Mem. Fac. Fish., Kagoshima Univ.
Vol. 27, No. 1, pp. 79~90 (1978)

Isolation and Characterization of *Vibrio parahaemolyticus* Bacteriophages in Sea Water*1

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Abstract

The authors carried out the isolation of *Vibrio parahaemolyticus* bacteriophages from sea water and determined the character of them. The isolated phages are detected in coastal sea water with terrestrial contamination. It does not seem that they are truly of marine origin. The phages vary widely in particle structure, host range, and physiological character, and are divided into four groups. The representative phage in each of the groups are Vp15P, Vp17P, Vp25P, and Vp33P. The phage particles of Vp15P and Vp25P consist of a head with a hexagonal outline and powerful tail with a contractile sheath. Vp17P and Vp33P have a head shaped a polyhedron, and a comparatively long, thin, and non-contracting tail. The tail of Vp17P is straight, and terminates in an end plate with shor lobate projections. The tail of Vp33P is considerably curved. According to the lytic patterns of the representative phages, it is possible to extend the classification of strains of *V. parahaemolyticus* by means of phage typing.

Several investigators have reported the isolation of marine bacteriophages (SPENCER, 1960; CHAINA, 1965; WIEBE and LISTON, 1968; JOHNSON, 1968; HIDAKA, 1971, 1973 and 1976) There are also a few extensive studies of bacteriophages from marine environment (ESPEJO *et al.*, 1968a, b, and 1969; COTA-ROBLES, 1968). However, few attempts have been made to estimate distribution, abundance, and the significance of bacteriophages in marine ecosystems (ZACHARY, 1976).

Vibrio parahaemolyticus is a pathogenic agent for food poisoning caused by ingestion of sea food. A number of papers describe the distribution and isolation of *V. parahaemolyticus*, and it is generally accepted that the incidence of *V. parahaemolyticus* is highest in estuarine or coastal areas of the world oceans. It is an interesting subject on microbial ecosystems in marine environment whether or not the bactriophages for this organism are in the environment.

Three bacteriophages active against *V. parahaemolyticus* have been isolated by NAKANISHI *et al.* (1966). It was decided to seek bacteriophages for the organism from the offshore sediments for phage-typing purposes. SKLAROW *et al.* (1973) also reported on the characteristics of a *V. parahaemolyticus* bacterio-

*1 This work was presented at the Annual Meeting of Japanese Society of Scientific Fisheries, Nagasaki, October 12, 1975.

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phage found in sediment samples from the Atlantic coastal regions.

We have isolated several *V. parahaemolyticus* bacteriophages from coastal sea water in Kagoshima Prefecture, Japan. This report characterizes the representative *V. parahaemolyticus* bacteriophages derived from it. It presents the evidence of distribution of these bacteriophages in coastal sea water with terrestrial contamination. It also examines comparing the isolated *V. parahaemolyticus* bacteriophages with the earlier isolated ones.

Materials and Methods

Bacterial strains. *Vibrio parahaemolyticus* strains as indicators for bacteriophage detection were kindly provided by Dr. IIDA at Faculty of Fisheries, Hokkaido University. The character of these strains has been described in other papers (IIDA *et al.*, 1973 a, b). Twelve set strains, O-1 to O-12, of *V. parahaemolyticus* were obtained from the Research Institute for Microbial Diseases, Osaka University. For estimating of host range of isolated phages *Vibrio anguillarum* NCMB 828, *V. piscium* var. *japonicus*, *V. ichthyodermis*, *V. fischeri*, *V. metschnikovii*, and *V. tyrogenus* were also used. These strains were kindly supplied by Dr. KIMURA, a Professor of Faculty of Fisheries, Hokkaido University.

Growth media. Sea water broth (SWB) was used for growth of the host bacteria and for multiplication of the bacteriophages. The medium consisted of 5 g polypeptone and 1 g yeast extract in a liter of Herbst's artificial sea water; final pH was adjusted to 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 sea water broth.

Detection and isolation of bacteriophage. This was experimented according to the indirect method described by SPENCER (1963). To each of a series of sterile shaking flasks was added 150 ml of SWB together with 1 ml of an overnight SWB culture of each of twelve indicator strains and shaken for 2-3 h. After then the young cultures were mixed with 200 ml of sample sea water. The mixtures were incubated with shaking for 2-3 h and were further incubated overnight without shaking. After a brief centrifugation at 4,500 G for 30 min, the cultures were filtered through HA Millipore filter membranes. The filtrates were spotted onto lawns prepared from the homologous bacterial culture on double agar layer plates. These preparations were incubated overnight and examined the appearance of lytic zone. Material from the center of a clear zone developing on lawn was transferred by a platinum wire to fresh SWB cultures of the appropriate bacterium and the inoculated cultures were incubated further 18 h at 25°C. Each of the cultures was then filtered through Millipore filter and a portion of the filtrate was mixed with the appropriate 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. Separated plaques arising by this method were picked and the cycle was

repeated three times to ensure their identity and to assist purification. An initial titer of $10^{9\sim10}$ plaque forming units (pfu) per ml of bacterial culture filtrate was obtained by this method for the single phage system isolated. The phage lysates were stored at 5–8°C.

Host range determination. This was done by spotting a drop of isolated phage lysates (10 × Routine Test Dilution, $10^{5\sim6}$ pfu/ml) on a double agar layer plate seeded with each strain to be tested. The plates were incubated at 25°C for 20 h and checked for lysis.

Phage assay. The bacteriophages were titrated using the double agar layer method of ADAMS (1959). The method was modified, and the following pre-adsorption technique was used. One ml samples of lysate diluted with SWB were mixed in sterile test tubes with 1 ml of the young culture containing about 10^8 indicator cells. After 5–10 min at room temperature, 25–28°C, 0.2 ml of the mixture was added to 3 ml of soft SWA, melted previously and kept in a 45°C water bath, and then all of the mixture were overlaid onto a fresh SWA plate. The double agar layer plates were incubated overnight at 25°C. By this modified technique the number of plaques that developed on each plate represented the number of plaque forming units (pfu) per 0.1 ml lysate diluted. The assay plates were made in duplicate. The plates were also examined for plaque morphology.

One-step growth experiment. This experiment was made according to the procedures described by ADAMS (1959). For the experiment, an inoculum of 1 ml from an overnight SWB culture of the host bacterium was transferred to 9 ml of SWB and incubated for about 3 h at 25° and 30°C with shaking. A 9 ml of each culture grown to a concentration of 2×10^8 cells/ml was mixed with 1 ml of the phage suspension containing 10^7 pfu/ml. The phage-bacterium mixture designated adsorption tube was incubated at decided temperature with gentle shaking for 20 min. At the end of 20 min, a portion of the adsorption mixture was diluted 1: 100 in ice-chilled SWB and then filted through a Millipore (HA, pore size 0.45 μ). Phage titers before and after the filtration were assayed for both total and unadsorbed phage. Another portion of the adosorption tube was diluted 1: 10,000 in prewarmed SWB and designated as the growth tube. The tube was incubated successively, and samples were taken from this tube periodically and assayed for plaque forming units.

Electron microscopy. A 50 ml of stock phage lysate prepared as above was centrifuged at 37,000 G at 0°C in superspeed refrigerated centrifuge for 90 min, and the pellet obtained from original suspension was resuspended in 1 ml of 2% ammonium acetate solution. The preparetions were negatively stained with phosphotungstic acid, namely, the phage suspensions were mixed with the same volume of 2% phosphotungstic acid solution at a pH of 7.2, and applied as droplets directly to the grids. Carbon-corted collodion grids of 150 mesh were used as the specimen supports. The preparations remained on the grids for 20 to 30

sec before the excess liquid was removed with filter paper. After drying, the grids were ready for examination in the electron microscope. The grids were examined in a Nihondenshi, JEM 100B type, electron microscope. Micrographs were taken at electronic magnification of 60,000.

Results

Detection and isolation of *Vibrio parahaemolyticus* bacteriophages in sea water. Field samplings for *V. parahaemolyticus* phage were carried out at eighteen stations in coastal sea around Kagoshima Prefecture, Japan. The sampling stations are shown in Fig. 1, and sampling data are described in Table 1.

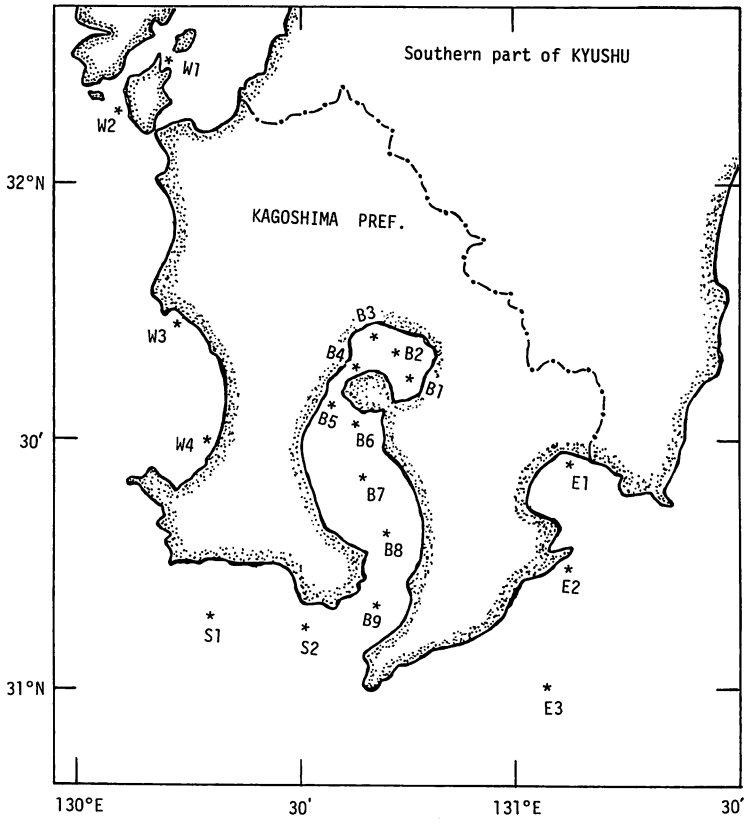

Fig. 1. Map showing the sampling stations for isolation of *Vibrio parahaemolyticus* bacteriophage systems. Star marks show the sampling points.

In this experiment, twelve strains of *V. parahaemolyticus* were used as indicator strains for detection of the bacteriopages in sea water samples. Bacteriophages were detected by lytic action for the indicators. The results are shown in Table 2. The short names of *V. parahaemolyticus* strains (serotypes) used as indicators are also written in addition in the table. It is decided in Table 2 that

Table 1. Sampling stations in coastal sea around Kagoshima Prefecture.

Date	Location	Station No.	Sampling layer (Depth, m)	Temp. (°C) of sea water
Sep. '74	Kagoshima Bay	B1	130	18
		B2	130	18
		B3	140	18
		B4	130	18
		B5	20	24
Jun. '75	Kagoshima Bay	B6	50	18
		B7	50	18
		B8	50	19
		B9	50	19
Jan. '75	East side			
	Shibushi Bay	E1	0.5–1	16
	Uchinoura	E2	0.5–1	17
	20 miles off Sata	E3	50	11
Jan. '75	West side			
	Azuma, Nagasima	W1	0.5–1	12
	Esashi, Nagashima	W2	0.5–1	14
	Shirahama, Kushikino	W3	0.5–1	14
	Fukiagehama	W4	0.5–1	12
Jul. '77	South side			
	10 miles off Makurazaki	S1	50	24
	5 miles off Nagasakibana	S2	50	25

Table 2. Detection of bacteriophages in sea water samples, for *Vibrio parahaemolyticus* strains tested as indicator strains.

Date	Sep. '74					Jan. '75							Jun. '75				Jul. '77	
Station / Indicator strain	B1	B2	B3	B4	B5	E1	E2	E3	W1	W2	W3	W4	B6	B7	B8	B9	S1	S2
Vibrio parahaemolyticus serotype																		
O 1: K25 (Vp25)*	−	−	+	+	+	−	−	−	+	−	−	−	−	−	+	−	−	−
O 1: K32 (Vp32)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
O 2: K28 (Vp28)	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
O 3: K 7 (Vp 7)	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
O 3: K33 (Vp33)	−	+	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−
O 4: K12 (Vp12)	−	+	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−	−
O 4: K34 (Vp34)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
O 5: K15 (Vp15)	−	−	−	+	+	−	−	−	+	−	−	−	−	−	+	−	−	−
O 5: K17 (Vp17)	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−
O 8: K20 (Vp20)	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−
O 9: K23 (Vp23)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
O11: K36 (Vp36)	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−

+, positive; ±, weak positive; −, negative

* Designation in parenthesis indicates the short name of indicator strain.

Table 3. Lytic action of the isolated bacteriophages for various bacteria tested.

Bacteria \ Phages	Vp25P	Vp15P	Vp12P	Vp20P	Vp33P	Vp36P	Vp17P
Vibrio parahaemolyticus							
serotype O 1: K25	+	+	+	−	−	−	−
O 5: K15	+	+	+	−	−	−	
O 2: K28	+	+	+	−	−	−	
O 4: K12	−	+	+	−	−	−	−
O 4: K34	−	+	+	−	−	−	−
O 8: K20	−	−	−	+	+	+	−
O 3: K33	−	−	−	+	+	+	−
O11: K36	−	−	−	+	+	+	−
O 5: K17	−	−	−	−	−	−	+
O 1: K32	−	−	−	−	−	−	−
O 3: K 7	−	−	−	−	−	−	−
O 9: K23	−	−	−	−	−	−	−
RIMD* O- 1	−	−	−	−	−	−	−
O- 2	+	+	+	−	−	−	−
O- 3	−	−	−	−	−	−	−
O- 4	+	+	+	−	−	−	−
O- 5	−	−	−	−	−	−	−
O- 6	+	−	−	+	+	+	−
O- 7	+	−	−	+	+	+	−
O- 8	+	−	−	+	+	+	−
O- 9	+	+	+	−	−	−	−
O-10	+	−	−	+	+	+	−
O-11	−	+	+	+	+	+	−
O-12	+	+	+	−	−	−	−
V. anguillarum NCMB 828	−	−	−	−	−	−	−
V. piscium var. *japonicus*	−	−	−	−	−	−	−
V. ichthyodermis	−	−	−	−	−	−	−
V. fischeri	−	−	−	−	−	−	−
V. metschnikovii	−	−	−	−	−	−	−
V. tyrogenus	−	−	−	−	−	−	−

Key: +, infection; −, non infection
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bacteriophages active against nine strains of *V. parahaemolyticus* are detected in six of eighteen sea water samples. The each detected bacteriophage is designated to annex P to short name of the indicator strain. The nine detected bacteriophages are tried to isolate, and seven positive strains, except two weak positive strains, in Table 2 are purified. They are as follows; Vp12P, Vp 15P, Vp17P, Vp20P, Vp25P, Vp33P, and Vp36P. They show different distribution each other in the stations.

Host range of the isolated bacteriophages. For host range determinations of the seven isolated bacteriophages *Vibio parahaemolyticus* (24 strains) and related vibrios (6 strains) were used. The lytic action of the phages for the various bacteria are shown in Table 3.

As shown in Table 3, it is found that the all phages are incapable of infection on bacteria other than various strains of *V. parahaemolyticus*. Vp17P lyses only an original indicator strain. Within the limits of this experiment, the host-specificity of Vp17 phage is very strict. And the others show wider host range than it. They lyse 7–10 strains in test strains of *V. parahaemolyticus*. These data also suggest that the bacteriophages are divided into four groups according to the lytic pattern for test bacteria. They are as follows: the first group, Vp25P; the second group, Vp12P and Vp15P; the third group, Vp20P, Vp25P, and Vp36P; the fourth group, Vp17P.

The authors introduce a representative phage strain in each of the groups, Vp15P, Vp17P, Vp25P, and Vp33P in following sections.

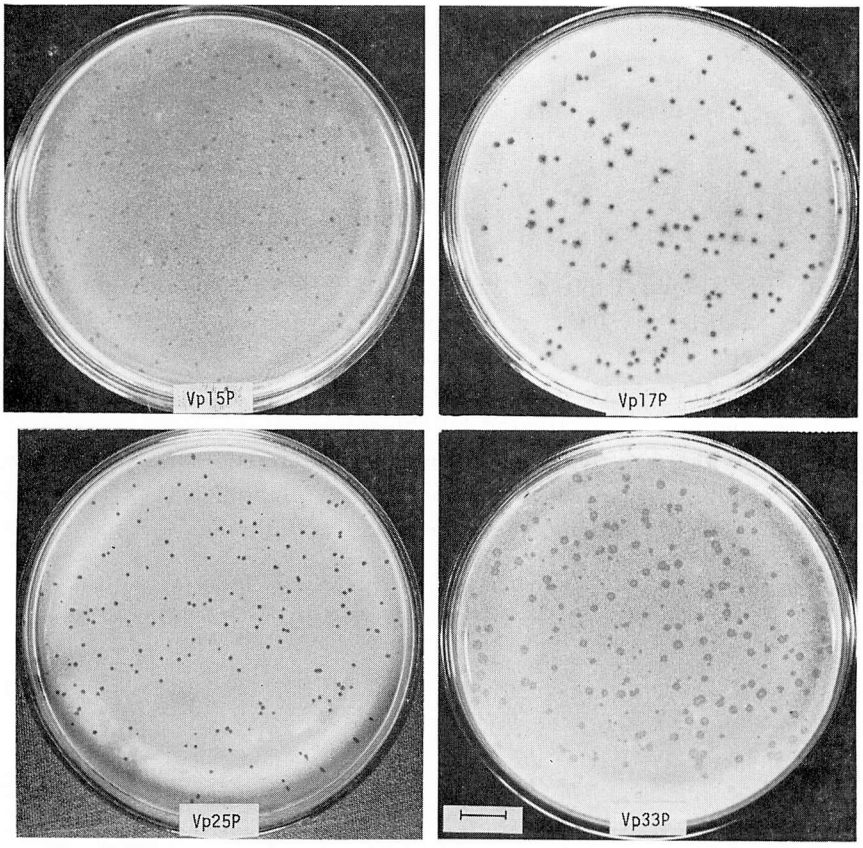

Fig. 2. Plaque morphology of the representative *Vibrio parahaemolyticus* bacteriophages, Vp15P, Vp17P, Vp25P and Vp33P.
Bar represents 10 mm.

Plaque morphology of *Vibrio parahaemolyticus* bacteriophages. The plaque appearances of representative *V. parahaemolyticus* phages are shown in Fig. 2.

Vp15 phage forms small turbid plaques of about 0.5 mm in diameter. Vp17 phage forms clear plaques, 0.8–1.0 mm in diameter, with irregular edges. Vp25 phage produces small clear plaques of about 0.5–0.7 mm in diameter with well-defined edges. Vp 33 phage gives rise to larger, turbid plaques of about 1.0–1.5 mm in diameter with more turbid center.

Sensitivity of the bacteriophages to heating or chloroform. Heating was examined by exposing the phage suspensions containing 10^7 pfu/ml in SWB, to temperature of 50°C for 30 min. Phage titrations were then made on these samples. The phage suspensions (10^7 pfu/ml) in SWB were added a one-tenth volume of chloroform, and after shaking vigorously for 1 min, were kept for 1 h. The samples were centrifuged breafly and titrated the survival phage concentration in the supernatant.

Table 4. Survival of representative *Vibrio parahaemoyticus* bacteriophages heated for 30 min at 50°C or treated with chloroform.

Treatment \ Phage	Plaque forming unit/ml			
	Vp15P	Vp17P	Vp25P	Vp33P
Control	1.6×10^7	1.3×10^7	5.6×10^7	2.0×10^7
Heating (for 30 min at 50°C)	1.4×10^7	1.3×10^7	9.0×10^3	1.3×10^7
Chroloform (after 1 h)	0	1.2×10^7	0	2.0×10^6

The results are shown Table 4. Vp15P, Vp17P, and Vp33P are not inactivated at all by the heating at 50°C for 30 min. The another, Vp25P shows marked inactivation by the heating. On the other hand, Vp17P is stable to treatment with chloroform. The phage titer of Vp33P decrease to one-tenth, and Vp15P and Vp25P are completely inactivated by chloroform.

Multiplication of the bactriophages in their host bacteria. One-step growth experiments were done with each phage in SWB at 25° and 30°C. The adsorption rate was measured by assying the free and total phages in adsorption tube

Table 5. Parameters of phage multiplication defined by the one-step growth experiments of the representative *Vibrio parahaemolyticus* bacteriophages at 25° and 30°C in sea water broth.

Parameter \ Phage	Vp15P		Vp17P		Vp25P		Vp33P	
Temperature (°C)	25	30	25	30	25	30	25	30
Adsorption rate (%)	100	98	92	98	96	98	98	97
Latent period (min)	95	70	80	55	120	90	110	65
Rise period (min)	40	25	70	25	60	45	65	35
Burst size (particles/cell)	5	7	300	350	7	18	150	200

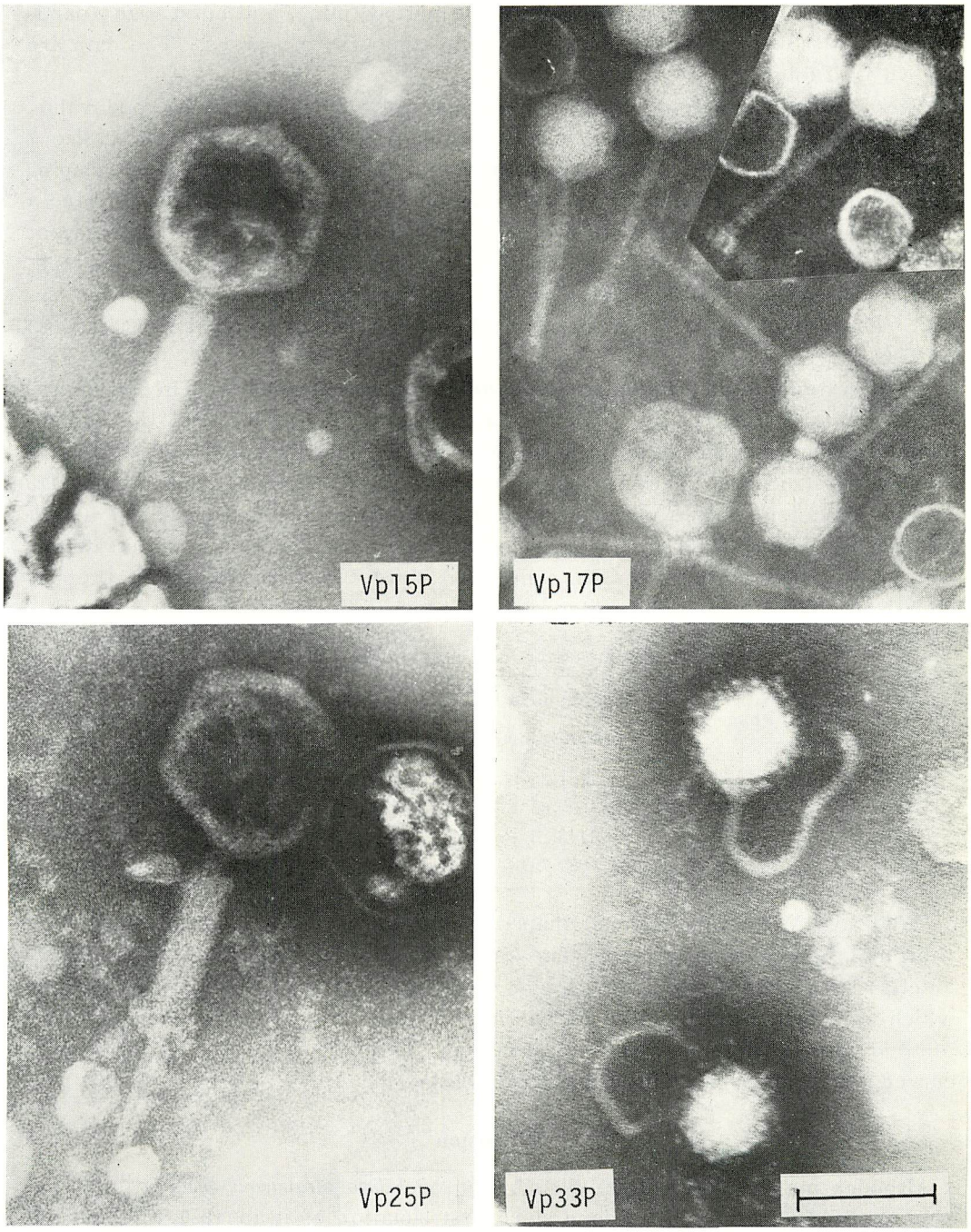

Fig. 3. Electron micrographs of the representative *Vibrio parahaemolyticus* bacteriophage particles, Vp15P, Vp17P, Vp25P and Vp33P, negatively stained with phosphotungstic acid. ×200,000
The marker bar represents 100 nm.

after 20 min. The latent period (min), rise period (min), and burst size (particles per cell) were determined from the one-step growth curves. The data are shown in Table 5.

The adsorption rates of them appear high rate over 90 %, it is good value. Vp15P has a latent period of 70 min, a rise period of 25 min, and burst size 7 particles per cell. Vp17P has a latent period of 55 min, a rise period of 25 min, and burst size of 350 particles per cell. Vp25P has a latent period of 90 min, a rise period of 45 min, and burst size of 18 particles per cell. Vp33P has a latent period of 65 min, a rise period of 33 min, and burst size of 200 particles per cell. The burst sizes of Vp15P and Vp25P are smaller than that of Vp17P and Vp-33P. The growth rate of the phages in their host cell are higher at 30°C than at 25°C.

Particle structure of *Vibrio parahaemolyticus* bacteriophages. The structures of *V. parahaemolyticus* phage particles are shown in Fig. 3, and the dimensions of them are presented in Table 6. The perticles of Vp15P and Vp25P are similar in structure. They consist of a head with a hexagonal outline, 110–125 nm in diameter, and a powerful tail with a contractile sheath, 225 nm length and 25 nm width. The tails consist of an outer sheath and an inner hollow rod. The tail sheath exists in contracted state. During contraction the tail sheath becomes shorter and wider, thereby exposing inner rod.

Table 6. Dimmensions of the phage particles tested.

Phage	Size of head	Size of tail	
	Diameter, nm	Length, nm	Width, nm
Vp15P	110–125	225	25
Vp17P	60– 65	125	8
Vp25P	110–125	225	25
Vp33P	65– 75	200	8

Vp17P and Vp33P have a head shaped a polyhedron, and a comparatively long thin, and non-contracting tail. The size of Vp17 page's head is 60–65 nm in diameter, and one of Vp33 phage's is 65–75 nm. The tail of Vp17P is straight which is 125 nm length and 8 nm width. The tail of this phage terminates in an end plate with short lobate projections. The tail of Vp33P is considerably curved which is 200 nm length and 8 nm width.

Discussion

Although many studies of the distribution of *Vibrio parahaemolyticus* in the marine environment have been done since first isolation of it in 1950, the natural habitat of this organism is not bully known. Now, the distribution of *V. parahaemolyticus* phage is determined to seek the ecological features of them, host bacterium-bacteriophage sytem, in their habitat. The authors isolated seven

strains of *V. parahaemolyticus* phage. The six of the eighteen samples examined contained *V. parahaemolyticus* phages. The six samples were collected from six stations as follows: B2, B3, B4, B5, and B8 station in Kagoshima Bay and W1 station in an inlet of Nagashima. They are in locations near city or town and polluted with some more or less city's sewage. The phages were not found in other stations, open sea. From these data, it could be seen that the distribution of *V. parahaemolyticus*-phage systems is limited to some coastal sea polluted with terrestrial matter. That is independent of season, because the phages have been isolated in January, June, and September. It does not seem they are not truly of marine origin. In four representative *V. parahaemolyticus* phages, two phages, Vp15P and Vp25P have very similar structure. They are, however, slightly different from each other in host range and sensitivity to heating and chloroform. The other two phages, Vp17P and Vp33P are very different from each other in particle structure, host range, and physiological character. NAKANISHI *et al.* (1966) have isolated three phages of *V. parahaemolyticus*, v6, v12, and v14. They have described the physical and chemical features of their isolates, and though incomplete, v12 and v14 phages are tadpole shaped and v16 is filamentous. SKLAROW *et al.* (1973) have isolated a *V. parahaemolyticus* phage and reported the character of it. The phage had a isometric icosahedron head, 60 nm diameter, and a thin tail, 6 nm width and 110 nm length. It also had a high specificity for host. When these phages are compared with new isolates in this paper, Vp17P is exactly similar to the phage isolated by SKLAROW *et al.* in particle structure and host range, but the v6, v12, and v14 isolated by NAKANISHI *et al.* are strikingly different from the new isolates. The all of *V. parahaemolyticus* phages vary widly in their host range. It is possible to extend the classification by means of phage typing.

Biochemical and serological tests are the two methods most commonly used in the detection and identification of bacteria. The classification of *V. parahaemolyticus* strains based on O antigen and K antigen has already been established.

In resent years interest has been revived in phage typing of bacteria. This may be due primarily to the fact that, although extensive serological typing has been accomplished, the technique is time consuming. The added advantage of typing bacteria by phage is that the method will detect differences between strains that are identical by serological and other tests.

As work progresses, it is hoped that data on more phages will be added, thus giving more patterns of lysis. The possibility exists of obtaining different patterns from a wider examination of cultures. It is our intention to expand our basic set of phages in an effort to develop an even greater potential for phage typing *V. parahaemolyticus* from marine environment.

Acknowledgement

The authors wish to express gratitude to Captain, Mr. K. Takahashi and

crews of Nansei Maru, a research ship of Kagoshima University, for their very able and kind assistance with the sampling of sea water.

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