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Abstract
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Restriction Analysis and Quantitative Estimation of Methylated Bases of Filamentous and Unicellular Cyanobacterial DNAs

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The DNAs of strains of three cyanobacterial genera (Anabaena, Plectonema, and Synechococcus) were found to be partially or fully resistant to many restriction endonucleases. This could be due to the absence of specific sequences or to modifications, rendering given sequences resistant to cleavage. The latter explanation is substantiated by the content of N\(^6\)-methyladenine and 5-methylcytosine in these genomes, which is high in comparison with that in other bacterial genomes. dcm- and dam-like methylases are present in the three strains (based on the restriction patterns obtained with the appropriate isochizomeric enzymes). Their contribution to the overall content of methyladenine and methylcytosine in the genomes was calculated. Partial methylation of GATC sequences was observed in Anabaena DNA. In addition, the GATC methylation patterns might not have been random in the three cyanobacterial DNA preparations, as revealed by the appearance of discrete fragments (possibly of plasmid origin) withstanding cleavage by DpnI (which requires the presence of methyladenine in the GATC sequence).

Cyanobacteria, performing oxygenic photosynthesis, are a diverse group of procaryotes, some of which fix atmospheric nitrogen aerobically (27, 30). Genetic studies have enabled the understanding of some developmental peculiarities (9, 14) and are required to expand the use of cyanobacteria as fertilizers and waste disposers (22, 23). The development of genetic transformation systems has to take into account that about 24 restriction endonucleases have been isolated from Nostoc/Anabaena cyanobacteria (a few of them are isochizomeric enzymes). Some of these endonucleases recognize sequences with insertions of ambiguous nucleotides (7, 16, 25). Indeed, cloning vectors were obtained after deletion of sites recognized by restriction enzymes present in the cyanobacterial host (4, 37).

Methylation of adenine or cytosine residues within specific recognition sequences is certainly the best-characterized means of protection against restriction enzymes (3, 16). Thus, modification of DNA might be expected, entailing the failure of restriction enzymes present within a cyanobacterial cell to digest chromosomal DNA. Yet, the modifications observed in different cyanobacterial DNAs could not be explained only by the methylation necessary to protect the DNA against the known type II restriction endonucleases possessed by the strains under study (18, 34). In addition, methylases without a direct nuclease counterpart (e.g., M· Eco dam, M· Eco dcmI, or bacteriophage-coded methylases) might contribute to modification of DNA. Indeed, the presence of dam-like methylases in cyanobacteria was suggested previously (1, 18). Alternatively, chromosomal DNA may be lacking recognition sites for certain endonucleases (13).

In this study, we selected three strains representative of filamentous heterocystic, filamentous nonheterocystic, and unicellular forms of photosynthetic bacteria. The relative resistance of their genomes to restriction enzymes, which could be due to the absence of recognition sites or the presence of methylated bases, was documented. To distinguish between these alternatives, the content of N\(^6\)-methyladenine (MeA) and 5-methylcytosine (MeC) was determined in DNA hydrolysates. These quantitative estimations were correlated with the observed restriction patterns, with special reference to dam and dcm methylation.

MATERIALS AND METHODS

Strains. The filamentous cyanobacteria Anabaena sp. strain PCC 7120 and Plectonema boryanum PCC 73110 and the unicellular cyanobacterium Synechococcus cedrorum R2 (obtained from L. A. Sherman, University of Missouri, Columbia) were used in the study. Cells were grown in batch cultures of 300 ml or 2 liters in modified Chu-10 medium, described elsewhere (23). Anabaena sp. strain PCC 7120 was grown in nitrate-free medium [0.38 mM CaCl\(_2\) · 2H\(_2\)O in place of Ca(NO\(_3\))\(_2\) · 4H\(_2\)O]. Illumination was provided by fluorescent lamps at 2,500 lx. Culture vessels were maintained at 24 ± 2°C.

DNA extraction. Wet packed cells (2 to 3 g) were washed repeatedly with 150 mM NaCl–100 mM EDTA (pH 7.5) and suspended in 10 ml of 200 mM Tris hydrochloride (pH 7.5)–50 mM EDTA. Lysozyme (40 mg/ml) was added (for 1 h at 37°C). Lysis of spheroplasts was achieved by adding 1% (final concentration) sodium dodecyl sulfate (for 30 min at 37°C), followed by freezing and thawing. After repeated phenol and chloroform-isooamyl alcohol extractions, DNA was precipitated by isopropanol. The pellet was suspended in 2 ml of 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA (TE solution). Further purification of DNA was performed by hydroxyapatite column chromatography as described by Coene and Cocito (5), with slight modifications. A 2-ml portion of DNA solution and 17 ml of lysing buffer (0.18 M phosphate buffer [pH 7.8], 9 M urea, 0.9% [wt/vol] sodium dodecyl sulfate) were mixed and loaded onto 3 to 5 g of hydroxyapatite. The column was washed with 0.18 M phosphate buffer and eluted with 0.48 M phosphate buffer (pH 7.8). The eluted DNA was dialyzed against TE solution. The purity of the DNA was assessed by standard spectrophotometric ratios (\(A_{260}/A_{280}\)).
Restoration endonucleases. The enzymes BamHI, BglII, Clal, EcoRI, HaeIII, HindIII, and HpaII were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany. Incubations (5 U/µg of DNA) were performed in 20-µl volumes at 37°C for 3 h. Enzymatic activity was terminated by heating at 65°C for 10 min. Electrophoresis in a 0.75 or 1% agarose gel was performed for 20 min at 150 V. The gel was then stained with ethidium bromide (1 µg/ml in TE buffer) and photographed under ultraviolet light.

Fractionation of DNA hydrolysates. Samples were prepared for high-pressure liquid chromatography (HPLC) by the method of Eick et al. (8). Purified DNA (450 to 480 µg) was lyophilized, dissolved in 100 µl of water, and dialyzed on membranes (pore size, 0.025 µm) against double-distilled water. The concentrated desalted DNA was lyophilized, and the pellet was dissolved in 300 µl of 90% formic acid for acid digestion at 180°C for 30 min under N2. The digest was lyophilized and dissolved in 50 to 80 µl of 50 mM NaH2PO4.

Hydrolysates were fractionated on a cation-exchange column (Partisil 10 SCX; Whatman) at 20°C with a mobile phase of 0.1 M NaH2PO4 adjusted to pH 3.3 with acetic acid. The UV detector was set at 270 nm, and peak surfaces were integrated. The absorbance spectra of different peaks in the DNA hydrolysates were recorded, allowing their identification by comparison with the spectra of the standard bases (5 mmol of each).

RESULTS

Cleavage of cyanobacterial DNA by restriction endonucleases. Cyanobacterial DNA was extracted, purified, treated with endonucleases, and analyzed by gel electrophoresis. Since preliminary results had revealed the inability of several endonucleases to cleave these DNAs, additional purification steps were used as described in Materials and Methods. It was ascertained that the DNA preparations contained no substances inhibiting restriction endonucleases and were devoid of contaminating nucleases.

Under these conditions, several restriction endonucleases only partially cleaved cyanobacterial DNA, yielding large fragments. This typical size distribution was scored P, for partial digestion (Table 1). Other endonucleases (scored + [Table 1]) yielded restriction fragments with an average size distribution expected on a statistical basis. Recognition sequences are indicated in Table 1 together with the effects of adenine and cytosine methylation within the relevant sequence on the activity of the endonuclease (16).

Methylated adenine was present within the GATC sequences of the three cyanobacterial DNAs, as well as in the DNA of the filamentous nonheterocystous organism Anacystis nidulans (results not shown). This can be deduced unambiguously from the restriction pattern obtained with three isoschizomeric enzymes (Table 1, no. 1 to 3; see Fig. 2). All three cyanobacterial DNAs (Table 1, no. 7) were partially hydrolyzed with Clal, confirming the presence of MeA within GATC sequences. MeA could occur in sequences other than GATC, as suggested by the inability of PsI (Table 1, no. 8) to cleave Anabaena DNA.

The internal cytosine of the sequence CCAG is methyalted in the three cyanobacterial DNAs. This conclusion stems from the results obtained with two isoschizomeric enzymes (Table 1, no. 22 and 23) which have been used in Escherichia coli to probe the sites methylated by the product of the dam gene (24). The presence of MeC within other sequences of Anabaena DNA was suggested by the restriction data in Table 1 for enzymes 4 to 6, 15, and 20.

The presence of MeC in Plectonema and Synechococcus DNAs is suggested by the same type of evidence, since only partial cleavage was observed upon treatment of these DNAs with the same restriction endonucleases (e.g., Table 1, no. 4, 5, 15, and 20).

The cytosine residue within the GGNCC sequence of Anabaena DNA might be methylated, since very limited cleavage was seen with Sau961 (Table 1, no. 19), which is known to be inhibited when the external cytosine is methylated. This enzyme was the only one analyzed in this study which has a recognition sequence shared by endonucleases isolated from Anabaena strains (AvAI, AfII, and Nsp7524 IV) (16).

Quantitative determination of MeA and MeC from cyanobacterial-DNA hydrolysates. To evaluate the overall extent of adenine and cytosine methylation in cyanobacterial genomes unambiguously, we turned to chromatographic analysis of DNA hydrolysates. Extensive purification of cyanobacterial DNA was required for this analysis and was performed as described in Materials and Methods. After formic acid hydrolysis, HPLC fractionation was performed under conditions described in Materials and Methods.

<table>
<thead>
<tr>
<th>No.</th>
<th>Restriction enzyme</th>
<th>Recognition sequence*</th>
<th>Cleavage of DNA of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anabaena sp. 7120</td>
</tr>
<tr>
<td>1</td>
<td>DpnI</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>MboI</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Sau3A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>BamHI</td>
<td>+</td>
<td>P</td>
</tr>
<tr>
<td>5</td>
<td>BglII</td>
<td>+</td>
<td>P</td>
</tr>
<tr>
<td>6</td>
<td>PvuII</td>
<td>+</td>
<td>P</td>
</tr>
<tr>
<td>7</td>
<td>ClaI</td>
<td>A</td>
<td>P</td>
</tr>
<tr>
<td>8</td>
<td>PsI</td>
<td>CT</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>SalI</td>
<td>GT</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>HpaII</td>
<td>GTTA</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>HindIII</td>
<td>AGGC</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>EcoRI</td>
<td>GAAT</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>AluI</td>
<td>AGCT</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>HpaII</td>
<td>CGGG</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>HaeIII</td>
<td>GCCG</td>
<td>P</td>
</tr>
<tr>
<td>16</td>
<td>HhaI</td>
<td>GCCG</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>TaqI</td>
<td>TCGA</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>Fnu4HI</td>
<td>GNGG</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>Sau96I</td>
<td>GNCC</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>Smal</td>
<td>CCCGG</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>KpnI</td>
<td>GTACC</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>BstNI</td>
<td>CCGG</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>EcoRI</td>
<td>CCGG</td>
<td>+</td>
</tr>
</tbody>
</table>

* Recognition sequences are specified for the 5'→3' strand. + or - indicates the presence of an endonuclease by an MeA or MeC residue within the recognition sequence; M, MeA residues are a prerequisite for the activity of DpnI; or +, digestion of the DNA is not influenced by MeA or MeC residues. If an adenine or cytosine residue is not identified by a symbol, the influence of methylation on the restriction activity is unknown (16).

P, Partial digestion (see text).
ditions affording a separation of MeA and MeC from the corresponding unmodified bases and from other purines and pyrimidines.

Bases were quantitated by spectrophotometric measurements of HPLC eluates, using standard samples as references (Fig. 1A). The mol% G+C values found in our experiments were in reasonable agreement with published values for Anabaena sp. strain PCC 7120 (42.5%), Plectonema sp. strain 594 (48%), and S. cedrorum R2 (56%) (12). Modified adenine and cytosine in cyanobacterial-DNA hydrolysates were identified by their retention times (Fig. 1B, C, and D) and by their absorption spectra, recorded during elution (data not shown). Their relative amounts are given in

![Image of HPLC separations](image-url)

**FIG. 1.** Fractionation of cyanobacterial DNA by HPLC of standard mixture of bases (5 mmol of hydroxymethyluracil, uracil, thymine, guanine, cytosine, adenine, MeC, and MeA) (a); hydrolysate of 100 µg of Anabaena variabilis DNA (b); Synechococcus DNA hydrolysate (c); and P. boryanum DNA hydrolysate. Spectrophotometric monitoring of the eluate was performed. A_{260} is shown on the ordinate, and elution time (minutes) is shown on the abscissa.

**FIG. 2.** Restriction patterns of cyanobacterial DNAs treated with isoschizomeric enzymes recognizing GATC sequences. Samples were treated with restriction enzymes or were untreated. After being labeled by nick translation (DNA polymerase I), the samples were subjected to agarose gel electrophoresis and autoradiography. Lanes: 1. λ HindIII on 1% agarose gel; 2, 3, and 4. Anabaena DNA untreated (lane 2) or treated with DpnI (lane 3) or MboI (lane 4) on 1% agarose gel; 5. Anabaena DNA, treated with Sau3A; 6 and 7, Plectonema DNA treated with DpnI (lane 6) or Sau3A (lane 7); 8 and 9, Synechococcus DNA treated with DpnI (lane 8) or Sau3A (lane 9); 10. λ HindIII; 11 and 12, untreated Plectonema DNA (lane 11) and S. cedrorum R2 DNA (lane 12) on 1.2% agarose gel.

Table 2. The three chosen cyanobacteria vary widely in their contents of methylated adenine and cytosine.

The calculated amounts of methylatable residues within two sequences, GATC and CCGG, recognized by the E. coli dam and dcm methylases, respectively, are also shown in Table 2. These values were obtained by the assumption of a random distribution of these sites along the genome. For Anabaena DNA, e.g., with a G+C content of 42.5%, there is a 21.25% probability for a randomly chosen base to be a G or a C and a 28.75% chance for it to be an A or a T. The probability of appearance of a GATC sequence is the product of the above values, i.e., 0.21252 × 0.28752 = 0.003732. The reverse of this probability yields the average distance separating two GATC sites: 268 base pairs (bp). Similar calculations were used for the CCGG sequence (Table 2) and other sequences (see Discussion). As revealed by the comparisons (Table 2), Anabaena DNA has a content of MeA (0.78% of the adenine content) which is not enough to account for the methylation of all GATC sequences, amounting to 1.30% of total adenine. This observation led to experiments described below.

Restriction patterns of cyanobacterial DNAs treated with isoschizomeric enzymes recognizing GATC sequences. Because of the results of experiments discussed above, Anabaena DNA restricted by DpnI, Sau3A, and MboI was submitted to gel electrophoresis to evaluate the average size of the restriction fragments (fragments of discrete size in Fig. 2, lanes 3, 6, and 8 will be dealt with further below). DpnI yielded fragments with an average size of 1,200 bp (Fig. 2, lane 3), in comparison with molecular size markers (Fig. 2, lane 1). A fivefold increase of DpnI yielded identical results, excluding partial hydrolysis of DNA. On the other hand, Sau3A yielded fragments of about 300 bp (Fig. 2, lane 5).
TABLE 2. Amounts of MeA and MeC in cyanobacterial-DNA hydrolysates and comparison with the calculated amounts, corresponding to dam- and dcm-methylated sequences

<table>
<thead>
<tr>
<th>Strain</th>
<th>MeA</th>
<th>Expected in GATC sequence</th>
<th>MeC</th>
<th>Expected in CC↓GG sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabaena sp. strain PCC 7120</td>
<td>0.78 ± 0.34 (6)</td>
<td>1.30</td>
<td>4.28 ± 1.96 (6)</td>
<td>0.55</td>
</tr>
<tr>
<td>Plectonema sp. strain 594</td>
<td>2.80 ± 0.82 (4)</td>
<td>1.50</td>
<td>2.90 ± 1.40 (4)</td>
<td>0.72</td>
</tr>
<tr>
<td>S. cedrorum R2</td>
<td>3.49 ± 2.05 (3)</td>
<td>1.72</td>
<td>1.30 ± 1.01 (3)</td>
<td>0.97</td>
</tr>
</tbody>
</table>

* Values are percentages of total adenine or cytosine ± standard deviation. The numbers of experiments are given in parentheses.

- The presence of one cytosine residue within the CC↓GG sequence was calculated as a percentage of total cytosine, as described in footnote b. (The dcm methylase of E. coli methylates only the internal cytosine of the sequence [33].)

Since hemimethylated and unmethylated GATC sequences are not cleaved by DpnI, this observation confirms that the Anabaena dam methylase might be limiting, providing a reduced number of fully methylated GATC sequences.

The amount of MeA in Plectonema and Synechococcus DNAs is well in excess of the calculated amount of adenine contained in GATC sequences which thus should be fully methylated. Both DNAs, treated with DpnI (Fig. 2, lanes 6 and 8) and Sau3A (lanes 7 and 9), yielded fragments of similar average size (about 300 bp). This value is close to the predicted size, suggesting that all GATC sites are indeed methylated.

In the course of these experiments, discrete bands were consistently seen in DpnI-treated cyanobacterial DNA samples. Anabaena DNA yielded a band corresponding to an average size of ca. 20,000 bp (Fig. 2, lane 3). Plectonema DNA (lane 6) yielded a fragment of 1,500 bp, whereas Synechococcus DNA yielded two fragments (10,000 and 1,500 bp). Longer incubation times or a fivefold increase of DpnI did not alter their mobilities, suggesting that they were not the result of partial hydrolysis. We conclude that they are discrete nucleotide sequences, lacking MeA within their GATC sites. These sequences contain GATC sites, since they were cleaved by Sau3A (Fig. 2, lanes 5, 7, and 9).

DISCUSSION

The genomes of three representative cyanobacterial strains were fully or partially resistant to restriction endonucleases from nonphotosynthetic bacteria (Table 1), as reported previously (18). This could have resulted from methylation, preventing cleavage by restriction enzymes, or to the absence or paucity of recognition sequences for certain endonucleases. In an effort to discriminate between these two possibilities, the amounts of modified bases were determined.

Although the values are different for the three cyanobacterial DNAs, as a whole they show a high content of both modified bases. This was concluded by comparing the present data with a survey of bacteria belonging to various taxonomic groups (35). E. coli C, for example, contains 2.09 mol of MeA and 0.95 mol of MeC per 100 mol of the corresponding unmodified base. The content of MeC seems to be strikingly high in Anabaena and Plectonema DNA (well above the highest value of 1.94 mol of MeC per 100 mol of cytosine reported in the cited study).

Both dcm- and dam-like methylases contribute to the presence of these modified bases, as indicated by the restriction patterns of all three cyanobacterial DNAs (Table 1, no. 1 to 3, 22, and 23). Their contribution to the contents of MeA and MeC was therefore calculated (Table 2). The MeC content of all three DNAs is well in excess of what is required for the methylation of the dcm sequence. For Anabaena DNA, however, the measured amount of MeA was below the amount expected in GATC sequences. The size of the Sau3A-generated fragments (ca. 300 bp) is close to the calculated size of fragments (268 bp), suggesting that GATC sequences are randomly distributed. DpnI, on the other hand, yielded fragments with an average size of 1,200 bp (Fig. 2, lane 3). This suggests that one of four GATC sequences is methylated on both strands, which is required for DpnI cleavage (17). If the other GATC sequences are unmethylated, they would be cleaved by MboI. In fact, MboI was unable to hydrolyze Anabaena DNA (Fig. 2, lane 4). We now believe that these potential MboI cleavage sites are hemimethylated, rendering them refractory to the action of this enzyme. This inference is substantiated by the quantitative estimation of MeA in Anabaena DNA (0.78% of the adenine content [Table 2]). Indeed, this value is close to the amount of MeA calculated on the assumption that of four randomly distributed GATC sites (corresponding to 1.30% of total adenine), one sequence is fully methylated and three are hemimethylated (respectively, 0.32% + 0.49% = 0.81% MeA). The intracellular level of the Anabaena dam methylase might thus be sufficient to fully methylate only a fraction of the GATC sequences. This enzyme might become even more limiting for methylation of GATC sequences of extrachromosomal DNA, as has been found for the genome of virus N-1 (work in progress). This observation is analogous to undermethylation patterns of plasmid pBR322 and bacteriophage λ observed under conditions of extensive replication in which the methylation level in E. coli becomes limiting (32).

We further attempted to evaluate the amount of methylated residues contributed by site-specific methylases, the counterparts of restriction enzymes in cyanobacteria. Therefore, it was assumed that methylases of this type, including a recently described cyanobacterial methyltransferase (15), modify a single residue, within the same sequence as the one recognized by the corresponding restriction enzyme (3, 10). In Anabaena sp. strain PCC 7120, these methylases supposedly modify only cytosine residues, since the measured amount of MeA was lower than what would be expected from dam methylation alone (Table 2). Based on the three known sequence-specific endonucleases of Anabaena sp. strain PCC 7120 (7), methylation of cytosine within these sequences was calculated to be 0.87% (for a measured amount of 4.28 MeC residues per 100 cytosines; Table 2). This strain, assigned to the same section of cyanobacteria as is Anabaena sp. strain PCC 7120 (12), contains five sequence-specific endonucleases (one is an isoschizomer to
Aval) (25). If one cytosine residue of each sequence is methylated, we calculated that this would amount to 2.39 MeC residues per 100 cytosines.

The measured amount of MeC in Anabaena sp. strain PCC 7120 could thus be partly accounted for by both dem methylase and methylases corresponding to known restriction endonucleases (6, 7, 25, 36). Of course, other restriction endonucleases might still be discovered, entailing the presence of additional methylases.

The inability of BamHI, BglII, and PvuI to cleave Anabaena sp. strain PCC 7120 DNA (Table 1, no. 4 to 6) was unexpected since these enzymes share an identical central tetranucleotide sequence, which itself is cleaved (by Sau3A; Table 1, no. 3). Most probably, these sequences are not present in the Anabaena genome, as already suggested (13). It is puzzling that the same enzymes are unable to cleave several bacterial DNAs (3).

Finally, we want to comment on the nature of discrete fragments resisting DpnI cleavage in the cyanobacterial DNA preparations (Fig. 2, lanes 3, 6, and 8). We hypothesize that they might originate from plasmids, which have been found in filamentous, as well as in unicellular, cyanobacteria (19, 20, 26, 28, 29). (The Plectonema DNA preparations [Fig. 2, lane 11] yielded a band with a lower electrophoretic mobility than that of the main chromosomal sequences.) Since we could show dam methylation to be limiting in Anabaena sp. strain PCC 7120 (Table 2; Fig. 2), it is reasonable to assume that this undermethylation would more specifically affect extrachromosomal elements (plasmid and viral DNAs). This might be a general pattern for cyanobacterial genes. This observation stresses the uniqueness of DpnI as a molecular tool to probe dam methylation, with its multiple roles and evolutionary relationships in different bacteria (1, 2, 11, 21, 31).

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