Determination of DNA Sequences Containing Methylcytosine in Bacillus subtilis Marburg

SIBAJYOTI GUHA
Laboratoire d’Enzymologie du Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France

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The methylcytosine-containing sequences in the DNA of Bacillus subtilis 168 Marburg (restriction-modification type BsuM) 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 methylation ability 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 AsuII digestion and the fact that in vivo-methylated DNA could not be digested by the enzyme XhoI 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′-PuTCGAPu-3′, the possibility that methylcytosine is present in the complementary sequences 5′-TTCGAG-3′ and 5′-CCTGAA-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 AsuII or XhoI recognition sequences. By analyzing the methyletable sites, it was found that in one of the two PuTCGAPu sequences, cytosine was methylated in vitro in both DNA strands. It is concluded that the sequence 5′-PuTCGAPu-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 Bacillus (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 BsuM 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 XhoI may be methylated came from the observation that B. subtilis 168 Marburg chromosomal DNA remains unrestricted by this enzyme (8). As XhoI 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 XhoI sites. It has also been reported that in strain Marburg, the only methylcytosine-containing site is the sequence recognized by XhoI (25).

Based on methylation experiments in vivo and in vitro, I found that in B. subtilis 168M the XhoI recognition site is not the only methylated sequence and that cytosine methylation occurs in 5′-PuTCGAPu-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 CaCl2 was infected with bacteriophage lambda cI857 Sam7 (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 MgSO4 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
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 CiaI, 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 electrodialuted in an ISCO electrodialation 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 [³H]SAM (specific activity, 81 Ci/mmole) and precipitated with alcohol after phenol extraction and the addition of 10 μg of yeast tRNA as carrier. *TaqI* 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 *XhoI*, *AsuII*, or *TaqI*. 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168M</td>
<td>trpC2</td>
<td>C. Anagnostopoulos</td>
</tr>
<tr>
<td>JH648</td>
<td>trpC2 phe-1, spo0B136</td>
<td>J. Hoch (21)</td>
</tr>
<tr>
<td>RM125</td>
<td>leuA8 argA15 SP90(s) hsdR168</td>
<td>Bacillus Genetic Stock Center (39)</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C600</td>
<td>F' leu thr supE gal thi</td>
<td>1</td>
</tr>
<tr>
<td>GM48</td>
<td>dam-3 dcm-6 derivative of</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>C600</td>
<td></td>
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obtained from New England Biolabs and Boehringer Mannheim and were used as specified by the suppliers. EcoRI methylase was purchased from New England Biolabs. EcoRII was purchased from Miles Laboratories. AsuII (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-32P]ATP (2,900 Ci/mmol) was purchased from New England Nuclear Corp., and [3H]SAM was from Amersham International.

Specificity of AsuII restriction. The specificity of AsuII restriction was determined by digesting lambda DNA either with AsuII alone or with AsuII followed by XhoI. It was found that digesting lambda DNA with AsuII gave rise to eight fragments (seven sites) of the sizes predicted by the sequence coordinates (9). A 3.9-kb AsuII fragment which contained the XhoI site remained undegraded. This fragment disappeared after further digestion with XhoI, 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 MboI and EcoRII, 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 H648-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 (HpaII and MspI), GGCC (HaeIII), GGC (HhaI), and GATC (SacI; see reference 13). Plasmid pGsOB2, as determined on the unmethylated form, did not contain any XhoI or XbaI 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 which recognize four-base sequences and expose a cytosine at the 5' end. This cytosine was labeled with 32P as described above and analyzed by two-dimensional chromatography for the presence of methylcytosine. Only the fragments generated by TaqI (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 TaqI sites was in methylated form (Fig. 1A).

Since the XhoI recognition sequence is a subsequence of TaqI cleavage sites, it seemed plausible that the partial methylation observed in TaqI sites was due to the methylcytosine present in the XhoI recognition sequence. In fact, by a different method of analysis, Jentsch (25) observed the occurrence of methylcytosine in TaqI sites and concluded that this is due to the methylation of XhoI 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 TaqI recognition sequence could really account for methylated bases being present only in XhoI sites. Moreover, Jentsch (25) did not try to determine whether any other DNA sequence containing TCAG was also methylated. Several six-base-pair-recognizing enzymes have the core sequence TCAG in their recognition sites (cleavage sites are shown by arrows; the endonuclease is given in parentheses): AT1CGAT (ClaI); G1TCGAC (SalI); C1TCGAG (XhoI); TT1CGAA (AsuII).

I have tried to analyze the restriction sites of these enzymes for the presence of methylcytosine by the 32P end-labeling method. The restriction sites of SalI and XhoI 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 SalI site and that this plasmid, when replicated in B. subtilis, still showed total linearization after SalI digestion, indicating that there was no cytosine methylation at this site. The absence of restriction
of B. subtilis DNA by XhoI (Fig. 2) would also suggest total methylation of this site. Moreover, Jentsch (25) has shown that DNA from phage 4105C propagated in an R-M-deficient strain can be restricted with XhoI. The CiaI site, analyzed for methylcytosine, totally lacked the modified base (data not shown). When chromosomal DNA from B. subtilis was restricted with AsuII, it was found that about 12% of the total cytosine at this site was methylated (Fig. 1B). The same proportion of methylcytosine in AsuII sites was observed in both exponential-phase and post-exponential-phase B. subtilis DNA. This finding also shows that the restriction capacity of AsuII 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 AsuII sites were found to contain methylcytosine, I digested the plasmid overnight with AsuII (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 AsuII 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 [3H]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 [3H]SAM, whereas pHV33 incorporated none (for comparison, 1 pmol of [3H]SAM was 15,870 cpm). It is interesting that these two plasmids did not contain any XhoI site. One AsuII 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 XhoI recognition sequence.

To determine whether the AsuII 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 AsuII, and the other half was digested with TaqI. The completeness of digestion was checked by agarose gel electrophoresis. A portion of each digest was analyzed for methylcytosine by 32P end labeling. About 63% of the cytosine in the AsuII sites was methylated in vitro; in the TaqI digest, 23% of the cytosine was methylated.

(ii) E. coli dam-3 dcm-6 chromosomal DNA. Further evidence of methylation in AsuII 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 AsuII sites and 15% of the cytosine in TaqI sites was methylated. In addition, it was observed that in vitro-methylated chromosomal DNA digested with XhoI showed less restriction than unmethylated DNA did.

(iii) 2.6-kb fragment of lambda DNA. The recognition sequences of AsuII and XhoI 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 PyTCApgu 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 CiaI digestion. This fragment lacked AsuII and XhoI sites but contained one EcoRI site. Each assay tube contained 0.5 μg of the 2.6-kb fragment, 5 μCi of [3H]SAM (specific activity, 73 Ci/mmol), and 28.9 μg of DEAE eluate from B. subtilis or 1 U of EcoRI 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 (24,273 cpm incorporated), indicating that sites other than those of AsuII or XhoI can also be methylated by the DNA methyltransferase of B. subtilis Marburg. With the EcoRI methylase, the 2.6-kb lambda DNA fragment incorporated 7,232 cpm.

To identify the methylated-base-containing sequences, the 3H-methylated 2.6-kb DNA was digested to completion with TaqI, which cleaves within the PyTCApgu sequences, allowing determination of whether the cytosines in the sequences TTCGAG and CTCGAA are methylated differently. In the PyGCGApgu sequence starting from nucleotide 43891, after digestion with TaqI 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 PyTGCApgu 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 TaqI 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 methylaccepting 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 *XhoI*, *AsuII*, or *TaqI*. The resulting restriction fragments were used as the substrate for subsequent methylation. Undigested DNA served as a control. Digestion with *XhoI* or *AsuII* 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 *TaqI* caused a total loss of methyl-accepting capacity, indicating that all methylatable cytosines are located within the *TaqI* site.

### 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 *XhoI* (25) and can serve as a methyl acceptor with the *BsuMI* 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 Gunther 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 SPB (42) indicates that this methylation enzyme is of chromosomal origin (25).

The facts that chromosomal DNA in strain Marburg was not restricted by *XhoI* and that the DNA of an R-M-deficient strain was susceptible to *XhoI* restriction clearly indicate the presence of a methylated base in the *XhoI* 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

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**TABLE 2. Effect of restricting DNA on in vitro methylation**

<table>
<thead>
<tr>
<th>DNA and digesting enzyme</th>
<th>[3H]SAM incorporated (μmol/mg of protein per 15 min)</th>
<th>% Activity remaining</th>
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<tr>
<td><em>E. coli</em> dam-3 dcm-6</td>
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<td></td>
</tr>
<tr>
<td>None</td>
<td>2.04</td>
<td>100</td>
</tr>
<tr>
<td><em>XhoI</em></td>
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<td>56</td>
</tr>
<tr>
<td><em>AsuII</em></td>
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<td>73</td>
</tr>
<tr>
<td><em>TaqI</em></td>
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<td>0</td>
</tr>
<tr>
<td>Lambda</td>
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<tr>
<td>None</td>
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</tr>
<tr>
<td><em>XhoI</em></td>
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<td>66</td>
</tr>
<tr>
<td><em>TaqI</em></td>
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</tr>
</tbody>
</table>

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 A260 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 *AsuII*-digested lambda DNA only 37% of the activity remained compared with that in the undigested control. *TaqI* digestion caused practically no loss of activity.

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**FIG. 3.** Autoradiography of the [3H]-labeled, *TaqI*-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 CTCGAAA, 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 *HpaII* digest of plasmid pBR322 (not shown).
(25) found that the internal cytosine of the XhoI recognition sequence is methylated, and he concluded that methylation occurs only in the XhoI site.

Several findings, however, suggest that the XhoI recognition sequence is not the only methylatable site in strain Marburg (BsuM). 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 XhoI site. Moreover, digesting unmethylated lambda DNA with XhoI resulted in only a 33% loss of methylatability. If the XhoI 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 XhoI 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 AsuII (TTGCA) is also methylated, both in vivo and in vitro. Since the possibility of methylation in CiaI and SalI sites is excluded by the results reported herein, my attention was drawn to the fact that AsuII and XhoI 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 PyTGCAPu (CTGCA and TTGAG) 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 CiaI fragment of lambda DNA without AsuII or XhoI 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. TaqI digestion was chosen because this enzyme, which is insensitive to cytosine methylation, produces a cut within the PyTGCAPu 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 TTGCA and TCTGAA are methylated differently. It was found that one of the two PyTGCAPu sequences was methylated in both strands, which indicates that the BsuM enzyme is able to methylate both TCTGAA and TTGCA sequences. The reason why the other PyTGCAPu 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 PyTGCAPu 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 PyTGCAPu sequences, have several PyTGCAPu 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 AsuII recognition sequences are methylated in vivo (12% in chromosomal and 33% in plasmid DNA), and the total absence of XhoI restriction of chromosomal DNA would suggest that all the XhoI 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 AsuII sites after in vitro methylation of both chromosomal and plasmid DNA clearly showed that AsuII sites are methylatable.

The observed partial methylation of AsuII 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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LITERATURE CITED