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Properties of a Marine RNA-containing Bacteriophage*

Tomio HIDAKA and Ken-ichi ICHIDA**

Abstract

A marine bacteriophage system isolated from sea water sample. The host bacterium has been characterized as a *Pseudomonas* species. The psychrophilic nature of the organism and its requirement for salts in sea water suggest that it is an indigenous marine bacterium. The bacteriophage active against for the bacterium is a hexagonal shape with particle diameter of 60 mμ and stable under in situ marine conditions. The phage is completely inactivated by treatments with organic solvents such as chloroform. The fact suggests that the phage can be interpreted as a phage contained lipids as a structural component. The phage also contains singlestranded RNA as its genetic material. The base composition (moles%) of the phage RNA are: adenylic acid, 24.0; guanylic acid, 33.5; cytidylic acid, 18.9; uridylic acid 23.6. The phage is a new type marine RNA-containing phage.

In the previous papers, the authors reported the isolation of marine bacteriophages (HIDAKA, 1971; HIDAKA and FUJIMURA, 1971; HIDAKA, 1973), and found that one of the thirty-two isolates contained ribonucleic acid (RNA) but no deoxyribonucleic acid (DNA) as its genetic material (HIDAKA, 1975).

A bacteriophage, f2, containing RNA was first isolated by LOEB and ZINDER (1961). Since then, some bacteriophages, R17, R23, MS2, etc., have been described their biophysical and serological characteristics related to f2. These RNA phages infect only male strains (Hfr and F^+) of *Escherichia coli* or other bacteria that have received the *E. coli* F^+ episome. These phages are small and circular and contain a single-stranded, linear RNA.

On the other RNA-containing bacteriophages, a phage 7s associated with a lysogenic strain of *Pseudomonas aeruginosa* (FEARY *et al.*, 1963) and a phage for *Caulobacter* sp. (SCHMIDT and STANIR, 1965) have also reported respectively. Recently a RNA-containing bacteriophage ϕ6 have been isolated from *Pseudomonas phaseolicola* (VIDAVER *et al.*, 1973). It has a double-stranded RNA as its nucleic acid and also contain lipids as a structural component like a phage PM2 isolated by ESPEJO and CANELO (1968).

The purpose of this report is to describe some of the properties of a new type of RNA-containing bacteriophage system isolated from sea water.

Materials and Methods

Phage system. The bacteriophage system, a bacteriophage and host bacterium

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** Laboratoty of Microbiology, Faculty of Fisheries, Kagoshima University.

for it, were isolated from the same sea water sample. The sea water was collected from a 50 meters of depth at a station (31°04'N–130°35'E) about 5 miles off the south of Kyushu, Japan, in June 1970. These are designated 06N–58 for the host bacterium and 06N–58P for the bacteriophage.

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

Microbiological tests of bacterium. The characterization tests of the host bacterium were done using standard methods (HARRIGN and McCANCE, 1966). Identification of it was carried out according to the systems outlined by HENDRIE and SHEWAN (1966). Incubations were at 25°C, unless otherwise stated.

Phage assay. The bacteriophage was assayed for plaque forming units (p. f. u.) by the agar-overlay technique as described by ADAMS (1959). The agar-overlay plates were incubated for 20 hr at 25°C and then examined for plaque counting and 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 taransferred to 9 ml of SWB and incubated for about 3 hr at 25°C with shaking. A 9 ml of this culture grown to a concentration of 2×10^8 cells/ml was mixed with 1 ml of the phage suspension containing 10^7 p. f. u./ml. The phage-bacterium mixture designated adsorption tube was incubated at 25°C with gentle shaking for 15 min. At the end of 15 min, a portion of the adsorption mixture was diluted 1 : 100 in ice-chilled SWB and then filterd through a Millipore (HA, Pore size 0.45μ). Phage titers before and after the filtration were assayed for both total and unadsorbed phage. Another portion of the adsorption tube was diluted 1 : 10,000 in prewarmed SWB and designated the growth tube. The tube was incubated continued, and samples were taken from this tube periodically and assayed for plaque forming units.

Preparation of high titer phage suspensions. The bacteriophage was concentrated and partially purified by alternate low-speed and high-speed centrifugation. The phage lysate was centrifuged at 4,500$\times$g for 30 min to remove bacterial cells and cell debris, and then the supernatant fluid was filtered through a HA Millipore. The filtrate was treated with DNase and RNase at a concentration of about 2 μg/ml for 2 hr at 30°C. After this, it was centrifuged at 37,000$\times$g with a superspeed centrifuge for 90 min at 0°C. The phage pellet obtained was washed and resuspended in appropriate medium at a concentration depended on following experimental purpose.

Electron microscopy. For observation of phage particle structure, the concentrated phage suspension (10^{11-12} p. f. u./ml) in a 1% ammonium acetate solution was mixed with an equal volume of 2% aqueous phosphotangstic acid adjusted

to pH 7.2 with NaOH. The mixture was applied as droplets 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.

For preparation of host cell, the bacterium cells grown on SWA plate were suspended in a 1% ammonium acetate solution, and a drop of the bacterial suspension placed on a microscope grid. After drying, the preparation was shadowed with chromium in an experimental evaporator. The grids were examined in a Hitachi, HU-11D type, electron microscope at instramental magnifications of 5,000 for bacterial cells and of 50,000 for phage particles.

Preparation of mononucleotides of phage RNA. The phage lysate of 5 liter was concentrated, purified and resuspended in 25 ml Tris buffer solusion (0.01 M EDTA, 0.14 M NaCl, 0.01 M Tris, pH 7.5). The phage suspension was shaken in a bottle with an equal volume of cold aqueous phenol. This was repeated several times until no proteins appear at the water-phenol interface after centrifugation. The aqueous layer is removed to a tube. Phenol dissolved in the aqueous layer was removed by several shaking with ether, and the ether was finally removed by bubbling nitrogen gas through it. The partially purified nucleic acids were precipitated with ethanol containing 0.1 M sodium acetate. The RNA was again dissolved in saline citrate. The RNA solution was treated with 0.3 N KOH at 37°C for 20 hr, permitting quantitative conversion of RNA into a mixture of mononucleotides. This was then acidified with 5% perchloric acid in the cold to precipitate proteins and DNA, if present. The sediment was washed with a small volume of cold 2% perchloric acid, and the supernatant was combined. The combined supernatant containing all RNA mononucleotides was then neutralized with 6 N KOH (pH 7–8). After the removal of precipitated $KClO_4$ by centrifugation in the cold, each nucleotide in the solution was separated by ion-exchange chromatography.

Chromatographic determination of base composition of phage RNA. The "formic acid system" of OSAWA *et al.* (1958) was used throughout. The eluting system was adapted for the complete separation of a mixture of 5 to 7 mg of RNA mononucleotides by using a 50 ml mixing flask and a 1.0×20 cm Dowex-1 column (Dowex-1, formate form, cross linkage 2%, 200–400 mesh). Adsorbed nucleotides were eluted with a continuously increasing concentration of eluent, that is, first with 30 ml of 1 N formic acid, followed by 200 ml of 4 N formic acid. Samples of the effluent containing 3 ml were collected automatically every 15 min. The extinction at 260 mμ and 280 mμ was measured for each tube. After the readings, the contents of the tubes in each peak were quantitatively transferred to a volumetric flask, and the extinctions were read again. For the calculation of each nucleotide content, the following millimolar extinction coefficients were used: adenylic acid, 14.2 at 260 μ; guanylic acid, 11.8 at 260 mμ; cytidylic acid, 13.0 at 280 mμ; uridylic acid, 9.9 at 260 mμ. As a control of mixed monoribonucleotides, hydrolysates of yeast nucleic

acid were used. Commercial adenylic, guanylic, cytidylic and uridylic acid were also employed for references.

Rasults

Characteristics of the host bacterium 06N-58

As shown in Fig. 1, the bacterium is a polar monotrichous Gram-negative rod.

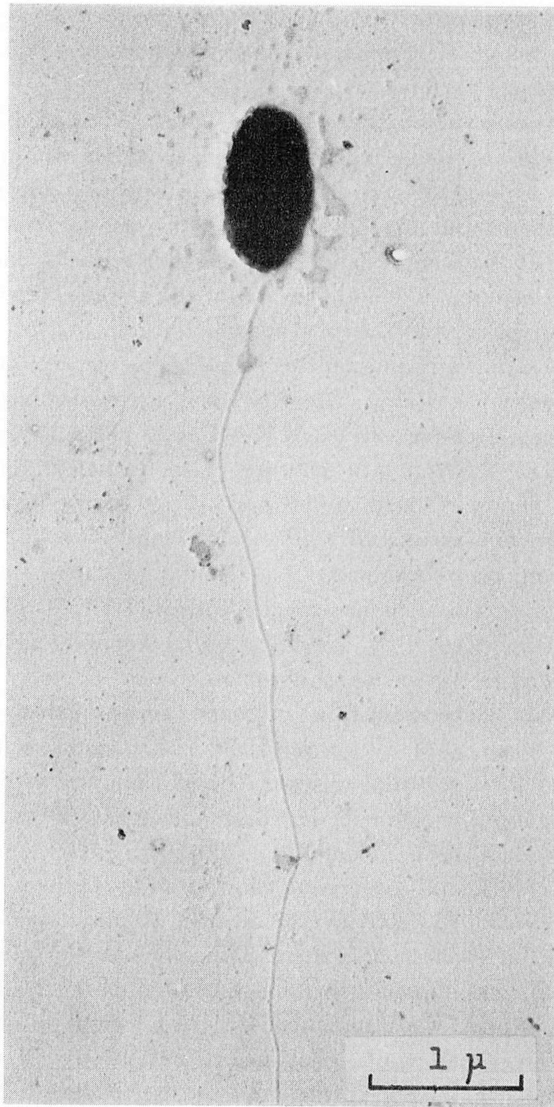


Fig. 1. Electron micrograph of a chromium-shadowed preparation of isolate 06N-58. × 20,000

It is also a aerobic and psychrophilic organism. A preliminary characterization of the organism was made by routine tests for the identification of Gram-negative polar flagellated bacteria. The results are shown in Table 1. The bacterium is oxidase positive, oxidative in Hugh & Leitson's medium (glucose), non-sensitive to the vibriostatic agent 0/129 (2,4-diamino 6,7-diisopropyl pteridine), arginine dihydrolase negative and so on. Most of the properties of 06N–58 are shared by many of the *Pseudomonas* species.

Table 1. Brief characterization of the host bacterium 06N–58.

Cell form	Rod	Nitrate reduction	+
Gram's stain	—	Indole production	—
Flagellation	Mono.	H_2S from cysteine	+
Kovacs oxidase	+	V. P. test	—
Hugh & Leifson test	O	M. R. test	—
Sensitivity to O/129	—	Growth in 7.5% NaCl	+
Arginine dihydrolase	—	Growth in 10% NaCl	+
Luminescence	—	Growth in 12% NaCl	—
Pigment	—	Growth at 37°C	weak
Gelatin hydrolysis	+	M-, H-, T-Typing*	M
Starch hydrolysis	+		

Key: +, positive; —, negative; Mono., monotrichous; O, oxidative; M, marine type.
* Three types, Marine (M-), Halophilic (H-) and Terrestrial (T-) type, were designated according to the requirements of bacteria for minerals in sea water by HIDAKA (1965).

The optimum temperature for its growth lies 25–28°C, and maximum is 37–39°C. The bacterium is a psychrophile.

The growth rate as a function of NaCl concentration was measured by use of SWB which contained varying amounts of NaCl but constant concentration of the other salts in artificial sea water. The optimal growth rate is attained in a medium with a NaCl concentration very similar to that of sea water. The growth is capable in SWB with a NaCl concentration ranging from 0.5% to 10%. The salt requirement for growth was tested by using the method of HIDAKA (1965). The results obtained indicate that the bacterium 06N–58 requir not only NaCl but also other minerals such as K-, Mg-, and Ca-salt in sea water. These facts suggest that 06N–58 is one of marine type bacteria.

Characteristics of the bacteriophage 06N–58P

Phage host range. The bacteriophage was incapable of plaque formation on other various strains of pseudomonads isolated from marine samples in our laboratory (HIDAKA, 1971, 1973).

Plaque morphology. Fig. 2 shows the plaque morphology of phage 06N–58P. It forms clear plaques of about 2–3 mm in diameter.

Structure of the phage particles. Fig. 3 shows the phage morphology as revealed by

Table 2. Survival of the phage after keeping at various temperatures in SWB.

Experimental condition	Survival (p. f. u./ml)
Control (initial titer)	10^8
50°C, 30 min	0
45°C, 10 min	0
45°C, 5 min	10^2
5°C, 3 month	10^5

Table 3. Survival of the phage after 24 hr at 5°C in SWB adjusted various pH.

pH	Survival (p. f. u./ml)
6.5	0
7.0	6×10^4
7.5	1×10^5
8.0	9×10^4
8.5	4×10^3

negative staining technique. The particles are extremely uniform in size and hexagonal shape with a particle diameter of 60 mμ. The particles can be interpreted as being icosahedral in shape. An apparently double-layer coat can be seen in some particles. No tail structure have been observed in these preparations of the phage.

Stability. After keeping at various conditions, stability of the phage was measured by assaying for survival plaque forming units. The results are shown in Table 2–6. The phage is very unstable. As shown in Table 2, the phage is marked or completely

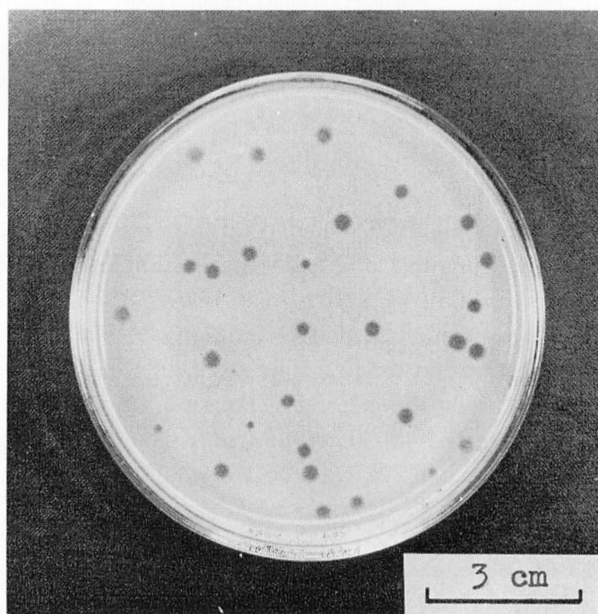
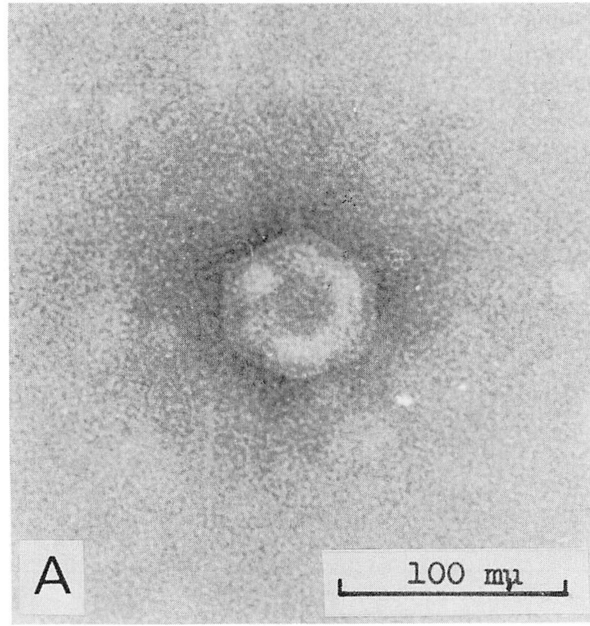


Fig. 2. Plaques formed on 06N–58 by phage 06N–58P. ×2/3

inactivated by heating for 5 min at 45°C. The phage is also inactivated during the storage at 5°C in SWB. The phage titer decreases to about one-tenth for a month. Table 3 shows that the phage is stable within the range of pH 7.0–8.0. At pH values



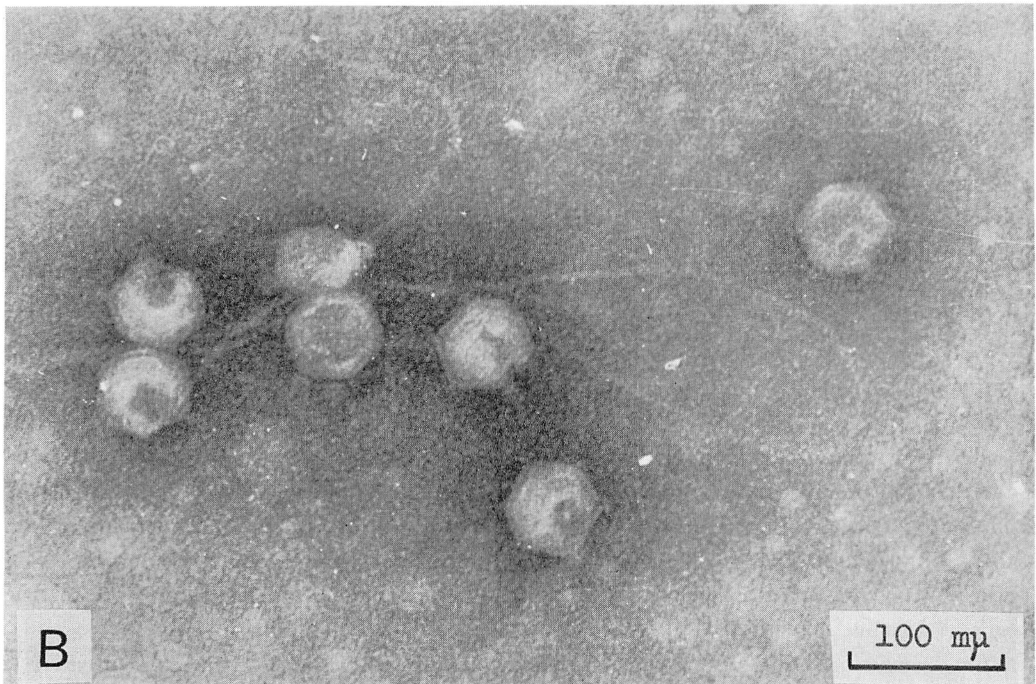


Fig. 3. Electron photo-micrographs of phage 06N–58P negative stained with 2% PTA. A, ×300,000; B, ×200,000

above 8.5 and below 6.5, infectivity is very rapidly lost. The phage is most stable in SWB as suspending media, but not in ASW and 1/6 ASW. Cmoplete inactivation of it appears on suspension in 3% NaCl solution, 0.5% NaCl solution and distilled water as shown in Table 4. The phage is completely inactivated by treatments with several organic solvents such as chloroform, carbon tetrachloride and toluene (Table 5) and some kinds of surface active agents (Table 6).

Table 4. Survival of the phage suspended in various media.

Suspension media	Survival (p. f. u./ml) after	
	3 hr at 20°C	24 hr at 5°C
SWB (control)	5×10^5	4×10^5
ASW *	2×10^5	3×10^5
1/6 ASW	5×10^4	4×10^3
0.5% NaCl soln	2×10^2	10
3% NaCl soln	5×10	0
distilled water	0	0

* Artificial sea water.

Table 5. Survival of the phage in shaking suspensions with certain organic solvents. (10 ml of phage suspension in SWB was shaken with 0.1–0.5 ml of solvent for one min followed by one hr standing before sampling).

Organic solvent	Survival (p. f. u./ml)
Chloroform	0
Carbon tetrachloride	0
Toluene	0

Table 6. Minimal inactive concentration of several surface active agents to the phage.

Surfactant	MIC (mg/ml) in SWB
Non-ionic phospholic surfactant	0.01
Anionic sulphonic surfactant	0.001
Sodium lauryl sulfate	0.1
Sodium laurylbenzen sulfonate	0.1
Sucrose laurate	100

One-step growth. The results of a one-step growth experiment with 06N–58P propagated on strain 06N–58 are shown in Fig. 4. Under conditions of single infection, phage 06N–58P is found to have a latent period of 35 min, a rise period of 25 min and a burst size of 170. When the adsorption rate was measured by assaying adsorption mixture for unadsorbed phage, 93% of phage particles was adsorbed to the host cells in 15 min.

Base composition of the phage RNA. Acridine orange staining of the concentrated and purifyed 06N–58P preparation using the method of BRADLEY (1966) revealed a flame red color similar to that of a control single-stranded RNA preparation (HIDAKA, 1975). Base ratio of the RNA was determined by a column chromatography in this

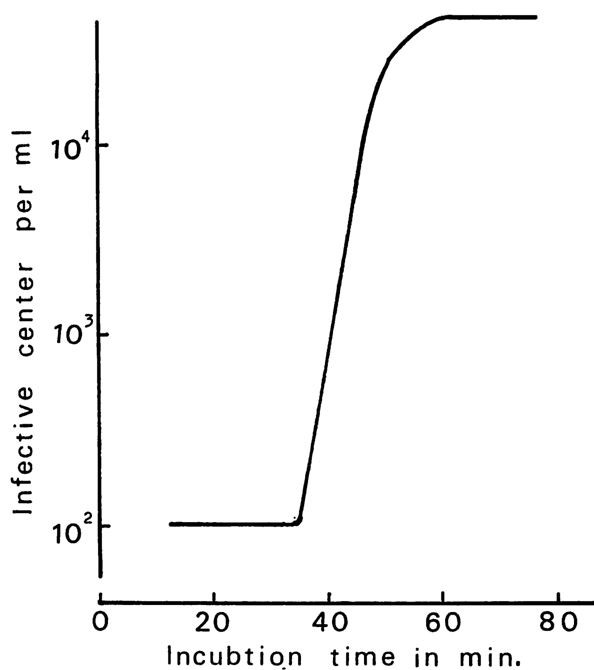


Fig. 4. One-step growthcurve with phage 06N-58P on host bacterium 06N-58 in SWB at 25°C. Bacterial cells, 10^8/ml; MOI, 0.01.

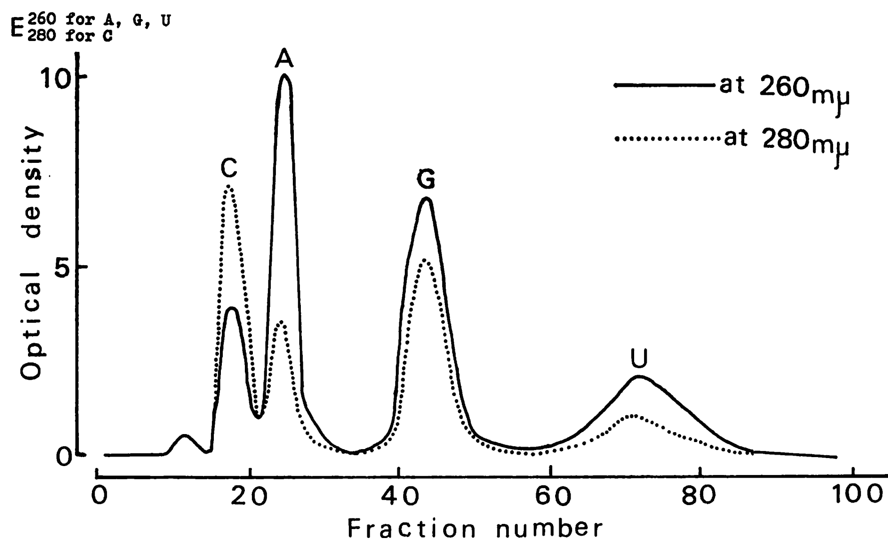


Fig. 5. Chromatographic separation of individual mononucleotides of RNA of phage 06N-58P.

C=Cytidylic acid; A=Adenylic acid

G=Guanylic acid; U=Uridylic acid

work. The mononucleotides obtained after alkaline hydrolysis of RNA of 06N–58P were quantitatively separated on a Dowex-1 column. The results obtained are shown in Fig. 5. The figure shows a sharp separation of four RNA mononucleotides and demonstrate the good reproducibility of this ion-exchange technique. The analytical data on the nucleotide composition of RNA from phage 06N–58P are given in Table 7. It is characterized by a higher guanylic acid and a lower cytidylic acid content as compared with that of reference RNA phages.

Table 7. Base composition of RNA phages.

Phage		Base composition, moles %			
		A	U	G	C
06N–58P		24.0	23.6	33.5	18.9
Reference*	R17	22.6	25.5	27.1	24.8
	R23	23.4	26.0	25.6	25.1
	R34	23.6	26.8	23.9	25.4
	R40	22.4	25.5	26.9	24.9
	f2	22.2	25.1	25.9	26.8
	fr	24.3	23.7	27.1	24.9
	QB	22.1	29.1	23.7	24.7
	B	23.3	28.0	21.6	27.2
	MS2	22.4	25.6	27.5	24.5

* Watanabe and August (1967).

Discussion

Cell morphology and the results of routine biochemical tests of the host bacterium 06N–58 indicate that the bacterium is a *Pseudomonas* species. The optimal temperature for its growth lies 25∼28°C, and the failure to grow at temperatures higher than 37°C, the facts define 06N–58 as a psychrophilic bacterium. MacLeod and Onofrey (1956) suggested that the requirement of Na^+ can be a point defining the nature of true marine bacteria. Hidaka (1965) divided the marine isolates into three types, "terrestrial", "halophilic" and "marine", based on their capacity to grow in media prepared with either freshwater, 0.5% NaCl, 3.0% NaCl, dilute or normal strength artificial sea water. The organic components for growth were supplied in each medium by 0.05% polypeptone and 0.01% yeast extract. Terrestrial types were considered to be those which could grow in all five media. Halophilic types were judged to be those lacking the capacity to grow in media prepared with fresh water or fresh water and 0.5% NaCl, while marine bacteria were defined as those able to grow only in the two media prepared with sea water. The bacterium 06N–58 belong to "marine type". The temperature range and the ionic requirement of 06N–58 clearly pointed to the marine origin of the bacterium. For immediate purpose, this bacterium is designated as *Pseudomonas* sp. 06N–58.

The bacteriophage 06N–58P appears to be a polyhedral object 60 mμ diameter,

and the coat seems to have an apparent double-layered wall can be observed in the electron microscope.

The phage is a very unstable one. It is completely inactivated by heating for 10 min at above 45°C, suspending in distilled water, or in media adjusted above 8.5 or below 6.5 of pH value. These facts suggest that the bacteriophage is stable under approximate in situ conditions, and that the nature of 06N–58P reflect the marine conditions where the phage isolated from. The environmental conditions for the phage-bacterium complex are more restrictive than for growth of the bacterium alone. The phage is also unstable under the shaking with organic solvent such as chloroform and surface active agents. SPENCER (1963) reported that certain marine bacteriophages which grew on marine pseudomonds (SPENCER, 1960) were inactivated by chloroform. In 1966 there was also a report of an apparently filamentus bacteriophage (V6) active against *Vibrio parahaemolyticus* being inactivated by chloroform (NAKANISH *et al.*, 1966). ESPEJO and CANELO (1968 a, b) isolated a phage PM2 which grows on a marine pseudomonad, *Pseudomonas* BAL–31 and reported some properties of it. The phage is hexagonal shape in diameter 60 mμ and also inactivated by chloroform and contains lipids as a structural component. HIDAKA (1971, 1973) isolated 32 strains of marine bacteriophage, and 7 strain of them appear some extent of inactivation by chloroform. In general, lipid-containing viruses can be inactivated by organic solvents due to extraction of the lipids and disruption of the physical particle (FRANKLIN, 1962). Much more detailed work has to be done, however, before the morphology and structure of phage 06N–58P is well established. The phage 06N–58P can be interpreted as a lipid-cantaining phage.

The DNA of bacteriophage PM2 is a closed circular double-stranded DNA of relatively low molecular weight, and the GC content of PM2 DNA is 42–43% (ESPEJO and CANELO, 1969). Recently a lipid-containing bacteriophage, ϕ6, was isolated from *Pseudmonas phaseolicola* infected bean straw (VIDAVER *et al.*, 1973). The host is a phytopathogenic pseudomonad and the virus has some interesting properties. The phage ϕ6 was completely inactivated by chloroform. The nucleic acid is a double-stranded RNA of total molecular weight 9.5×10^6 and the high GC content of 58% make this RNA unique among the known viral double-stranded RNA species. Most RNA phages infect only male strains of *E. coli*. Although they are all similar in single-stranded RNA, small size and circular shape, and male specificity, RNA bacteriophages present in nature need not all exhibit these same characteristics. A phage 7s isolated from a lysogenic strain of *Pseudomonas aeraginosa* (FEARY *et al.*, 1963) and a phage for *Canlobacter* sp. (SCHMIDT and STANIR, 1965) are also RNA-containing bacteriophage. Phage 7s is small size particle in diameter 25 mμ, and the sensitivity of it to RNase offers additional proof that this virus contains RNA as its nucleic acid component. As far as I know, no further work has been done with phage 7s.

On the other hand, the nucleic acid of phage 06N–58P is a single-stranded RNA (HIDAKA, 1975). The molar ratios of the bases of 06N–58P are: adenylic acid, 24.0; guanylic acid, 33.5; cytidylic acid, 18.9; uridylic acid, 23.6. The comparable

ratios for many RNA phages active against male strains of *E. coli* are: 25; 25; 25; 25. The RNA of 06N–58P is characterized by a higher guanylic acid and a lower cytidylic acid content as compared with that of *E. coli*-RNA phages. The phage 06N-58P is a new type of RNA-containing phage. The phage 06N–58P have a latent period of 35 min and a bust size of 170 in situ marine conditions.

The probable indigenous marine origin of 06N–58P is suggested by the high salinity and low temperature requirement for survival and reproduction of the phage and host bacteria. The fact that some phages isolated from sea water are sensitive to organic solvents poses an interesting ecological problem.

References

Adams, M. A. (1959): "Bacteriophages", Interscience Publishers, Inc., New York.

Bradley, D. E. (1966): The fluorescent staining of bacteriophage nucleic acids. *J. gen. Microbiol.*, **44**, 383–391.

Espejo, R. T. and E. S. Canelo (1968a): Properties of bacteriophage PM2: A lipid-containing bacterial virus. *Virology*, **34**, 738–747.

Espejo, R. T. and E. S. Canelo (1968b): Properties and characterization of the host bacterium of bacteriophage PM2. *J. Bacteriol.*, **95**, 1887–1891.

Espejo, R. T. and E. S. Canelo (1969): The DNA of bacteriophage PM2. Ultracentrifugal evidence for a circular structure. *Virology*, **37**, 495–498.

Feary, T. W., E. Jr. Fisher and T. N. Fisher (1963): A small RNA-containing *Pseudomonas aeruginosa* bacteriophage. *Biochem. Biophys. Res. Commun.*, **10**, 359–365.

Franklin, R. M. (1962): The significance of lipids in animal viruses. An essay on virus multiplication. In "Progress in medical virology", vol. 4, p. 1–53, ed. by Berger, E. and J. L. Melnick, Basel-New York, S. Karger.

Harrigan, W. F. and M. E. McCance (1966): "Laboratoly Methods in Microbiology", Academic Press, New York.

Hendrie, M. S. and J. M. Shewan (1966): The identification of certain *Pseudomonas* species. in "Identification Methods for Microbiologist, Part A" (B. M. Gibbs and F. A. Skinner, ed.), 1–7, Academic Press, New York.

Hidaka, T. (1965): Studies on the marine bacteria. II. On the specificity of mineral requirements of marine bacteria. *Mem. Fac. Fish. Kagoshima Univ.*, **14**, 127–180.

Hidaka, T. (1971): Isolation of marine bacteriophages from sea water. *Bull. Jap. Soc. Fish.*, **37**, 1199–1206.

Hidaka, T. and T. Fujimura (1971a): A morphological study of marine bacteriophages. *Mem. Fac. Fish. Kagoshima Univ.*, **20** (1), 141–154.

Hidaka, T. and T. Fujimura (1971b): On the heat- and chloroform-resistance of marine bacteriophages. *Mem. Fac. Fish. Kagoshima Univ.*, **20** (1), 155–158.

Hidaka, T. (1972): On the stability of marine bacteriophages. *Bull. Jap. Soc. Sci. Fish.*, **38**, 517–523.

Hidaka, T. (1973): Characterization of marine bacteriophages newly isolated. *Mem. Fac. Fish. Kagoshima Univ.*, **22** (1), 47–61.

Hidaka, T. (1975): Identification of the type of nucleic acid in marine bacteriophages with acridine orange staining. *Mem. Fac. Fish. Kagoshima Univ.*, **24**, 133–138.

Loeb, T. and N. D. Zinder (1961): A bacteriophage containing RNA. *Proc. Natl. Acad. Sci.*, **47**, 282–289.

MACLEOD, R. A. and E. ONOFREY (1965): Neutrition and metabolism of marine bacteria. II. Observation on the relation of sea water to growth of marine bacteria. *J. Bacteriol.*, **71**, 661–667.

NAKANISHI, H., IIDA, Y. MAESHIMA, K., and TERAMOTO, T. (1966): Isolation and properties of bacteriophages in *Vibrio parahaemolyticus*. *Biken's J.*, **9**, 149–157.

OSAWA, S., K. TAKATA, and Y. HOTTA (1958): Nuclear and cytoplasmic ribonucleic acid of calf thymus. *Biochim. Biophys. Acta*, **28**, 271–277.

SCHMIDT, J. M. and R. Y. STANIER (1965): Isolation and characterization of bacteriophages active against stalked bacteria. *J. gen. Microbiol.*, **39**, 95–107.

SPENCER, R. (1960): Indigenous marine bacteriophages. *J. Bacteriol.*, **79**, 614.

SPENCER, R. (1963): Bacterial viruses in the sea. In "Symposium on marine microbiology", p. 350–365, ed. by OPPENHEIMER, C. H., Charles C. Thomas, Springfield, Illinois.

VIDAVER, A. K., R. K. KOSKI and J. L. VAN ETTEN (1973): Bacteriophage ϕ6: A lipid-containing virus of *Pseudomonas phaseolicola*. *J. Virol.*, **11**, 799–805.

WATANABE, M. and J.T. AUGUST (1967): Methods for selecting RNA bacteriophage. In Methods in virology. Vol. 3, Edited by MARAMOROSCH, K. and H. KOPROWSKI. Academic Press Inc., New York. pp. 337–350.