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

RABINDRANATH N. PADHY,† FRANÇOISE G. HOTTAT, MARC M. COENE, AND PHILIPPE P. HOET*

*Microbiology and Genetics Unit, International Institute of Cellular and Molecular Pathology,
University of Louvain Medical School, 1200 Brussels, Belgium*

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 isoschizomeric 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 isoschizomeric 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 $CaCl_2 \cdot 2H_2O$ in place of $Ca(NO_3)_2 \cdot 4H_2O$]. 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-isoamyl 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}).

---

* Corresponding author.

† Present address: Department of Botany, Khallikote College, Berhampur 760001, India.

Restriction endonucleases. The enzymes *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hae*III, *Hind*III, and *Hpa*II 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 UV 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 N_2. The digest was lyophilized and dissolved in 50 to 80 μl of 50 mM $NH_4H_2PO_4$.

Hydrolysates were fractionated on a cation-exchange column (Partisil 10 SCX; Whatman) at 20°C with a mobile phase of 50 mM $NH_4H_2PO_4$ 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 nonheterocystic 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 *Cla*I, confirming the presence of MeA within GATC sequences. MeA could occur in sequences other than GATC, as suggested by the inability of *Pst*I (Table 1, no. 8) to cleave *Anabaena* DNA.

The internal cytosine of the sequence $CC_{\overline{T}}^{A}GG$ is methylated 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 *dcm* 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 *Sau*961 (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 (*Ava*II, *Afl*I, and *Nsp*7524 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 con-

TABLE 1. Restriction enzyme cleavage of cyanobacterial DNA

No.	Restriction enzyme	Recognition sequence[a]	Cleavage of DNA of:		
			Anabaena sp. 7120	*Plectonema* sp. strain 594	*S. cedrorum* R2
1	*Dpn*I	G$\overset{M}{A}$TC	+	+	+
2	*Mbo*I	GATC	−	−	−
3	*Sau*3A	GATC	+	+	+
4	*Bam*HI	GGATCC	−		P[b]
5	*Bgl*II	AGATCT	−	P	P
6	*Pvu*I	CGATCG	−		
7	*Cla*I	ATCGAT	P	P	P
8	*Pst*I	CTGCAG	−		+
9	*Sal*I	GTCGAC	−		+
10	*Hpa*I	GTTAAC	+	+	
11	*Hind*III	AAGCTT	+		P
12	*Eco*RI	GAATTC	+		+
13	*Alu*I	AGCT	+		+
14	*Hpa*II	CCGG	+	+	+
15	*Hae*III	GGCC	−	P	+
16	*Hha*I	GCGC	+		+
17	*Taq*I	TCGA	+		+
18	*Fnu*4HI	GCNGC	+		+
19	*Sau*961	GGNCC	P		+
20	*Sma*I	CCCGGG	−		P
21	*Kpn*I	GGTACC	−		P
22	*Bst*NI	$CC_{\overline{T}}^{A}GG$	+	+	+
23	*Eco*RII	$CC_{\overline{T}}^{A}GG$	−	−	−

[a] Recognition sequences are specified for the 5′ → 3′ strand. $\overset{+}{A}$ or $\overset{+}{C}$, Inhibition of an endonuclease by an MeA or MeC residue within the recognition sequence; $\overset{M}{A}$, MeA residues are a prerequisite for the activity of *Dpn*I; $\overset{o}{A}$ or $\overset{o}{C}$, 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).

[b] 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
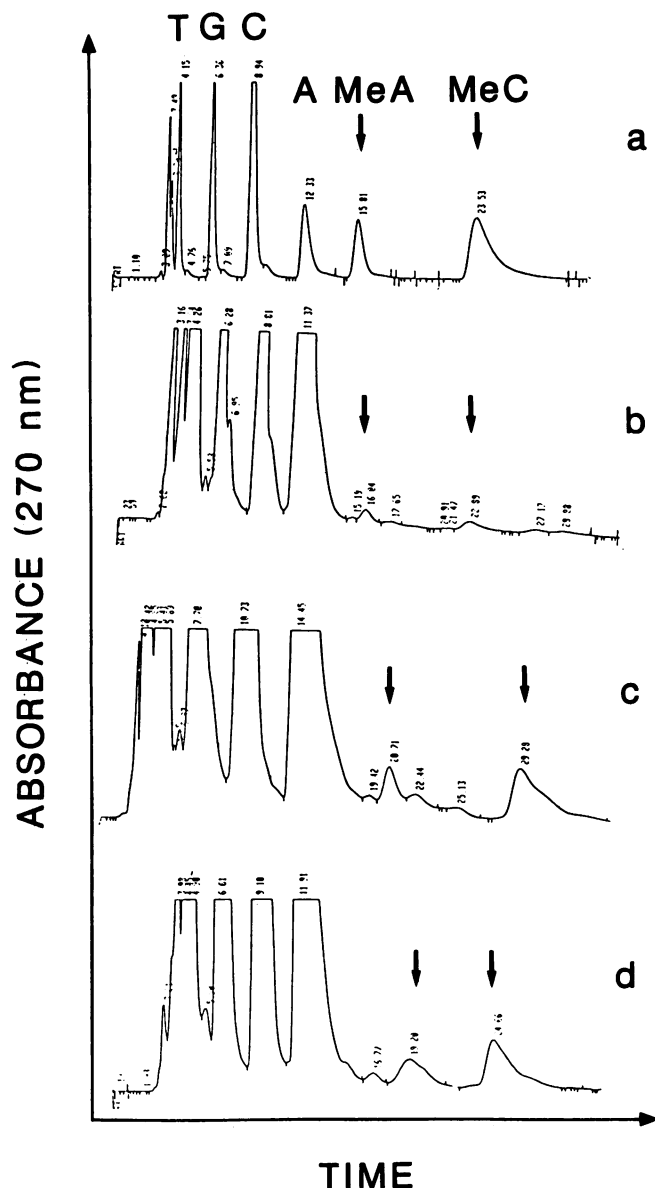

FIG. 1. Fractionation of cyanobacterial DNA by HPLC of standard mixture of bases (5 mmol of hydroxymethyluracil, uracil, thymine, guanine, cytosine, adenine, MeC, and MeA) (a); hydrolysate of 100 μg of *Anabaena variabilis* DNA (b); *Synechococcus* DNA hydrolysate (c); and *P. boryanum* DNA hydrolysate. Spectrophotometric monitoring of the eluate was performed. A_{270} is shown on the ordinate, and elution time (minutes) is shown on the abscissa.
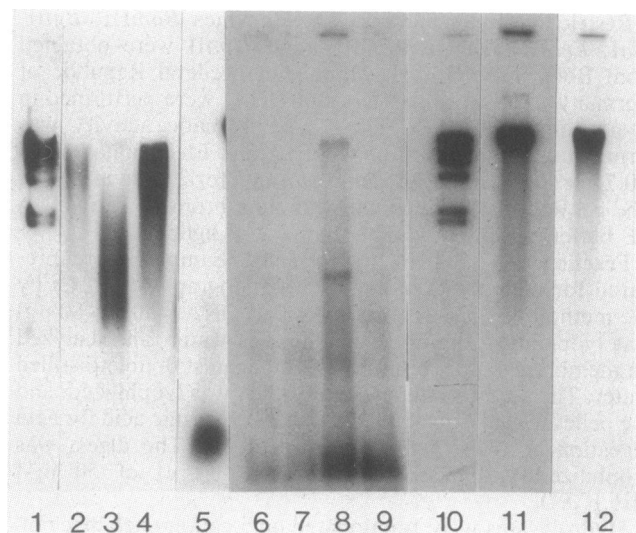

FIG. 2. Restriction patterns of cyanobacterial DNAs treated with isoschizomeric enzymes recognizing GATC sequences. Samples were treated with restriction enzymes or were untreated. After being labeled by nick translation (DNA polymerase I), the samples were subjected to agarose gel electrophoresis and 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 CC^A_TGG, 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., $0.2125^2 \times 0.2875^2 = 0.0037324$. The reverse of this probability yields the average distance separating two GATC sites: 268 base pairs (bp). Similar calculations were used for the CC^A_TGG 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

Strain	MeA		MeC	
	Expl[a]	Expected in GATC sequence[b]	Expl[a]	Expected in CC$\frac{A}{T}$GG sequence[c]
Anabaena sp. strain PCC 7120	0.78 ± 0.34 (6)	1.30	4.28 ± 1.96 (6)	0.55
Plectonema sp. strain 594	2.80 ± 0.82 (4)	1.50	2.90 ± 1.40 (4)	0.72
S. cedrorum R2	3.49 ± 2.05 (3)	1.72	1.30 ± 1.01 (3)	0.97

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

[b] Values are percentages of total adenine contained in GATC sequences, assuming their random distribution within genomes, having given percent G+C contents (see the text).

[c] The presence of one cytosine residue within the CC$\frac{A}{T}$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 *Dpn*I, 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 *Dpn*I (Fig. 2, lanes 6 and 8) and *Sau*3A (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 *Dpn*I-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 *Dpn*I 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 *Sau*3A (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 methylations, 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 *Sau*3A-generated fragments (ca. 300 bp) is close to the calculated size of fragments (268 bp), suggesting that GATC sequences are randomly distributed. *Dpn*I, 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 are methylated on both strands, which is required for *Dpn*I cleavage (17). If the other GATC sequences are unmethylated, they would be cleaved by *Mbo*I. In fact, *Mbo*I was unable to hydrolyze *Anabaena* DNA (Fig. 2, lane 4). We now believe that these potential *Mbo*I 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 methylase 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). Another 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

*Ava*I) (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 *dcm* 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 *Bam*HI, *Bgl*II, and *Pvu*I 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 *Sau*3A; 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 *Dpn*I 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 *Dpn*I as a molecular tool to probe *dam* methylation, with its multiple roles and evolutionary relationships in different bacteria (1, 2, 11, 21, 31).

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Barbeyron, T., K. Kean, and P. Forterre.** 1984. DNA adenine methylation of GATC sequences appeared recently in the *Escherichia coli* lineage. J. Bacteriol. **160:**586–590.

2. **Brooks, J. E., R. M. Blumenthal, and T. R. Gingeras.** 1983. The isolation and characterization of the *Escherichia coli* DNA adenine methylase (*dam*) gene. Nucleic Acids Res. **11:**837–851.

3. **Brooks, J. E., and R. J. Roberts.** 1982. Modification profiles of bacterial genomes. Nucleic Acids Res. **10:**913–934.

4. **Buzby, J. S., R. D. Porter, and S. E. Stevens, Jr.** 1983. Plasmid transformation in *Agmenellum quadruplicatum* PR-6: construction of biphasic plasmids and characterization of their transformation properties. J. Bacteriol. **154:**1446–1450.

5. **Coene, M., and C. Cocito.** 1985. A microanalytical procedure for determination of the base composition of DNA. Eur. J. Biochem. **150:**475–479.

6. **De Waard, A., C. P. Van Beveren, M. Duyvesteyn, and H. Van Ormondt.** 1979. Two sequence-specific endonucleases from *Anabaena oscillarioides*. FEBS Lett. **101:**71–76.

7. **Duyvesteyn, M. G. C., J. Korsuize, A. de Waard, A. Vonshak, and P. Wolk.** 1983. Sequence-specific endonucleases in strains

8. **Eick, D., H. J. Fritz, and W. Doerfler.** 1983. Quantitative determination of 5-methylcytosine in DNA by reverse-phase high-performance liquid chromatography. Anal. Biochem. **135:**165–171.

9. **Haselkorn, R., J. W. Golden, P. J. Lammers, and M. E. Mulligan.** 1986. Developmental rearrangement of cyanobacterial nitrogen-fixation genes. Trends Genet. **2:**255–259.

10. **Hattman, S., H. van Ormondt, and A. de Waard.** 1978. Sequence specificity of the wild-type (*dam*⁺) and mutant (*dam*ʰ) forms of bacteriophage T2 DNA adenine methylase. J. Mol. Biol. **119:**361–376.

11. **Hattman, S., J. Wilkinson, D. Swinton, S. Schlagman, P. M. MacDonald, and G. Mosig.** 1985. Common evolutionary origin of the phage T4 *dam* and host *Escherichia coli dam* DNA-adenine methyltransferase genes. J. Bacteriol. **164:**932–937.

12. **Herdman, M., M. Janvier, J. B. Waterbury, R. Rippka, and R. Y. Stanier.** 1979. Deoxyribonucleic acid base composition of cyanobacteria. J. Gen. Microbiol. **111:**63–71.

13. **Herrero, A., J. Elhai, B. Hohn, and C. P. Wolk.** 1984. Infrequent cleavage of cloned *Anabaena variabilis* DNA by restriction endonucleases from *A. variabilis*. J. Bacteriol. **160:**781–784.

14. **Herrero, A., and C. P. Wolk.** 1986. Genetic mapping of the chromosome of the cyanobacterium, *Anabaena variabilis*. Proximity of the structural genes for nitrogenase and ribulose-biphosphate carboxylase. J. Biol. Chem. **261:**7748–7754.

15. **Karreman, C., N. Tandeau de Marsac, and A. de Waard.** 1986. Isolation of a deoxycytidylate methyl transferase capable of protecting DNA uniquely against cleavage by endonuclease R · *Aqu* I (isoschizomer of *Ava* I). Nucleic Acids Res. **14:**5199–5205.

16. **Kessler, C., P. S. Neumaier, and W. Wolf.** 1985. Recognition sequences of restriction endonucleases and methylases—a review. Gene **33:**1–102.

17. **Lacks, S., and B. Greenberg.** 1977. Complementary specificity of restriction endonucleases of *Diplococcus pneumoniae* with respect to DNA methylation. J. Mol. Biol. **114:**153–168.

18. **Lambert, G. R., and N. G. Carr.** 1984. Resistance of DNA from filamentous and unicellular cyanobacteria to restriction endonuclease cleavage. Biochim. Biophys. Acta **781:**45–55.

19. **Lau, R. H., and W. F. Doolittle.** 1979. Covalently closed circular DNAs in closely related unicellular cyanobacteria. J. Bacteriol. **137:**648–652.

20. **Lau, R. H., C. Sapienza, and W. F. Doolittle.** 1980. Cyanobacterial plasmids: their widespread occurrence, and the existence of regions of homology between plasmids in the same and different species. Mol. Gen. Genet. **178:**203–211.

21. **Mannarelli, B. M., T. S. Balganesh, B. Greenberg, S. S. Springhorn, and S. A. Lacks.** 1985. Nucleotide sequence of the *Dpn* II DNA methylase gene of *Streptococcus pneumoniae* and its relationship to the *dam* gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **82:**4468–4472.

22. **Padhy, R. N.** 1985. Cyanobacteria employed as fertilizers and waste disposers. Nature (London) **317:**475–476.

23. **Padhy, R. N., and P. K. Singh.** 1978. Genetical studies on the heterocyst and nitrogen fixation of the blue-green alga *Nostoc muscorum*. Mol. Gen. Genet. **162:**203–211.

24. **Razin, A., S. Urieli, Y. Pollack, Y. Gruenbaum, and G. Glaser.** 1980. Studies on the biological role of DNA methylation. IV. Mode of methylation of DNA in *E. coli* cells. Nucleic Acids Res. **8:**1783–1792.

25. **Reaston, J., M. G. C. Duyvesteyn, and A. de Waard.** 1982. *Nostoc* PCC7524, a cyanobacterium which contains five sequence-specific deoxyribonucleases. Gene **20:**103–110.

26. **Reaston, J., C. A. M. J. J. Van den Hondel, G. A. Van Arkel, and W. D. P. Stewart.** 1982. A physical map of plasmid pDU1 from the cyanobacterium *Nostoc* PCC 7524. Plasmid **7:**101–104.

27. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.

28. **Roberts, T. M., and K. E. Koths.** 1976. The blue-green alga *Agmenellum quadruplicatum* contains covalently closed DNA

circles. Cell 9:551–557.

29. **Simon, R. D.** 1978. Survey of extrachromosomal DNA found in the filamentous cyanobacteria. J. Bacteriol. 136:414–418.

30. **Stanier, R. Y., and G. Cohen-Bazire.** 1977. Photosynthetic prokaryotes: the cyanobacteria. Annu. Rev. Microbiol. 31:225–274.

31. **Sternberg, N.** 1985. Evidence that adenine methylation influences DNA-protein interactions in *Escherichia coli*. J. Bacteriol. 164:490–493.

32. **Szyf, M., K. Avraham-Haetzni, A. Reifman, J. Shlomai, F. Kaplan, A. Oppenheim, and A. Razin.** 1984. DNA methylation pattern is determined by the intracellular level of the methylase. Proc. Natl. Acad. Sci. USA 81:3278–3282.

33. **Urieli-Shoval, S., Y. Gruenbaum, and A. Razin.** 1983. Sequence and substrate specificity of isolated DNA methylases from *Escherichia coli* C. J. Bacteriol. 153:274–280.

34. **Van den Hondel, C. A. M. J. J., R. W. Van Leen, G. A. Van Arkel, M. Duyvesteyn, and A. de Waard.** 1983. Sequence-specific nucleases from the cyanobacterium *Fremyella diplosiphon*, and a peculiar resistance of its chromosomal DNA towards cleavage by other restriction enzymes. FEMS Microbiol. Lett. 16:7–12.

35. **Vanyushin, B. F., A. N. Belozersky, N. A. Kokurina, and D. X. Kadirova.** 1968. 5-Methylcytosine and 6-methylaminopurine in bacterial DNA. Nature (London) 218:1066–1067.

36. **Whitehead, P. R., and N. L. Brown.** 1982. *Aha*III: a restriction endonuclease with a recognition sequence containing only A:T basepairs. FEBS Lett. 143:296–300.

37. **Wolk, C. P., A. Vonshak, P. Kehoe, and J. Elhai.** 1984. Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. Proc. Natl. Acad. Sci. USA 81:1561–1565.