Restriction Pattern of the Nucleic Acid of *Synechococcus* sp. Cyanophage

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The nucleic acid of *Synechococcus* sp. cyanophage was identified as a double-stranded DNA by the result of digestion with enzymes such as exonucleases, DNase, RNase, and S1 nuclease, and by acridine orange staining. The cyanophage DNA was cleaved with several restriction endonucleases such as *Apa*I, *Bam*HI, *Bgl*II, *Hae*III, *Eco*RI, *Hin*dIII, *Pst*I, and *Sma*I, which produced sets of fragments with wide size distributions. Among the enzymes, *Eco*RI, *Hin*dIII, *Bam*HI, *Pst*I, and *Apa*I gave the clearest sets of bands on agarose gels and the fragment numbers for each were 12, 20, 29, 20, and 7, respectively. The sums of the sizes from *Bam*HI and *Pst*I digestions were estimated approximately 227± 4 kb, which are in agreement with the result of the pulsed field gel electrophoresis. This virus is thought to have the largest genome among those of known cyanophages, which corresponds to the largest head of 90 nm when compared with the head sizes of cyanophages discovered since 1963.

Key words: nucleic acid, restriction endonucleases, cyanophage

Cyanophages are viruses which attack photosynthetic cyanobacteria and are very similar to bacteriophage both in structure and their infection cycle (12). Because the photosynthesis of cyanobacteria is like that of higher plants, the alga-cyanophage system can be adapted as a model to study plant photosynthesis under viral infection (14). The possible uses of cyanophages for biological control and for cyanobacteria taxonomy in confusing state have been suggested since the discovery of these viruses were made by Dr. Safferman in 1963 (13). Nevertheless, much has yet to be learned in the genetics of the cyanophages.

Generally, the cyanophages that have been characterized possess a single piece of double-stranded DNA. The genome sizes of cyanophages which has been reported are 40 kb (A-4L, AN-23), 67 kb (A-1L, AN-10), 76~81 kb (LPP-1M, LPP-2), and 97 kb (SM-1) (1, 11, 12). Apart from several spontaneous mutants, neither mutagenesis nor recombination has been reported so far for cyanophages, although complementation and recombination in the temperature-sensitive mutants (LPP-2-spI) have been indicated in some work. The spontaneous mutants, reported for LPP cyanophage, include acriflavine resistance and rapid lysis rate. Host-range mutants of LPP cyanophages have also been isolated (14).

The cyanophage of *Synechococcus* sp. used throughout this study was isolated from a Baekwoon reservoir in Korea (7, 8) and studied in our laboratory. The differences of morphology, growth cycle, and physiological characteristics with those of AS-I which is the most related group with this phage have suggested that this virus is new (9, 10). This study has been attempted to primarily analyze the nucleic acid of the *Synechococcus* sp. cyanophage for the prospective study of the nature of regulatory process involved in the interaction with the host system at the molecular level.

Materials and Methods

Host cyanobacterium and cyanophage

The cyanobacterium used throughout this study was *Synechococcus* sp. and the cyanophage was isolated by Kim *et al.* (7, 8). *Synechococcus* sp. cells were grown in modified Hughes medium (2) illuminated with cool white fluorescence light of 40 μmol $s^{-1}m^{-2}$ at 28~30°C. For preparation of crude lysate, cells of 1 l grown to early exponential phase (10^7 cells/ml) were centrifuged at 6,

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Table 1. The volume activities of *Eco*RI, *Hin*dIII, *Bam*HI, *Bgl*II, *Apa*I, *Sma*I, *Xba*I, and *Pst*I

R.E.	Units											
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	6.0	7.0	8.0	10.0
*Eco*RI	−	−	±	±	±	±	±	±	±	±	+	+
*Hin*dIII	−	−	±	−	±	−	±	±	±	±	+	+
*Bam*HI	±	±	+	+	+	+	+	+	+	+	+	+
*Bgl*II	±	±	+	+	+	+	+	+	+	+	+	+
*Sma*I		+		+				+	+		+	+
*Apa*I		+		+				+	+		+	+
*Xba*I		−		±				±	+		+	+
*Pst*I		−		±				±	+		+	+

−: Not digested
±: Partially digested
+: Completely digested

600×g for 50 min and was infected at a multiplicity of infection (m.o.i.) of 0.01 after cell pellet was resuspended in two-fold concentrated modified Hughes medium of 60 ml (10). The host and virus suspension of 3 ml was mixed with 1.3% molten agar of 3 ml (top agar) and was overlayed on the bottom agar of modified Hughes medium. After incubating for 2~3 days in the above condition, the titer of virus stock was determined (5, 11).

Purification of cyanophage and the isolation of the nucleic acid of the virus

Crude lysate was clarified by the use of 0.2 μm pore size cellulose acetate membrane filter. Lysate was treated with DNase and RNase (2 μg/ml of each) at room temperature for 30 min in order to remove the nucleic acid of host and then chloroform was added to the final concentration of 0.2% (18). Viruses were pelleted by centrifugation for 2 hr at 160,000×g. The virus pellet was resuspended in 500 μl of CP buffer (5 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM NaCl, 10 mM N-tris (hydroxymethyl)-methyl-2-aminoethane sulfonic acid, pH 7.0). The virus suspension was retreated with 10 μg/ml of DNase and 5 μg/ml of RNase, and incubated at 37℃ for 1 hr to allow digestions of the nucleic acid of host to go to completion. DNA preparation was carried out according to the methods of Sambrook *et al.* (17).

Determination of the nucleic acid type

DNase and RNase were added to 1 μg of the cyanophage nucleic acid to determine whether the nucleic acid was DNA or RNA. Also, 1 μg of the cyanophage nucleic acid was treated with S1 nuclease and exonuclease III in order to investigate whether the genome is single- or double-stranded (6). Meanwhile, 3 μg of the cyanophage nucleic acid was treated with Na_2HPO_4 solution after staining with acridine orange and was examined using fluorescent microscope (Leitz DIALUX 20 EB, excitation wave length 350 nm, emission wave length 450 nm) (16). The enzymes were purchased from Boehringer Mannheim and was used according to the suggestions of the suppliers.

Volume activities of restriction endonucleases

Cyanophage DNA treated with several restriction endonucleases such as *Eco*RI, *Hin*dIII, *Bam*HI, *Bgl*II, *Sma*I, *Apa*I, *Xba*I, *Pst*I of each Units given in Table 1 were incubated at 37℃ for 1 hr. The reactions were then stopped by the addition of 0.5 M EDTA at a final concentration of 10 mM. The enzymes were purchased from Boehringer Mannheim and was used according to the suggestions of the supplier.

Agarose gel electrophoresis

One μg of cyanophage DNA was cleaved with each restriction enzyme to digest the DNA for the investigation above. The resulting fragments were analyzed by employing agarose gel electrophoresis (17). The concentrations of agarose and running conditions for 1 kb, 1~2 kb, and 20 kb fragments were 1.5%~2.0% at 1.5 V/cm for 5 hr 30 min, 0.8%~1.0% at 0.75 V/cm for 24 hr, 0.3%~0.4% at 0.75 V/cm for 24 hr or 48 hr, respectively.

In order to estimate the total size of the nucleic acid of the cyanophage, pulsed field gel electrophoresis (PFGE) was performed with a contour-clamped homogeneous electric field (CHEF) system (CHEF-Mapper, BIO-RAD) using 0.5X TBE as a running buffer at 15℃ (3). The concentration of agarose, field strength, angle, switch interval time, and running time were 1.0%, 6 V/cm voltage, 120°, 55 sec, and 15 hr, respectively.

The standards to determine the sizes of cleaved products were lambda DNA/*Hin*dIII marker (Poschochem), lambda DNA/*Eco*RI+*Hin*dIII marker (Promega), pGEM® DNA marker (Promega), high molecular weight DNA marker (BRL), ProMega-Markers® Delta 39 (Promega), and *Saccharomyces cerevisiae* DNA marker (BIO-RAD).

After completion of electrophoresis, the gels were stained with ethidium bromide (0.5 mg/ml), visualized with a UV transilluminator (LKB 2011 marcrowave), and photographed with a Polaroid camera system (SL II, Seoulin Scientific Co.) using a red filter. The sizes of fragments were determined by manual interpolation using a plot of log Mr vs. mobility constructed by the use of the standard fragments after image analysis (Bio-profil, Sulim Co.).

Results and Discussion

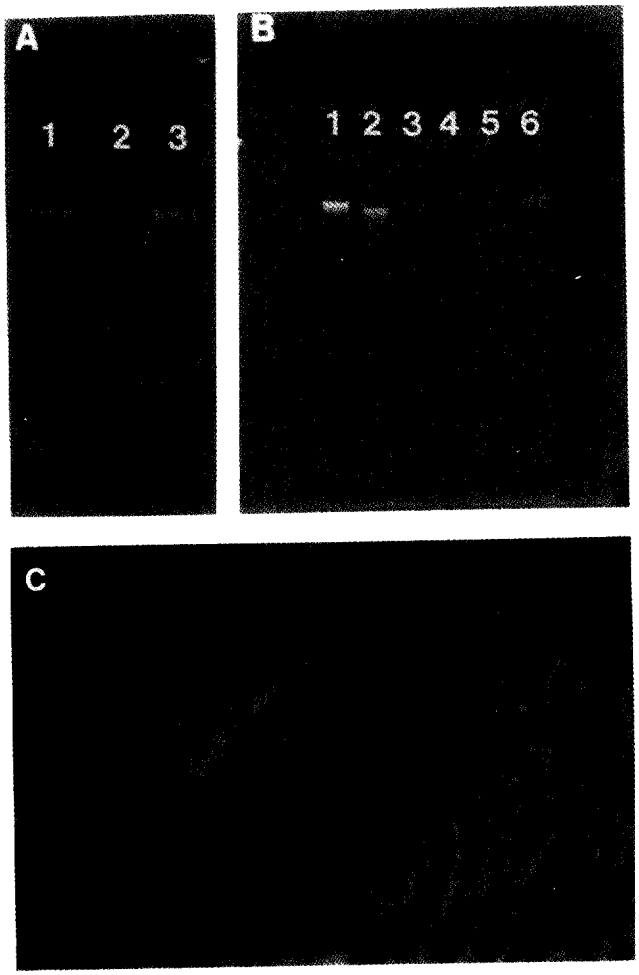

Fig. 1. The determination of the nucleic acid type of *Synechococcus* sp. cyanophage. (A) The cyanophage DNA treatment: +DNase (lane 2), +RNase (lane 3), negative control (lane 1). (B) The DNA was digestions: +nuclease S1 (lane 2), +exonuclease III for 120 min at 37℃ (lane 3), +exonuclease III for 90 min at 37℃ (lane 4), +exonuclease III for 60 min at 37℃ (lane 5), +exonuclease III for 30 min at 37℃ (lane 6), negative control (lane 1). (C) The virus DNA was stained with acridine orange and photographed by fluorescence microscope system (X250). The green color indicates that DNA is double-stranded.

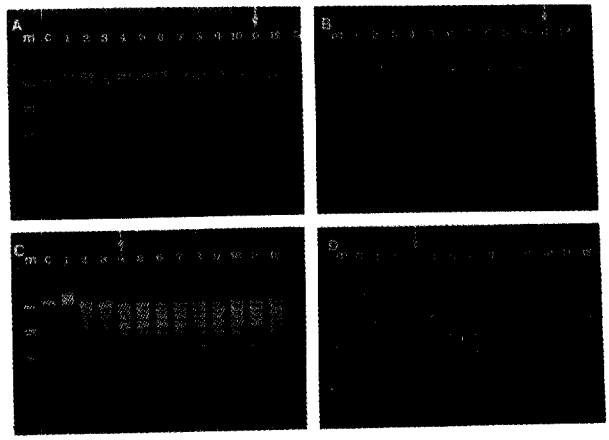

Fig. 2. The volume activities of *Eco*RI (A), *Hin*dIII (B), *Bam*HI (C), and *Bgl*II (D) on cyanophage DNAs. Lane m: DNA size markers of lambda DNA digested with *Eco*RI and *Hin*dIII, lane c: cyanophage DNA (control), lane 1~12: the cyanophage DNA treated with each Units of each R.E. Arrows indicate the cyanophage DNA were completely digested with each R.E. The DNAs were throughly cleaved when the Units of *Eco*RI and *Hin*dIII were 8 Units, and those of *Bam*HI and *Bgl*II were 1.5 Units.

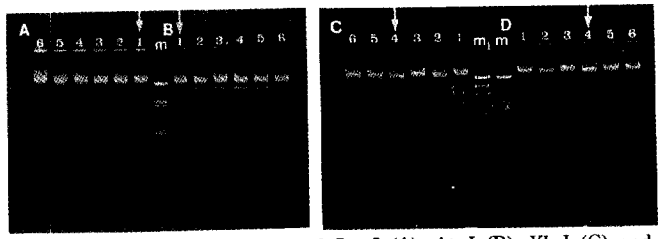

Fig. 3. The volume activities of *Sma*I (A), *Apa*I (B), *Xba*I (C) and *Pst*I (D) on cyanophage DNA. Lane m: DNA size markers of lambda DNA digested with *Eco*RI and *Hin*dIII, lane m_1: DNA size markers of lambda DNA cleaved with *Hin*dIII, lane 1~6: DNA treated with each Units of each R.E. Arrows indicate the cyanophage DNA completely digested by each R.E. The cyanophage DNAs were thoroughly cleaved when the Units of *Sma*I and *Apa*I were 1 Unit, and those of *Xba*I and *Pst*I were 1.5 Units.

Determination of the nucleic acid type of the *Synechococcus* sp. cyanophage

The nucleic acid of the *Synechococcus* sp. cyanophage was completely digested with DNase, showing no band on agarose gel and was not attacked by either RNase or nuclease S1. The treatment with exonuclease III gave more obscure bands as time went by (Fig. 1). In the meanwhile, the genome was stained with acridine orange in which double-stranded DNA has been reported to be dyed green under the short wave length, 260 nm, of ultraviolet and single-stranded DNA by dyed red (16). The result of the staining was green (Fig. 1). The above results showed that the nucleic acid of the virus is a double-stranded DNA. The presence of double-stranded DNA in cyanophages conforms with Bradley's prediction (4) that the head-tail bacteriophages contain only this type of nucleic acid.

Volume activities of restriction endonucleases

The nature of the DNA to be restricted is also important, since supercoiled or complex DNA may not be comparable to the lambda DNA used for unit definition (15). It is therefore advisable to titrate each enzyme preparation and recalculate the unit number before proceeding with experiments. The enzyme quantities needed for complete digestion of 1 μg of the phage DNAs are given in Table 1, Fig 2, 3.

Digestion with restriction endonucleases

The results of restriction endonuclease digestions are

Table 2. The sizes of cyanophage DNA fragments treated with *Eco*RI, *Hin*dIII, *Bam*HI, *Pst*I, *Hae*III, *Bgl*II, and *Apa*I

	*Eco*RI	*Hin*dIII	*Bam*HI	*Pst*I	*Hae*III	*Bgl*II	*Apa*I
1	50.7[a]	40.3	31.3	44.9	7.7	14.1	53.8[b]
2	21.5	24.7	19.2	40.5	5.9	13.1	11.3
3	16.7	21.9	18.5	29.1	5.6	10.7	6.6
4	8.2	19.3	13.5	20.6	4.9	8.3	6.1
5	6.8	14.8	12.5	17.7	4.2	7.8	4.2
6	6.2	12.1	11.3	15.3	3.8	6.0	2.0
7	5.2	11.7	10.3	11.8	3.7	5.7	1.6
8	4.7	6.5	9.6	12.4	3.3	5.5	
9	4.5	5.6	9.1	8.2	3.1	5.1	
10	3.2	4.4	8.5	7.9	3.0	4.9	
11	0.9	4.3	8.0	2.8	2.9	4.6	
12	0.7	3.5	7.6	2.6	2.8	4.5	
13		3.4	6.5	2.4	2.7	4.2	
14		2.9	6.1	2.0	2.4	3.8	
15		2.5	5.8	1.8	2.3	3.6	
16		2.3	5.4	1.5	2.3	3.6	
17		1.6	5.0	1.3	2.2	3.5	
18		1.4	4.6	1.1	2.2	3.3	
19		1.2	4.3	0.9	2.2	3.1	
20		0.9	4.2	0.8	2.2	3.0	
21			4.1		2.0	3.0	
22			3.8		1.9	2.8	
23			3.4		1.8[c]	2.8	
24			2.9			2.6	
25			2.7			2.5	
26			2.6			2.4	
27			2.3			2.2	
28			1.3			2.1	
29			0.6			2.0	
30						2.0	
31						1.9	
32						1.7[d]	
Sum			225.0	223.0			

[a,b]: Digested DNA fragments got out of the marker range and the size were calculated from image analysis system only, [c,d]: The smaller fragments were not determined due to band smearing.

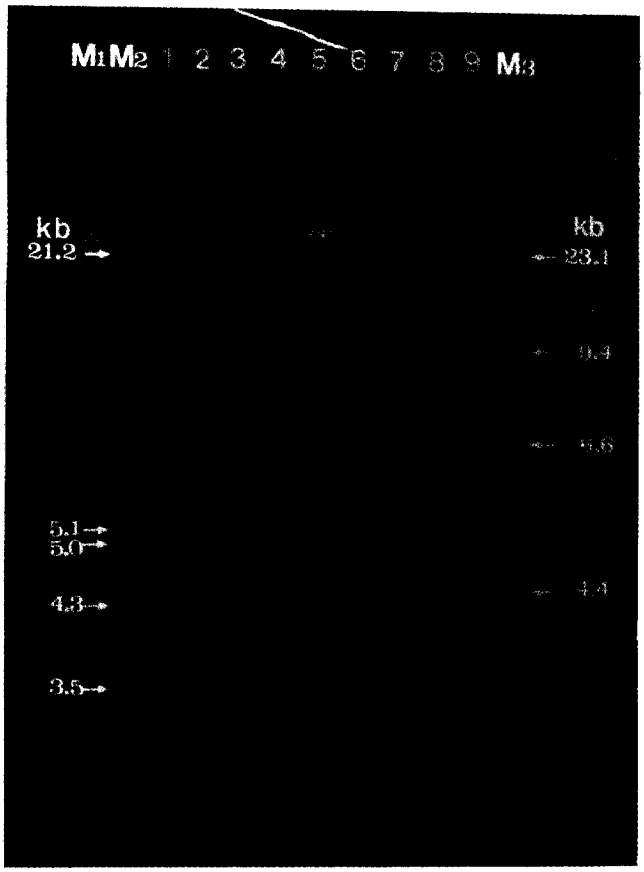

Fig. 4. Agarose gel (0.8%) electrophoresis of cyanophage DNA fragments digested with *Bgl*II (lane 1), *Xba*I (lane 2), *Kpn*I (lane 3), *Hae*III (lane 4), *Apa*I (lane 5), *Bam*HI (lane 6), *Pst*I (lane 7), *Hin*dIII (lane 8), and *Eco*RI (lane 9) for 45 hr 20 min at 0.75 V/cm. Lane M_1: high molecular weight DNA marker, lane M_2: DNA size markers of lambda DNA digested with *Eco*RI and *Hin*dIII, lane M_3: DNA size markers of lambda DNA treated with *Hin*dIII.

shown in Table 2, Fig. 4, 5. Because treatment with *Hae*III and *Bgl*II generated extremely varied fragment sizes with smearing on agarose gel, the exact numbers and sizes of the cleaved products could not be detected below 1 kb. *Bam*HI and *Pst*I digestion produced 29 and 20 fragments, respectively, from which the sums of the sizes of each fragment set was calculated to be 225 kb by *Bam*HI and 223 kb by *Pst*I. The total size of phage DNA was 231 kb by PFGE (Fig. 6). However, the genome size of the phage calculated from *Eco*RI and *Hin*dIII digestion were far below the values provided by the digestion with *Bam*HI, *Pst*I, and PFGE. As expected from the result of *Eco*RI and *Hin*dIII cleavage, some fragments might have migrated as doublets or beyond the upper limit of DNA marker. The genomes of IL-3A and NC-1A which are viruses of eukaryotic algae have been reported to be over 300 kb (19, 20). It has been known that the largest genome of cyanophage is the SM-1 DNA of 97 kb (15). However, this *Synechococcus* sp. cyanophage has been proved to have the largest genome of 227± 4 kb among cyanophages discovered so far and this value corresponds to the large size of the 90 nm phage head containing the nucleic acid, which is the largest size among the known cyanophages.

On the basis of this result and related data acquired by Kim *et al.* (8, 9, 10), it is thought that the genomic function of this cyanophage is more complicated than expected. Nevertheless, this outcome is thought to be helpful for further study of the phage DNA at the molecular level.

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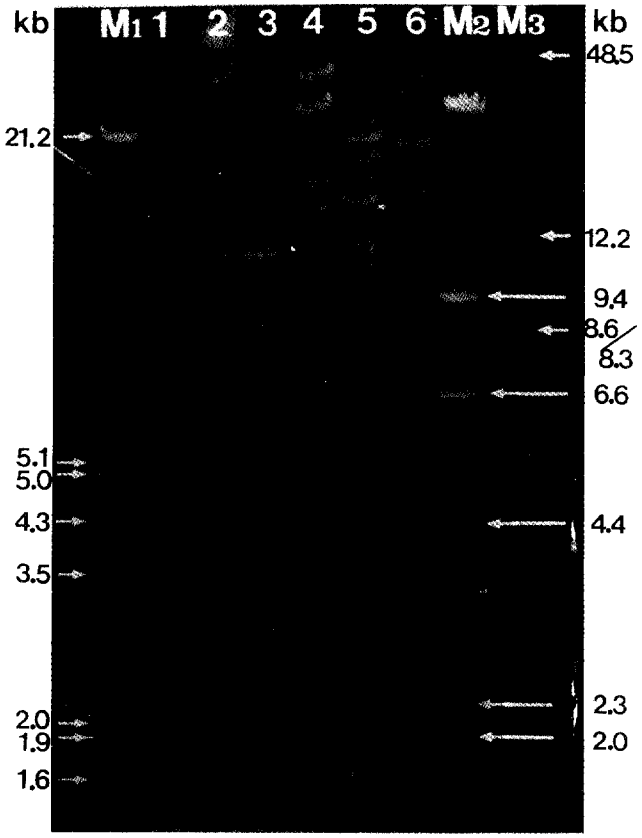

Fig. 5. Agarose gel (0.4%) electrophoresis of cyanophage DNA fragments digested with *Hae*III (lane 1), *Apa*I (lane 2), *Bam*HI (lane 3), *Pst*I (lane 4), *Hin*dIII (lane 5), and *Eco*RI (lane 6) at 0.75 V/cm for 24 hr. Lane M_1: DNA size markers of lambda DNA digested with *Eco*RI and *Hin*dIII, lane M_2: DNA size markers of lambda DNA treated with *Hin*dIII, lane M_3: high molecular weight DNA marker.

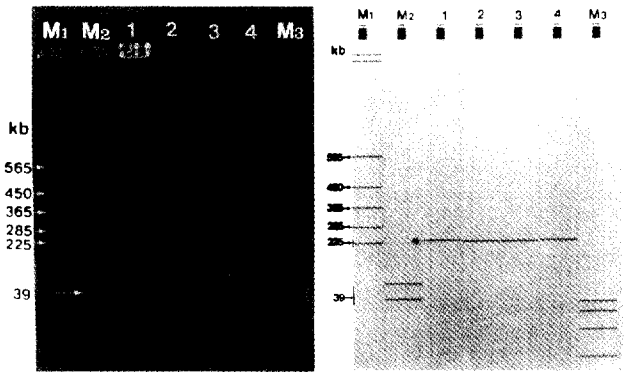

Fig. 6. PFGE of the cyanophage DNA (lane 1), and DNA fragments digested with *Not*I (lane 2), *Apa*I (lane 3), and *Sma*I (lane 4). Electrophoresis was performed for 24 hr on a agarose gel of 1.0% in 0.5x TBE at 15C at field strengths of 6 V/cm, and the switch interval of 55 sec. The size of cyanophage DNA was estimated 231 kb. *Not*I could not cut the cyanophage DNA. The fragments digested with *Apa*I, and *Sma*I were not shown here.

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