Identification of the Type of Nucleic Acid in Marine Bacteriophages with Acridine Orange Staining

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Identification of the Type of Nucleic Acid in Marine Bacteriophages with Acridine Orange Staining

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Abstract

The experiments were carried out the identification of the type of nucleic acid in test marine bacteriophages with acridine-orange staining. A total of 32 test phages have been isolated from sea water and marine mud samples. The acridine-orange staining and post-stain treatments were used the procedures described by BRADLEY (1966). The results were then confirmed with RNase and DNase digestion tests. It is found that a large majority of test marine phages cantain double-stranded DNA, and only one phage (06N–58P) of them is a single-stranded RNA-containing phage. There are no single-stranded DNA or double-stranded RNA-containing phages in the test ones. The host of 06N–58P is a marine *Pseudomonas* sp. The phage system is a new type of RNA-containing phage.

In the previous papers, the author isolated thirty-two strains of marine bacteriophages from sea water and marine mud samples collected from the several stations off the south of Kyushu, Japan, and investigated the morphological and biological character of the isolates (HIDAKA, 1971; HIDAKA and FUJIMURA, 1971a; HIDAKA and FUJIMURA, 1971b; HIDAKA, 1972; HIDAKA and ICHIDA, 1972; HIDAKA 1973; HIDAKA and SHIRAHAMA, 1974).

In the preliminary characterization of a bacteriophage it is also important to ascertain the type of nucleic acid which it contains as its genetic material. However, the biochemical analysis of nucleic acid in bacteriophage particles is a long and involved process. The use of the fluorescent stain with acridine orange for the quick identification of bacteriophage nucleic acids had been described by MAYOR and HILL (1961). Since the procedure of MAYOR and HILL had involved numerous treatments and the use of fluorescence microscope, BRADLEY (1966) have devised modifications which simplify them and avoid the use of the fluorescence microscope, an ordinary ultraviolet lamp being substituted. Also, it has been found that certain post-staining treatments cause colour changes which are related to the strandedness and type of nucleic acid and therefore are valuable confirmations of the normally used nuclease sensitivity test. With the new procedures, the type of nucleic acid contained in a bacteriophage can be definitely established with a very small quantity of suspension in a comparatively short time.

In this paper, the author dealt with the establish of the type of nucleic acid in the isolated marine bacteriophages with the BRADLEY's procedures.

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Materials and Methods

Bacteriophages. A total of 32 phage strains used in this experiments have been isolated from sea water and marine mud samples. The sources of them and their host bacteria are listed in Table 1. The procedures for the phage experiments; used media, phage propagation, phage assay etc., were essentially similar to that described previously (HIDAKA, 1971; HIDAKA, 1972).

Table 1. Soures and original host bacteria of test marine bacteriophages.

Phage	Host bacterium	Source	
		Location	Material
06N – 12P	*Flavobacterium* sp.		
06N – 21P	*Vibrio* sp.		
06N – 22P	*Vibrio* sp.		
06N – 24P	*Flavobacterium* sp.	31°04′N–130°35′E	Sea water
06N – 25P	*Pseudomonas* sp.		
06N – 34P	*Vibrio* sp.		
06N – 52P	*Pseudomonas* sp.		
06N – 58P	*Pseudomonas* sp.		
0XN– 32P	*Pseudomonas* sp.		
0XN– 36P	*Achromobacter* sp.		
0XN– 52P	*Vibrio* sp.		
0XN– 69P	*Vibrio* sp.	31°06′N–130°18′E	Sea water
0XN– 72P	*Vibrio* sp.		
0XN– 85P	*Vibrio* sp.		
0XN– 86P	*Vibrio* sp.		
0XN–100P	*Vibrio* sp.		
07T – 12P	*Pseudomonas* sp.	31°26′N–130°39′E	Marine mud
15N – 5P	*Pseudomonas* sp.		
15N – 11P	*Flavobacterium* sp.	31°00′N–131°03′E	Sea water
15N – 12P	*Pseudomonas* sp.		
25N – 12P	*Vibrio* sp.	31°07′N–130°38′E	Sea water
25N – 27P	*Achromobacter* sp.		
25N – 35P	*Pseudomonas* sp.	31°17′N–130°45′E	Sea water
25N – 42P	*Achromobacter* sp.		
25N – 61P	*Flavobacterium* sp.		
25N – 66P	*Aeromonas* sp.		
25N – 69P	*Pseudomonas* sp.	31°24′N–130°39′E	Sea water
25N – 70P	*Aeromonas* sp.		
25N – 73P	*Vibrio* sp.		
2ZT – 6P	*Vibrio* sp.		
2ZT – 33P	*Vibrio* sp.	30°26′N–131°11′E	Sea water
2ZT – 36P	*Vibrio* sp.		

Preparation of test specimens. The bacteriophage suspensions were prepareted according to the methods of ADAMS (1959) using both broth lysates and confluently lysed plates. Crude phage suspensions were treated with DNase and RNase at a

concentration of about 30 μg/ml for 1–2 hr at 37°C. The phages were concentrated and partially purified by alternate high-speed and low-speed centrifugation. Small droplets of a phage suspension (10^{10}–10^{12} particles/ml in phosphate-buffered saline, pH 7.2) are dried down on to microscope slides. The resulting spots were fixed in Cornoy's fluid (1 part glacial acetic acid, 3 parts chloroform; 6 parts ethanol) for 5 min at room temperature. They were then rinsed in absolute alcohol and dried in a stream of warm air.

Acridine-orange staining and post-stain treatments. The dried fixed slides were stained with acridine orange for 5 min in the following modified McIlvaine's buffer: 6 ml 0.1 M citric acid; 4 ml 0.15 M Na_2HPO_4; 0.1 ml 1% (w/v) acridine orange; pH 3.8. The slides were rinsed twice, briefly, in two separate baths of McIlvaine's buffer: 6 ml 0.1 M citric acid +4 ml 0.15 M Na_2HPO_4, pH 3.8, and soaked in 0.15 M Na_2HPO_4 for 15 min. Excess liquid was shaken off and the colours of the spots observed under U. V. radiation, wave-length 2537Å, from U. V. lamp. This treatment indicated whether the phage contained 2–DNA or 2–RNA on the one hand, or 1–DNA or 1–RNA on the other (see Table 2).

Further differentiation was achived as follows. A dish of 0.1 per cent molybdic acid solution was placed beneath the U. V. lamp. The slide from above was dipped in and out of the solution, the colour changes being continuously observed. The time required for the completion of these changes was between 15 sec and 90 sec. The spots of double-stranded nucleic acids remained the same colour, but those of the single-stranded types changed as described in Table 2, thus permitting their differentiation. Confirmation of the results was obtained as follows. A dish of 0.1 M tartaric acid was placed beneath the U. V. lamp. The another slide treated with 0.15 M Na_2HPO_4 was placed in the solution. Colour changes were observed over 2–5 min without removing the slide from the solution, which is transparent to U. V. radiation.

Digestion tests with Ribonuclease and Deoxyribonuclease. RNase and DNase digestion tests were a valuable conformation of results obtained with staining.

Ribonuclease. Digestion with RNase was done as follows. Carnoy-fixed spots were incubated for 2 hr at 37°C in 0.1% (w/v) RNase in modified McIlvaine's buffer at pH 3.8. The Controls were incubated in the buffer alone. The both control and treated spots were then stained in acridine orange in the normal way, and colours were observed under U. V. irradiation before and after Na_2HPO_4 treatment as described above. Spots which are susceptible to RNase, i.e. which give no colour after treatment, as compared with the control spot, will contain RNA and those resistant will contain DNA, with the exceptions of spots containing 2–RNA.

Deoxyribonuclease. DNase digestion was done as follows. Carnoy-fixed spots were incubated for 2 hr at 37°C in 0.02% (w/v) DNase in phosphate+acetate buffer (pH 5.5) added 0.003 M magnesium acetate to improve the efficiency of the DNase. The controls were incubated in the buffer alone. After removal, the slides were soaked in modified McIlvaine's buffer (pH 3.8) for 5–10 min and then stained as

described above. Colours were observed under U. V. radiation before and after treatment with Na_2HPO_4 solution. As with RNase digestion, appropriate susceptibility to DNase indicated the type of nucleic acid present.

BRADLEY (1966) has summarized the results of acridine-orange staining and nuclease digestion tests on various viral nucleic acids according to nucleic acid type. It is shown in Table 2.

Table 2. Colours for different types of nucleic acid.

Nucleic acid	Treatment				
	Na_2HPO_4	Molybdic acid	Tartaric acid	RNase	DNase
2-DNA	Green	Green	Orange	−	+
2-RNA	Green	Green (fading)	Red	−	−
1-DNA	Red	Paler green	Paler green	−	+
1-RNA	Red	Paler red	Paler red	+	−

+, sensitive; −, resistant (BRADLEY, 1966)

Results and Discussion

The results of the acridine-orange staining technique for identification of the type of nucleic acid contained in test marine bacteriophages are shown in Table 3. As shown in Table 3, a large majority of test specimens fluoresced a green under U. V. radiation after acridine-orange staining and post-stain treatment with phosphate, and one specimen (06N–58P) of them fluoresced a red 'flame-red' under same condition. By comparison it with Table 2, it is estimated that the former specimens contain double-stranded nucleic acids and the nucleic acid in the latter specimen is single-stranded type. According to the colour changes after the other post-stain treatments with molybdic acid and tartaric acid, it is obvious that the type of nucleic acid in 06N–58P is a single-stranded RNA, and those in the other 31 bacteriophages are double-stranded DNA.

RNase and DNase digestion tests to the specimens were carried out for confirmation of the results obtained with the staining. It is important to compare the colour obtained with the control spot with that of the treated spot. The susceptivilities of test specimens to nucleic acid enzymes are shown also in Table 3. As shown in Table 3, the specimen of 06N–58P is susceptible to RNase but not to DNase. On the other hand, the others are susceptible to DNase but not to RNase. These confirm the above results obtained with acridine-orange staining. In this experiments, the author found a new single-stranded RNA-containing marine bacteriophage in 32 isolates, but not any single-stranded DNA or double-stranded RNA-containing ones.

Only three species of bacteria have been reported to serve as hosts for RNA-containing bacteriophages: *Escherichia coli* (LOEB and ZINDER, 1961; BRADLEY, 1964), *Pseudomonas aeruginosa* (FEARY *et al.*, 1963), and *Caulobacter* species (SCHMIDT and

Table 3. Colours of acridine-orange staining test and susceptibility to RNase and DNase of test specimens.

Specimen	Post-staining treatment			Susceptibility to		Nucleic acid type
	After Na_2HPO_4	Molybdic acid	After tartaric acid	RNase	DNase	
06N – 12P	Green	Green	Orange	–	+	2–DNA
06N – 21P	Green	Green	Orange	–	+	2–DNA
06N – 22P	Green	Green	Orange	–	+	2–DNA
06N – 24P	Green	Green	Orange	–	+	2–DNA
06N – 25P	Green	Green	Orange	–	+	2–DNA
06N – 34P	Green	Green	Orange	–	+	2–DNA
06N – 52P	Green	Green	Orange	–	+	2–DNA
06N – 58P	Red	Paler red	Paler red	+	–	1–RNA
0XN– 32P	Green	Green	Orange	–	+	2–DNA
0XN– 36P	Green	Green	Orange	–	+	2–DNA
0XN– 52P	Green	Green	Orange	–	+	2–DNA
0XN– 69P	Green	Green	Orange	–	+	2–DNA
0XN– 72P	Green	Green	Orange	–	+	2–DNA
0XN– 85P	Green	Green	Orange	–	+	2–DNA
0XN– 86P	Green	Green	Orange	–	+	2–DNA
0XN–100P	Green	Green	Orange	–	+	2–DNA
07T – 12P	Green	Green	Orange	–	+	2–DNA
15N – 5P	Green	Green	Orange	–	+	2–DNA
15N – 11P	Green	Green	Orange	–	+	2–DNA
15N – 12P	Green	Green	Orange	–	+	2–DNA
25N – 12P	Green	Green	Orange	–	+	2–DNA
25N – 27P	Green	Green	Orange	–	+	2–DNA
25N – 35P	Green	Green	Orange	–	+	2–DNA
25N – 42P	Green	Green	Orange	–	+	2–DNA
25N – 61P	Green	Green	Orange	–	+	2–DNA
25N – 66P	Green	Green	Orange	–	+	2–DNA
25N – 69P	Green	Green	Orange	–	+	2–DNA
25N – 70P	Green	Green	Orange	–	+	2–DNA
25N – 73P	Green	Green	Orange	–	+	2–DNA
2ZT – 6P	Green	Green	Orange	–	+	2–DNA
2ZT – 33P	Green	Green	Orange	–	+	2–DNA
2ZT – 36P	Green	Green	Orange	–	+	2–DNA

STANIER 1965). These facts suggest that the natural phage populations may contain an usually small proportion of RNA-containing bacteriophages. The discovery of a new type of RNA-containing marine bacteriophage provides a somewhat broader basis for drawing conclusions about the properties of this interesting category of bacterial vivuses.

The host bacterium of 06N–58P is a marine *Pseudomonas* sp. Like the type which have been earlier described, the 06N–58P is small and apparently simple in structure. It shares the property of susceptibility to the action of RNase in the presence of host bacterium. More detailed study on the RNA-containing marine bacteriophage, 06N–58P will be reported another paper.

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