG and CTCGAA are methylated differently. It was found that one of the two PyTCGAPu sequences was methylated in both strands, which indicates that the *Bsu*M enzyme is able to methylate both CTCGAA and TTCGAG sequences. The reason why the other PyTCGAPu sequence in the 2.6-kb DNA fragment remained totally unmethylated is not clear. It is possible that the lambda DNA used in this study had undergone a mutation within this site, causing loss of recognition by the methyltransferase.

The possibility of the occurrence of methylcytosine in the sequence PyTCGAPy was ruled out by examination of the nucleotide sequence of plasmid pHV33 (30). This composite plasmid was constructed by joining plasmids pBR322 and pC194, whose complete sequences are known (22, 37). The component plasmids of pHV33, devoid of PyTCGAPu sequences, have several PyTCGAPy sequences (four in pBR322 and two in pC194), and pHV33 was not a methyl acceptor for the *B. subtilis* enzyme (see above).

It was found that only some of the *Asu*II recognition sequences are methylated in vivo (12% in chromosomal and 33% in plasmid DNA), and the total absence of *Xho*I restriction of chromosomal DNA would suggest that all the *Xho*I sites are methylated. Whether the methyltransferase of strain Marburg methylates some sites in preference to others is yet to be determined by using a DNA of known sequence as the substrate. However, analysis of *Asu*II sites after in vitro methylation of both chromosomal and plasmid DNA clearly showed that *Asu*II sites are methylatable.

The observed partial methylation of *Asu*II sites can also be explained by the existence of partial in vivo methylation of DNA in *B. subtilis*. In fact, it was found that *B. subtilis* 168M DNA can be methylated in vitro (about 1% compared with unmethylated DNA) with a homologous methyltransferase. Vanyushin and Dobrista (41) have reported a similar observation for *Bacillus brevis*. This indicates that in *B. subtilis*, DNA sites susceptible to methylation are not fully saturated. An alternative explanation is possible. With *Haemophilus influenzae* type d, a portion of the cells in a culture at any time are deficient in the R-M system (15). In fact the strain of *H. influenzae* from which the restriction enzymes and methyltransferases were isolated was found to contain about 50% $HsdR^-$ $HsdM^-$ cells in any given culture, which explains why the DNA of this strain is methylatable by a homologous enzyme (32). Because in *B. subtilis* a certain percentage of cells of the clear-plaque mutant of phage ϕ105 escape restriction, it is possible that in a culture of strain Marburg some cells are $HsdR^-$ $HsdM^-$. Consequently, the chromosomal DNA of these cells remains unmethylated and serves as the substrate for in vitro methylation with a homologous enzyme.

A cytosine-methylating enzyme is apparently induced in competent *B. subtilis* cells (14). I have also observed that methylation, although very low, occurs during the sporulation phase. It is not known whether methylation under these conditions occurs in a sequence(s) different from those identified so far. In *B. subtilis*, no correlation has been observed between the state of methylation and gene expression. Any role for methylation apart from R-M is yet to be determined.

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