Characteristics of DNA and Multiple *rpoD* Homologs of *Microcystis* (*Synechocystis*) Strains

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The base compositions of DNAs from nine *Microcystis* strains, as determined by high-performance liquid chromatography, were 41 to 42 mol% G+C. Chromosomal DNAs derived from these strains were found to be extremely resistant to many restriction endonucleases, and a restriction analysis revealed the presence of a *dam*-like methylase or both *dam*- and *dcm*-like methylases in all of the strains examined. Genomic Southern hybridization in which a synthetic oligonucleotide probe (*rpoD* probe) was used showed that members of the genus *Microcystis* might have multiple *rpoD* homologs, and the hybridization signal patterns observed with the DNAs of *Microcystis aeruginosa* strains were different from each other.

Members of the genus *Microcystis* are unicellular colony-forming cyanobacteria (blue-green algae) which produce surface blooms in eutrophic water; some strains have been found to possess a peptide toxin, microcystin (13, 19). The taxonomy of *Microcystis* species traditionally has been based on colony morphology and cell arrangement (10). However, these morphological features are easily changed in culture (16). Rippka proposed that, as prokaryotes, cyanobacteria are divisible into five major groups (sections) on the basis of culture characteristics (16), and *Microcystis* strains have been classified as members of the *Microcystis* cluster of the genus *Synechocystis* (26). At the present time, data for chemical, genetic, and physiological characteristics based on the use of axenic cultures are required for the taxonomy of cyanobacteria. The importance of G+C contents in microbial classification has been established. However, the data available on the G+C contents of *Microcystis* clones are limited. Chromosomal DNAs from different filamentous and unicellular cyanobacteria have been shown to be surprisingly resistant to cleavage by a number of restriction endonucleases (5, 11, 15). This resistance could be due to the absence of specific sequences or to modification (methylation). Resistance of *Microcystis* DNAs to restriction endonuclease cleavage has been little examined. RNA polymerase holoenzymes contain the principal sigma factors that play a central role in the initiation of transcription (12). Tanaka et al. designed a synthetic oligonucleotide probe (*rpoD* probe) from an amino acid stretch conserved among the principal sigma factors in *Escherichia coli* and *Bacillus subtilis* (24) and, using it, detected four or five signals from the DNAs of *Anabaena* sp. strain PCC 7120, *Synechococcus* sp. strain PCC 7942, and *Synechocystis* sp. strain PCC 6803 by genomic Southern hybridization (23). Recently, we developed a technique for isolating axenic clones of *Microcystis* by using a combination of the agarose plate technique and two-step centrifugation and isolated nine axenic *Microcystis* clones (13, 18, 19). In this paper we describe the G+C contents, restriction endonuclease cleavage characteristics, and multiple *rpoD* homologs of the DNAs of *Microcystis* isolates.

Cultures. Axenic *Microcystis aeruginosa* strains K-17,

K-79, K-81, K-139, M-20, B-19, B-47, and S-77 and *Microcystis viridis* S-70 were isolated from Lake Kasumigaura, Ibaraki Prefecture, Japan (13, 18, 19). *Synechocystis* sp. strain PCC 7820 was provided by R. Rippka (Pasteur Institut, Paris, France), and *M. viridis* NIES-102 was obtained from the Microbial Culture Collection of the National Institute for Environmental Studies, Tsukuba, Japan. All cyanobacterial strains were grown in CB medium under continuous illumination (19).

DNA extraction. Cells at the late logarithmic growth phase were sonicated for 10 s with a water bath type of sonicator (200 W, 36 kHz) to disrupt the gas vacuoles, spun down by centrifugation at 8,000 × g for 10 min, and then washed in TES buffer (0.05 M Tris-HCl, 0.1 M NaCl, 0.05 M EDTA; pH 8.0). The washed cells (about 2 g [wet weight]) were resuspended in 5 ml of SET buffer (0.05 M Tris-HCl, 0.05 M EDTA, 25% [wt/vol] sucrose; pH 8.0) and centrifuged, and the resulting cell pellet was frozen at -80°C. After thawing at 30°C, the cells were suspended in 4 ml of SET buffer containing 8 mg of lysozyme (Sigma) per ml and incubated at 37°C for 30 min. The cells were lysed by adding 2% (final concentration) sodium dodecyl sulfate and incubating the preparation for 20 min at 60°C. The DNA was extracted from each lysate with an equal volume of phenol-chloroform (1:1, vol/vol) and then with chloroform-isoamyl alcohol (24:1, vol/vol). After the DNA was recovered by ethanol precipitation, it was incubated in 2 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) containing 200 µg of pancreatic RNase (Sigma) at 37°C for 30 min. After phenol and chloroform-isoamyl alcohol extractions, the DNA was spooled onto a glass rod during ethanol precipitation and rinsed with 70% ethanol. The spooled DNA was suspended in TE buffer and dialyzed against the same buffer. The UV absorbance spectra between 190 and 300 nm were as predicted for high-purity nucleic acid, and the ratios of A_{260} to A_{280} were 1.8 to 2.0.

DNA base composition. DNAs were hydrolyzed with nuclease P1 by using a DNA-GC kit (Yamasa Shoyu Co., Ltd., Choshi, Japan) (27). The standard solution and hydrolysate were analyzed with a Nihon Bunko high-performance liquid chromatography system (model 880 pump and model 875 UV detector; Nihon Bunko Co., Ltd., Tokyo, Japan) by using a Inertsil octyldecyl silane column (250 by 4.6 mm; GL Sciences, Inc., Tokyo, Japan) and 0.2 M ammonium dihy-

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TABLE 1. DNA base compositions and restriction enzyme cleavage of *Microcystis* strains

Restriction enzyme	Recognition sequence ^a	<i>M. aeruginosa</i> strains ^b								<i>M. viridis</i> strains ^b		<i>Synechocystis</i> sp. strain PCC 7820
		K-17	K-79	K-81	K-139	B-19	B-47	S-77	M-20	S-70	NIES-102	
	M											
<i>DpnI</i>	GATC	+	+	+	+	+	+	+	+	+	NT	NT
<i>MboI</i>	GATC	-	-	-	-	-	-	-	-	-	NT	NT
<i>Sau3AI</i>	GATC	+	+	+	+	+	+	+	+	+	NT	NT
<i>BstNI</i>	CCAGG	+	+	+	+	+	+	+	+	+	NT	NT
<i>EcoRII</i>	CCAGG	-	-	-	+	+	+	+	+	+	NT	NT
<i>BamHI</i>	GGATCC	-	-	-	-	-	-	-	-	-	NT	NT
<i>BglII</i>	AGATCT	-	-	-	-	-	-	-	-	-	NT	NT
<i>EcoRI</i>	GAATCC	-	-	-	-	-	-	-	-	-	NT	NT
<i>HindIII</i>	AAGCTT	+	+	+	+	+	+	+	+	+	NT	NT
<i>PstI</i>	CTGCAG	-	-	-	-	-	-	-	-	-	NT	NT
<i>SalI</i>	GTCGAC	-	-	-	-	-	-	-	-	-	NT	NT
<i>SmaI</i>	CCCGGG	-	-	-	-	-	-	-	-	-	NT	NT
<i>XbaI</i>	TCTAGA	+	+	+	+	+	+	+	+	+	NT	NT

^a Recognition sequences are specified for the 5'→3' strand. $\overset{+}{A}$ or $\overset{+}{C}$, inhibition of an endonuclease by a methyladenine or methylcytosine residue within the recognition sequence; $\overset{+}{A}$, methyladenine residues are a prerequisite for the activity of *DpnI*; $\overset{+}{A}$ or $\overset{+}{C}$, digestion of the DNA which is not influenced by methyladenine or methylcytosine residues. *SmaI* cuts C^{m5}CCGGG but not C^{m4}CCGGG (m5, 5-methylcytosine; m4, 4-methylcytosine). If an adenine or cytosine residue is not identified by a symbol, the influence of methylation on the restriction activity is not known (17). +, cleaved; -, not cleaved; NT, not tested.

^b The G+C contents of *M. aeruginosa* K-17, K-79, K-81, K-139, B-19, B-47, S-77, and M-20 are 42, 41, 41, 41, 41, 42, 42, and 41 mol%, respectively; the G+C contents of *M. viridis* S-70 and NIES-102 are 42 and 41 mol%, respectively; and the G+C content of *Synechocystis* sp. strain PCC 7820 is 42 mol%.

drogen phosphate as the mobile phase. The various *Microcystis* strains examined had fairly similar base compositions (41 to 42 mol% G+C), and there was no difference in G+C content between *M. aeruginosa* and *M. viridis* (Table 1). All measurements of G+C content used were mean values obtained from at least three individual digestions of DNA. The average, maximum, and minimum values were 0.92, 0.98, and 0.88, respectively, for the A/T ratio, 1.04, 1.08, and 1.00, respectively, for the G/C ratio, and 0.97, 1.00, and 0.94,

respectively, for the purine/pyrimidine ratio. These ratios suggest that the analytical method used was adequate for estimating the G+C contents of DNAs (7). The G+C content of the *Escherichia coli* K-12 DNA used as a control was 51 mol%, in agreement with the value reported previously (14). The G+C content of *Synechocystis* sp. strain PCC 7005 (*Microcystis* cluster) has been reported to be 45 mol% (26). Recently, Fahrenkug et al. reported that the G+C contents calculated from the 50% thermal denaturation temperature

FIG. 1. Restriction analysis of chromosomal DNAs prepared from *M. aeruginosa* K-81 (A) and S-77 (B). The DNA (2 μ g in 20 μ l of buffer) was digested with an enzyme (10 to 40 U per μ g of DNA) for 3 h at each optimum temperature, and the restricted DNA was analyzed by electrophoresis on an 0.8% agarose gel. Lanes 1, *HindIII* digest of DNA (molecular weight markers); lanes 2, untreated DNA; lanes 3, *DpnI* digests; lanes 4, *MboI* digests; lanes 5, *Sau3AI* digests; lanes 6, *BamHI* digests; lanes 7, *PstI* digests; lanes 8, *SalI* digests; lanes 9, *HindIII* digests; lanes 10, *EcoRI* digests; lanes 11, *EcoRII* digests.

FIG. 2. Southern hybridization analyses of genomic DNAs from *Microcystis* strains performed with the ^{32}P -labeled *rpoD* probe (A and C) and the ^{32}P -labeled *rpoD1* gene of *Synechococcus* sp. strain PCC 7942 (B and D). The DNAs in panels A and B were digested with *Xba*I, and the DNAs in panels C and D were digested with *Hind*III. After DNA blotting, the filters used in panels A and C were washed to remove the probe and then rehybridized with the cloned 2.4-kb *Eco*RI fragment containing the *rpoD1* gene of *Synechococcus* sp. strain PCC 7942 (panels B and D). All lanes except lanes 4 contained strains of *M. aeruginosa*; lanes 4 contained a strain of *M. viridis*. Lanes 1, strain K-139; lanes 2, strain K-17; lanes 3, strain B-19; lanes 4, strain S-70; lanes 5, strain K-81; lanes 6, strain M-20; lanes 7, strain B-47; lanes 8, strain K-79; lanes 9, strain S-77.

data for *Microcystis* sp. strains P1-15, C3-40, C5-34, and AK1-6 were 38, 41, 41, and 43 mol%, respectively (3). Both these data and our results show that the G+C contents of all currently characterized *Microcystis* strains range from 38 to 45 mol%.

Cleavage of *Microcystis* DNAs by restriction endonucleases. DNAs of *Microcystis* isolates were treated with type II restriction endonucleases and analyzed by gel electrophoresis (Fig. 1) (17). The DNAs were found to be fully resistant to a number of restriction endonucleases (Table 1). The presence of *dam*- and *dcm*-like methylases or site-specific methylase counterparts of the restriction systems in some cyanobacterial strains has been reported previously (2, 5, 11, 15, 25).

Since the DNAs of all nine *Microcystis* strains were hydrolyzed by *Dpn*I and *Sau*3AI, but not by *Mbo*I, *dam*-like methylases may be present in these strains (4). The results of a cleavage analysis of the DNAs in which two isoschizomeric enzymes, *Bst*NI and *Eco*RII, were used suggested that *M. aeruginosa* K-17, K-79, and K-81 had *dcm*-like methylases. Since restriction endonuclease activities were detected in these *Microcystis* strains (data not shown), members of the genus *Microcystis* might have site-specific methylase counterparts of the restriction system. On the other hand, the chromosomal DNA of an *Anabaena* strain might lack the recognition sites for certain endonucleases (6). However, it is not known whether the present members of the genus *Microcystis* lack some endonuclease recognition sites.

Multiple *rpoD* homologs. Southern hybridization with the *rpoD* probe (5'-GCTTGICIIATCCACCAIGTIGCITAIGT3') was performed as described previously (23, 24). A 20- μg portion of chromosomal DNA was completely digested with the restriction endonuclease, and the restricted DNA was analyzed by electrophoresis in an 0.8% agarose gel (13.5 by 13.5 cm). Three signals were detected in every *Xba*I-cleaved DNA sample, and three to five signals, including weak signal bands, were detected in *Hind*III-cleaved DNAs (Fig. 2A and C). Most of the hybridization signal patterns were different from each other; the exceptions were the patterns of *M. aeruginosa* K-17, K-79, and K-81. The signal patterns of both *Xba*I- and *Hind*III-cleaved DNAs of strains K-17, K-79, and K-81 were extremely similar. We are currently cloning and sequencing the DNA regions corresponding to the hybridization signals (1). Our results indicate that *M. aeruginosa* K-81 has at least four *rpoD* homologous genes. Four or five Southern hybridization signals with the *rpoD* probe were detected with the DNAs of the following three cyanobacterial strains: *Anabaena* sp. strain PCC 7120, *Synechococcus* sp. strain PCC 7942, and *Synechocystis* sp. strain PCC 6803 (low-G+C-content cluster) (23). The putative products of four *rpoD*-related genes of *Synechococcus* sp. strain PCC 7942 were very similar in basic structure to the principal sigma factors of eubacterial strains (23). Furthermore, one of the four *rpoD*-related genes (*rpoD1*) was very similar to the *sigA* gene of *Anabaena* sp. strain PCC 7120 and might be the functional homolog of the principal sigma factors (22). Therefore, Southern hybridization was performed with a 2.4-kb *Eco*RI fragment containing the *rpoD1* gene of *Synechococcus* sp. strain PCC 7942 as the probe (23). The 2.4-kb *Eco*RI fragment exhibited strong hybridization with one of the *rpoD* homologs from each *Microcystis* DNA digested with *Xba*I or *Hind*III (Fig. 2B and D). These observations indicate that one of the *rpoD* homologs of *Microcystis* strains was highly homologous with *rpoD1* of *Synechococcus* sp. strain PCC 7942. The results of a Southern hybridization analysis in which the *rpoD* probe was used suggest that cyanobacteria may generally have multiple *rpoD* homologs. However, the biological role of each *rpoD* homolog is not known.

The differences in the hybridization signal patterns among *M. aeruginosa* strains indicate that the genotypes of these strains may differ from each other. An analysis of alloenzyme divergence performed by Kato et al. showed that *M. aeruginosa* was highly polymorphic and that there were 12 different genotypes among the 19 strains examined (8). Furthermore, these authors reported that two morphotypes (large and small cell types) were recognized in *M. aeruginosa* on the basis of differences in average cell size and colony formation (8). On the other hand, Komárek reported that some strains of *M. aeruginosa* identified by Kato et al.

should belong to *Microcystis ichthyoblabe* on the basis of light microscopic features (9). Thus, the taxonomy of the genus *Microcystis* remains confused. Tanaka found that the signal patterns of the chromosomal DNA digests of 12 *Streptomyces* species after Southern hybridization with the *rpoD* probe were different from each other, but that those of *Streptomyces lividans* 66 and *Streptomyces coelicolor* A3(2), which is very closely related to *S. lividans*, were identical (21). Southern hybridization analysis in which the *rpoD* probe is used may thus be useful for the identification and classification of cyanobacterial species.

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