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Detection and Isolation of Marine Bacteriophage Systems in the Southwestern Part of the Pacific Ocean*

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Abstract

During the cruise for the marine ecological survey in the southwestern part of Pacific Ocean, 47 of sea water samples were obtained from various depth layers at 13 stations between Kagoshima and Guam via Palau. A total of 576 cultures of bacteria isolated from the sea water samples, of which 72 cultures were sensitive to the bacteriophage lysates enriched from the sea water. It is found that the bacteriophages are not present in high concentrations in the ocean, or occur only sporadically in water layers, from surface to 50 m depth, harboring large bacterial population.

The host bacteria of the isolated marine bacteriophage systems belong to *Pseudomonus* (2 strains), *Vibrio* (3 strains), *Photobacterium* (1 strain), and *Lucibacterium* (1 strain). These bacteriophage systems form clear or turbid plaque of about 1–2 mm in diameter. The all of them are virulent phage systems.

Bacteriophages could exert a considerable influence on controlling natural bacterial populations in limiting the numbers, types, and duration of active population growth and ultimately, through possible genetic exchange mechanisms, on the biochemical capabilities of bacteria. These phenomena are of considerable potential importance because of the prominent role of bacteria as mineralizing and chemical transforming agents within the environment. However, the information on marine bacteriophages is relatively scant. The ecological significance of bacteriophages in the marine environment is not known. The author has been isolating marine bacteriophages from sea water and marine mud samples collected from several stations in some ten miles off the south coast of Kyushu, Japan, and investigated the morphological and biological characters 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; HIDAKA, 1975; HIDAKA and ICHIDA, 1975). Now, it is very interesting to compare with marine bacteriophages in coast and in ocean. During the third cruise of KEITEN MARU as part of the special research project for the marine ecological survey in the southwestern part of Pacific Ocean, the author dealt with investigations into the ecological system of microorganisms, especially bacteriophage systems in the ocean. The present paper de-

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scribes the detection and isolation of marine bacterium-bacteriophage systems in sea water samples collected from the ocean.

Materials and Methods

Media used. The sea water broth (SWB) contained 5 g polypeptone and 1 g yeast extract in a liter of HERBST's artificial sea water. The pH of the medium was adjusted to 7.6–7.8. Solid media were prepared by adding agar at either 1.5% for sea water agar (SWA) or 0.5% for soft sea water agar (sSWA) to the sea water broth.

Sea water samples. The sea water samples were collected with steril bacteriological J–Z water samplers from five layers, 1–3, 50, 100, 200, and 300 m of depth, at each of 13 stations. The samples of water were immediately examined in a laboratory aboard.

Estimation and isolation of marine heterotrophic bacteria in the sea water samples. Most probable number technique was used for the estimating bacterial population in the sea water samples, using five SWB tubes for three sample sizes, 1 ml, 0.1 ml, and 0.01 ml, respectively. The tubes were incubated for 3 days and observed growth. The numbers of positive tubes in each set of five were tabulated and consulted the appropriate table. The most plobable number of bacteria for each sample was calculated for 100 ml of sample. On the other hand, a 0.2 ml of each sample was also cultured on a SWA plate for 6 days. Five plates were used for each sample to be tested. The colonies developed on the agar plates were counted, and then the viable cell counts per 1 ml of the samples were calculated. After that the all colonies were transferred to agar slopes of the same composition. The incubations were done in air-conditioned room temperature, 23–25°C.

Microbiological tests of isolated bacteria. The characterization tests of the host bacteria were done using standard methods (HARRING and MCCANCE, 1966). Identifications of them were carried out according to the systems outlined by HENDRIE and SHEWAN (1966) and BERGEY's Manual (8th ed). Incubations were at 25°C, unless otherwise stated.

Detection of bacteriophage. This was experimented according to the enrichment method. To each of sterile shaking flasks was added 200 ml of SWB together with 5 ml of SWB culture inoculated 1 ml of each sea water sample and incubated for 2–3 hours. After then the young culture was mixed with 250 ml of the sea water sample. The mixtures were incubated overnight, and were then put into a cold chamber to bring back to land. At the university laboratory, the cultures were centrifuged at 4,500 G for 30 min and filtered through HA Millipor membrane filters. The filtrates were spoted onto double agar layer plates seeded each of marine bacteria isolated. These preparations were incubated overnight and examined the appearance of lysis zone.

Isolation of the bacteriophages. Material from the center of clear zones developing on lawns was transferred by a platinum wire to fresh SWB cultures of the

appropriate bacterium and the inoculated cultures were incubated for a further 18 h at 25°C. Each of the cultures was then filtered and a portion of the filtrate was mixed with the appropriated bacterium in 3 ml of soft SWA melted and cooled to 45°C and the mixture was layered onto the surface of an agar plate. The double agar layer plates were incubated overnight. Single plaques arising by this method were picked and the cycle was repeated three times to ensure their identity and assist purification. The phage lysates were stored at 5–8°C.

Results and Discussion

In the cruise of the Keiten Maru from Kagoshima to Guam via Palau during the period from October 25 to November 19, 1976, microbiological samplings were carried out at 13 stations. The track and the microbiological stations are shown in Fig. 1 and Table 1.

Distribution of heterotrophic bacteria in the sea water. In sea water at various depths of the stations, the numbers of heterotrophic bacteria as host for marine bacteriophages are shown in Table 2. The numbers indicate viable cell counts

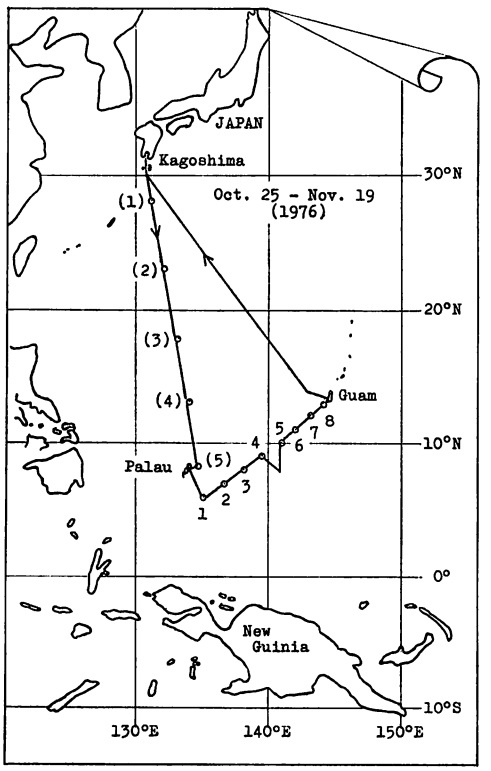

Fig. 1. The track and microbiological stations in the cruise of the Keiten Maru, 1976.

Table 1. Microbiological stations in southwestern part of the Pacific Ocean.

Station No.	Position		Date and time of sampling	
	Latitude	Longitude	Date (in 1976)	Time
(1)	27°46.5′N	131°12.0′E	Oct. 26	10:00 — 10:20
(2)	22°57.6′N	132°10.2′E	Oct. 27	10:00 — 10:20
(3)	18°12.6′N	133°03.3′E	Oct. 28	10:00 — 10:20
(4)	13°29.6′N	134°07.5′E	Oct. 29	10:00 — 10:20
(5)	08°43.0′N	134°46.0′E	Oct. 30	10:00 — 10:20
1	06°00.1′N	134°59.8′E	Nov. 4	19:30 — 21:00
2	07°00.0′N	136°36.4′E	Nov. 5	07:00 — 08:30
3	07°59.0′N	138°12.9′E	Nov. 5	19:00 — 20:30
4	09°00.4′N	139°50.6′E	Nov. 6	07:30 — 09:00
5	10°02.3′N	141°09.7′E	Nov. 7	11:30 — 13:00
6	11°00.3′N	142°06.4′E	Nov. 7	20:30 — 22:00
7	12°00.0′N	143°06.4′E	Nov. 8	07:30 — 09:00
8	13°00.0′N	143°56.0′E	Nov. 8	17:00 — 18:30

Table 2. Numbers of heterotrophic bacteria in sea water at various depths of the microbiological stations.

Station No.	Depth in meters									
	1–3		50		100		200		300	
	c.f.u.	M.P.N.	c.f.u.	M.P.N.	c.f.u.	M.P.N.	c.f.u.	M.P.N.	c.f.u.	M.P.N.
		$\times 10^2$		$\times 10^2$		$\times 10^2$		$\times 10^2$		$\times 10^2$
(1)	10	22	5	17	/	/	/	/	/	/
(2)	5	17	5	17	/	/	/	/	/	/
(3)	25	35	25	35	/	/	/	/	/	/
(4)	55	92	75	160	/	/	/	/	/	/
(5)	35	17	35	35	/	/	/	/	/	/
1	20	17	25	24	10	17	5	7	5	7
2	50	54	70	54	15	18	15	14	10	9.5
3	100	92	110	92	15	13	5	9.5	5	7.9
4	30	28	20	35	5	7.9	10	17	8	7
5	30	22	30	17	5	7	8	9.5	6	4.9
6	20	28	25	35	15	18	10	14	3	4.6
7	10	17	10	28	5	7	2	4.6	2	3.3
8	10	14	10	22	/	/	/	/	/	/

Key: c.f.u. = colony forming units per 1 ml of sea water sample
M.P.N. = Most Probable Numbers of bacteria per 100 ml of sea water sample
/ = not experimented

(colony forming units, c.f.u.) per 1 ml of sample and most probable number (M. P. N.) of bacteria per 100 ml of sample. The measured values by two defferent methods almost agree. It is decided in Table 2 that the heterotrophs are found at every depth below the surface to 300 m at the stations and usually more abundant at a depth of 50 m than in water near the surface. Even at a depth of 50 m, the range of the numbers of bacteria per ml of sea water is from a few to a hundred. The samples from the 100 m depth contain from 5 to 18 bacteria per ml. Beginning with the 100 m level, the number of bacteria gradually decrease with increasing depth. The

data recorded in Table 2 suggest that the bacterial population is generally most abundant in the photosynthetic zone near depth 50 m and then decreases with depth at various stations. Table 2 also shows that the quantity of heterotrophs is more abundant at three stations (4), 2, and 3 than at other stations. An increase in the numbers of heterotrophs usually occurred at the upper layers of water at the junction of currents, where the amount of organic substances was relatively high. One must assume that these conditions are reproduced to some extent at the interface of some layers in a water mass.

Detection of marine bacteriophage systems. The marine bacteriophages in the sea water samples were detected by lytic action for the bacteria isolated from the same samples. A total of 576 cultures of bacteria isolated from the sea water samples, of which 72 cultures were sensitive to the bacteriophage lysates enriched from the sea water. However, it was difficalt to detect of phage action to the isolated bacteria by direct method from the sea water samples. These 72 bacteriophage systems were divided broady into seven systems (tentative names: A, B, C, D, E, F, and G group) by host-phage cross infection method. The distribution of the seven marine bacteriophage systems in the every stations are shown in Table 3. As in

Table 3. Distribution of marine bacteriophage systems isolated.

Depth (meters) / Station No.	1–3	50	100	200	300
(1)	A, D	A, D	/	/	/
(2)	A, D, E	A, D, E	/	/	/
(3)	C, D	C, D	/	/	/
(4)	D, E	C, D, E	/	/	/
(5)	D	C, D	/	/	/
1	A, D, G	A, D	G	—	—
2	A, D, E	A, C, D, E	A	—	—
3	A, D, E	A, D, E	F	—	—
4	A, B, C	A, B, C, E	G	C	—
5	B, C, E	B, C, E, F	—	—	—
6	A, C	A, C, E	—	—	—
7	D	A, D	—	—	—
8	A, D	A, D	/	/	/

A and B, *Pseudomonas*; C, D and E, *Vibrio*; F, *Photobacterium*; G, *Lucibacterium*.
—, not detected; /, not experimented.

Table 3, bacteriophages are detected in the layers from surface to 50 m depth at all of the stations, and the composition of them is almost same in the layers, but it doesn't always follow that it is same at the every stations. In the layers below 100 m depth at the majority of the station bacteriophages are not found. It is indicated that the bacteriophage is not present in high consentrations in the ocean or that it occurs only sporadically in water layers harboring large bacterial populations.

Some characters of the isolated marine bacteriophage systems. The

Table 4. Brief characterization of the representative host bacterium strain of each seven groups.

Group	A	B	C	D	E	F	G
6YK-strain No.	3212	5201	1102	2202	3210	3306	1303
Cell form	R	R	R	R	R	R	R
Gram's stain	−	−	−	−	−	−	−
Flagellation	M	M	M	M	M	M	P
Kovacs' oxidase	+	+	+	+	+	−	+
Hugh & Leifson test	NC	O	F	F	F	F	F
Sensitivity to 0/129	−	−	+	+	+	+	−
Arginine dihydrolase	+	+	−	−	+	+	−
Gelatin hydrolysis	+	+	+	+	+	−	+
Starch hydrolysis	−	+	+	−	+	−	+
Growth in 7.5% NaCl	+	+	+	+	+	+	+
Growth at 37°C	−	−	−	−	−	−	−
Luminescence	−	−	−	−	−	+	+
Pigments	−	−	−	−	−	−	−
Indole production	−	−	−	−	+	−	+
Nitrate reduction	+	+	+	+	+	+	+
H_2S production	−	+	−	−	−	−	−
V. P. test	−	−	−	−	−	+	−
M. R. test	−	−	+	+	−	−	+

Key: R, rods; M, monotrichous; P, peritrichous; −, negative; +, positive; O, oxidative; F, fermentative; NC, growth with no change in reaction.

brief characterization of the representative host bacterium strain of each seven groups is shown in Table 4. All of them are aerobic or facultative anaerobic Gram-negative rods and psychrophilic bacteria. They are typical marine bacteria. The two strains of them, 6YK–3212 and 6YK–5201, are motile by polar monotrichous; oxidase (Kovacs'), positive; and carbohydrate metabolism, respiratory. Other three strains, 6YK–1102, 6YK–2202, and 6YK–3210, are motile by a single polar flagellum; oxidase (Kovacs'), positive; carbohydrate metabolism, fermentative; and sensitive to vibriostatic compound (0/129). The rest, 6YK–3306 and 6YK–1303, are characterized by luminescence. And one of the two strains, 6YK–3306 is motile by polar flagella; oxidase (Kovacs'), negative; sensitive to 0/129. On the other hand, 6YK–1303 is motile by peritirichous flagella; oxidase (Kovacs'), positive; and insensitive to 0/129. They belong to the following genera: 6YK–3212 and 6YK–5201, *Pseudomonas*; 6YK–1102, 6YK–2201, and 6YK–3210, *Vibrio*; 6YK–3306, *Photobacterium*; and 6YK–1303, *Lucibacterium*.

The plaque morphorogy of the isolated marine bacteriophage systems was examined by the double agar layer technique. The appearances of plaque formed by the seven representative bacteriophage systems are shown in Fig. 2. The bacteriophage systems produce clear or turbid plaque of about 1–2 mm in diameter.

The titers of these phage lysates are of 10^{8-9} plaque forming units (p.f.u.) per ml of them. The all of the isolated bacteriophages are virulent pages.

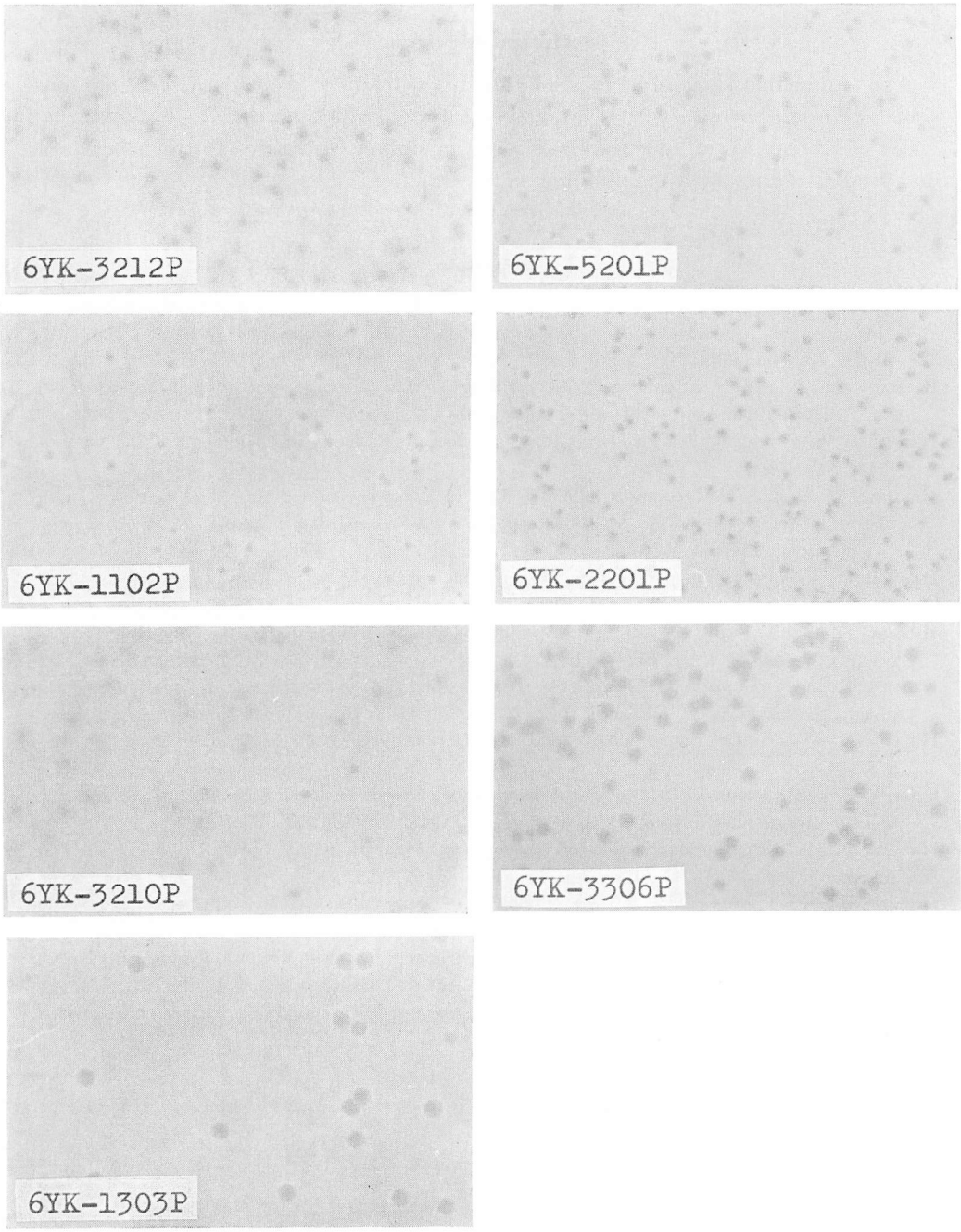

Fig. 2. Plaque morphology of the isolated marine bacteriophage systems. ×1

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