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Isolation and characterization of a marine bacteriophage

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Abstract

A bacteriophage active against a marine *Aeromonas* sp. was isolated from surface sediments of the North Pacific Ocean at 825 m depth. The sensitive *Aeromonas* sp. grew between 0° and 33 °C but plaques were formed only between 0° and 23 °C with a maximum zone of lysis at 5° to 12 °C. The phage was rapidly inactivated at temperatures above 45 °C. The characteristic plaque formation occurred only on media made with sea-water but some lysis was obtained in distilled water media supplemented with 0.085 M NaCl and 0.05 M $MgCl_2$. Phage replication occurred in cultures of *Aeromonas* sp. growing under applied hydrostatic pressures of up to 200 atmospheres. Electron microscopy revealed that the phage has an hexagonal head 530 Å in diameter, a thin tail 1200 Å in length and a terminal base plate 400 Å in diameter.

Introduction

Bacteriophages have been isolated from cultures of bacteria representing most of the commonly encountered heterotrophic genera (ADAMS, 1959) and as free virus from a wide variety of terrestrial environments including soil, milk, air, animal intestine, etc. The isolation of bacteriophages lytic for indigenous marine bacteria has been infrequent though the occurrence of phages active against adventitious species such as the coliforms has often been reported in polluted and estuarine waters (BUTTIAUX et al., 1955; CARLUCCI and PRAMER, 1960; ZOBELL, 1946).

KRISS and RUKINA (1947) and KRISS (1963) reported numerous isolations of bacteriophages from the Black Sea which lysed species of the genera *Bacillus*, *Sarcina*, *Micrococcus*, *Mycobacterium* and *Bacterium*. However, since the lytic activity was observed as plaque (taches vierges) formation on bacterial lawns and no isolation or characterization of the "phages" was attempted, the possibility that bacteriocins, antibiotic effects or even *Bdellovibrio* or protozoa rather than phage were responsible for the plaque formation cannot be ruled out. Furthermore, the sensitive bacteria involved are rather atypical of the typical heterotrophic microflora of the sea, which is mainly composed of non-coliform Gram-negative rods.

The only well documented reports of marine phage isolation were published by SPENCER (1955, 1960, 1963) who isolated phage active against several types of salt requiring marine bacteria including species of *Pseudomonas*, *Photobacterium* and (probably) *Cytophaga*. Characteristically, lysis was only obtained when Na^+ and Mg^{++} concentrations roughly equivalent to sea-water were included in non-sea-water media. The phages were inactivated at temperatures between 50° and 60 °C, which are lower than for most terrestrial phages. These results lead SPENCER to conclude that the bacteriophages reflected their marine origin, that is they had a high environmental fidelity. Electron micrographs of one of SPENCER's phages have recently been published (VALENTINE and CHAPMAN, 1966; VALENTINE et al., 1966). Other authors who have reported isolation of bacteriophages active against marine bacteria include JOHNSON (1966) and SMITH and KRUEGER (1954); in this latter case, however, the isolation was from San Francisco Bay which is an estuarine rather than a truly marine environment.

The present paper describes the first confirmed isolation of a bacteriophage from ocean sediments and the characterization of the marine properties of the virus with reference to temperature, salinity and pressure.

Materials and methods

Isolation of test-bacteria: A mud-water slurry from sediment obtained by a gravity corer (the plastic liner rinsed thoroughly with 70% ethanol prior to descent) from sediment at 825 m on the bed of the Pacific Ocean (August 1963, water temperature 4.5 °C, salinity 34.31‰) off the Oregon-Washington Coast (45° 52′ N, 124°, 50′ W) was serially diluted and appropriate dilutions cultured in sea-water agar (SWA: 0.1% Difco peptone, 0.1% Difco yeast extract, 1.1% Bacto agar in sea-water, salinity 32%, pH 7.4) at 5° to 8 °C for approximately 3 weeks. 25 colonies were picked at random from the agar plates and purified by three consecutive streakings of isolated colonies on SWA.

Demonstration of bacteriophage: 0.5 ml of each purified bacterial isolate grown in still culture for 24 h at 12 °C in sea-water broth (SWB: 1.0% peptone, 0.1% yeast extract in sea-water, pH 7.4) was added to 1 ml of sterile SWB and 5.0 ml of the original mud-water slurry (held at 0° to 2 °C until required) in a 100 ml Erlenmeyer flask. The mixture was incubated without shaking at 12 °C for 5 to 7 days, then filtered through an HA millipore filter. The filtrate was spotted onto a lawn prepared from the homologous bacterial culture by the overlay agar technique (ADAMS, 1959) using SWA containing 0.6% agar. These preparations were incubated at 12 °C and examined daily for plaque formation for up to 1 week.

Isolation of the bacteriophage: Material from the center of clear zones developing on lawns was trans-

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ferred by a platinum wire to fresh 24 h (12 °C) SWB cultures of the homologous bacterium and the inoculated cultures incubated a further 24 h at 12 °C. The culture was then filtered and a portion of the filtrate mixed with the homologous bacterium and the mixture layered as lawn on the surface of an agar plate. Single plaques arising by this method were picked and the cycle repeated three times to ensure their identity and assist purification. An initial titer of 5×10^7 plaque forming units (pfu) per ml of bacterial culture filtrate was obtained by this method for the single phage system isolated. The phage preparation was stored at 0 °C without addition of a bacteriocidal agent since chloroform, most commonly employed in the storage of phage preparations, was found to inactivate the marine phage.

Electron microscopy: A stock phage suspension prepared as above was centrifuged at 8,000 g at 2 °C in a Servall superspeed refrigerated centrifuge for 1 to 2 h and the pellet obtained from original suspension was resuspended in 1 ml of sea-water. The preparation was positively stained with a 3% solution of uranyl acetate.

Cation effects: Studies of the effects of various concentrations of Na^+, Mg^{++} and Ca^{++} on growth of the sensitive bacterium and operation of the lytic system were carried out by adding appropriate amounts of reagent grade NaCl, $MgCl_2$ and $CaCl_2$ to a basal medium containing 0.1% peptone, 0.1% yeast extract, triple distilled water and, where appropriate, 1.1% agar; pH was adjusted to 7.4 to 7.5 using 1 N NaOH.

Temperature effects: The effect of temperature on growth and lysis was tested using the media described above and by incubating the bacterium-phage system in controlled water baths, or, in some cases, by using a Thermal Gradient Block modified from the design of the apparatus described by LANDMAN et al. (1962). The block consists of a solid piece of aluminum 18″ × 36″ × 2″ (ca. 457 mm × 914 mm × 51 mm) scored lengthwise by machined channels 1″ (25.4 mm) deep and 1″ in width. The agar medium (SWA) was poured into the channels, permitted to solidify, and brought to the proper temperature gradient before the bacteria and phage were inoculated. The entire assembly was encased in an insulating styrofoam structure. Temperature gradients were monitored using thermocouples (copper-constantin) attached to a six channel automatic BROWN recorder. Heat inactivation data were obtained by heating small volume suspensions (1 cc) of the bacteriophage in sea-water in controlled water baths.

Pressure effects: Pressure chambers and culture techniques similar to these described by ZOBELL and MORITA (1959) were used to study pressure effects except that SWB was used in place of ZOBELL's 2216 medium (ZOBELL, 1946).

Results

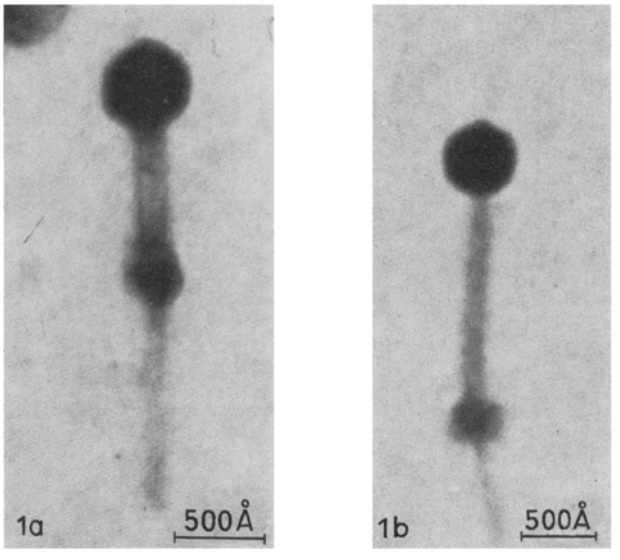
There proved to be only one organism sensitive to phage in the sediment sample among the 25 isolates tested. This was a bacterium designated 8-450-5 [cruise no. 8, 450 fathoms (ca. 832 m) depth, strain number 5] which appears to be an *Aeromonas* species (Table 1), since it is a polarly flagellate, oxidase positive, fermentative, Gram negative, rod shaped organism which is insensitive to the vibriostatic compound 0/129 (COLWELL and LISTON, 1961; SHEWAN et al., 1954). However, unlike terrestrial *Aeromonas* the strain has an apparent requirement for Na^+ and to a lesser extent Mg^{++} and possibly Ca^{++} and it is moderately psychrophilic, growing well at 0 °C but failing to grow at 37 °C.

Table 1. *Characterization of bacterium 8-450-5*

Gram negative	Alanine as sole carbon source: +
Polar flagella	Koser's citrate: +
Rod with tapered ends	NH_3 production: +
Penicillin: resistant	$NO_3 \rightarrow NO_2$: +
Pteridin 0/129: insensitive	Catalase: +
Litmus milk: peptonization	Kovac's oxidase: +
Gelatin: liquefaction	Agar digestion: —
Indole: —	Chitin digestion: —
Methyl red: doubtful +	Cellulose digestion: —
Urease: +	Glucose: acid 24 h — 3 day
H_2S production: —	Galactose: acid 24 h — 3 day
O-F Medium (Leifson):	Cellobiose: acid 24 h — 3 day
Aerobic +	Mannitol: acid 24 h — 3 day
Anaerobic +	
Cystine as sole carbon source: +	

Electron microscopy

The morphology of the lytic bacteriophage is similar to that of *Pseudomonas* phages reported by



Figs. 1a and b. a: Electron micrograph of a positively-stained preparation of marine phage showing overall morphology of head, partially contracted sheath, base plate and core. 300,000 ×. Magnification mark on all pictures is 500 Å. b: Electron micrograph of marine phage showing sheath in uncontracted state. 225,000 × (Photographs by E. S. BOATMAN)

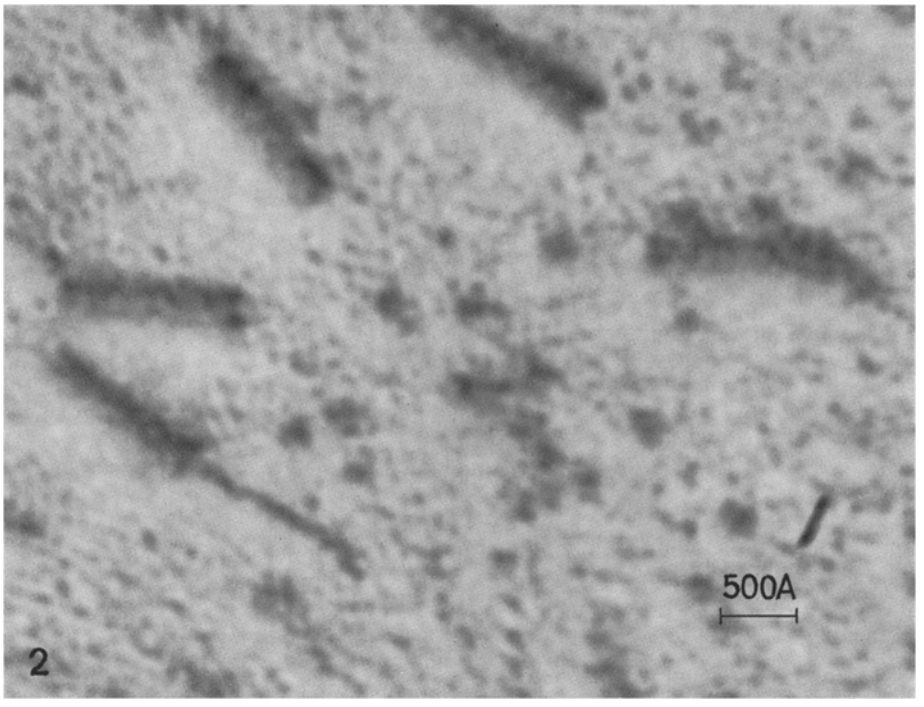


Fig. 2. Electron micrograph of sedimented phage tails after high speed centrifugation. 200,000 ×

other investigators (BRADLEY and KAY, 1960; FEARY et al., 1964). Electron micrographs of the purified phage are shown in Fig. 1a, b. The virus has a hexagonal head of 530 Å diameter, a thin tail 1600 Å in length, consisting of a dense core 100 Å × 1200 Å surrounded by an outer sheath (150 Å expanded, 270 Å contracted diameter), a terminal base plate (400 Å diameter). High speed centrifugation used for the concentration of coliphage (e.g. 50,000 g) disrupted the phage structure so that only tail portions were obtained from the pellet (Fig. 2); thus speeds of 8,000 g for 2 h were routinely used in concentrating this phage.

Temperature effects

The temperature range for growth of the bacterium, 8-450-5, and for its lysis by the phage is shown in Table 2. The bacterium grew well from 0° to 30 °C, poorly from 31° to 33 °C and not at all above 33 °C. Plaque formation, however, could only be demonstrated within the temperature range 0° to 23.5 °C. It is noteworthy that active lysis occurred at 0 °C since previously only SPENCER (1963) and ELDER and TANNER (1928) have demonstrated phage activity at temperatures approaching 0 °C. At the upper temperature limit, not only is there no evidence of lysis above 23.5 °C but apparently irreversable adsorption does not take place above this temperature since, as can be seen from the data in Table 3, there is only a limited reduction in phage titer as a result of exposure of a mixed phage-bacterium preparation to 30 °C for 24 h.

Table 2. *Temperature range of growth of bacterium 8-450-5 and lysis by the marine phage on sea-water agar (SWA). + growth or plaque produced; ∅ slight growth or few plaques produced; — no growth or no plaques produced*

Temperature (°C)	Growth of 8-450-5	Plaque production
0	+	+
5	+	+
10	+	+
15	+	+
20	+	+
23	+	∅
25	+	—
30	+	—
32.5	∅	—
37	—	—

Table 3. *Cell growth and phage production in sea-water broth (SWB) at three temperatures. Initial phage concentration 3 × 10⁶ plaque forming units (pfu)/ml; initial O.D.: 0.005 (660 mμ)*

Incubation temperature	O.D. cells[a]	O.D. cells and phage	Final phage production (pfu)
(°C)	(660) mμ	(660) mμ	
10	.29	.15	1×10^8
20	.50	.24	4×10^8
30	.66	.63	1×10^6

[a] 24 h culture.

Temperature inactivation of the phage is shown in Table 4; rapid activation occurs at temperatures above 45 °C.

Table 4. *Heat inactivation of the phage suspended in buffered sea-water. c: confluent lysis, initial count 10^5 pfu/ml; p: confluent plaques*

Temperature (°C)	Plaque count/ml after indicated exposure time (min) 0	1	2	3	4	5	10	15	20	24 h
45	c	c	c	c	c	c	c	c	c	c
50	c	p	100	10	5	4	0	0	0	0
55	c	300	20	4	0	0	0	0	0	0
60	c	8	0	0	0	0	0	0	0	0

Cation effects

The effects of various concentrations of the cations Na^+, Mg^{++}, and Ca^{++} on growth of 8-450-5 and its lysis by the marine bacteriophage at two temperatures is shown in Table 5. The bacterium grows in 0.085 M NaCl supplemented media, without added Mg^{++} or Ca^{++}, at both 10° and 22 °C, but does not grow in the unsupplemented medium. Supplementation of 0.017 M NaCl with 0.05 M Mg^{++} permits sparse but positive growth at 22 °C but no lysis occurs. While reasonably vigorous growth can be obtained in media containing 0.085 M Na^+, and 0.05 M Mg^{++} or 0.01 M Ca^{++} growth in these media is less vigorous than in sea-water media. Consistent plaque formation occurs only in sea-water media at 10° and 22 °C or in the 0.05 M Mg^{++} supplemented 0.085 M NaCl medium at 10 °C. Limited lysis occurs in the 0.085 M NaCl — 0.01 M Ca^{++} supplemented medium at 10 °C. At 22 °C no lysis was determined in any of the combinations of salts other than sea-water. Sea-water or a more extensively supplemented salts solution is required for lysis at this temperature.

Table 5. *Effect of Na^+, Mg^{++} and Ca^{++} on the growth of bacterium 8-450-5 and lysis by a marine phage. Basal medium: 0.1% peptone; 0.1% yeast extract. + positive growth or lysis; ∅ slight growth or poor lysis; — no growth or lysis*

Salt concentration	Temperature of incubation 10 °C Growth	10 °C Lysis	22 °C Growth	22 °C Lysis
No added NaCl	—	—	—	—
+ 0.05 M Mg^{++}	—	—	—	—
+ 0.01 M Ca^{++}	—	—	—	—
0.017 M NaCl	—	—	—	—
+ 0.05 M Mg^{++}	—	—	∅	—
+ 0.01 M Ca^{++}	—	—	—	—
0.085 M NaCl	∅	—	∅	—
+ 0.05 M Mg^{++}	+	+	+	—
+ 0.01 M Ca^{++}	+	∅	+	—
Sea-water-broth	++	+	+	+

Pressure effects

The hydrostatic pressure at the depth from which the sediment sample was obtained is approximately 1200 p.s.i. (82.5 atmospheres). The results given in Table 6 confirm that the organism can grow and phage replication can occur at this pressure. While there is some decrease in the rate or extent of phage production as pressure is increased, at applied pressures of up to 200 atmospheres, the number of infective particles produced showed a significant increase over the initial concentration of pfu.

Table 6. *Effect of pressure on growth of Aeromonas sp. and on the production of phage*

Pressure (atmospheres)	Final-count organism no./ml	Final-count-phage no. infecting particles/ml
1	2.4×10^7	3.0×10^6
90	2.5×10^7	1.4×10^5
200	1.8×10^7	3.7×10^4

Initial cell count: 9×10^6 cells/ml; initial phage count: 3×10^2 pfu/ml.

Discussion and conclusions

The marine origin of this sediment bacteriophage, is, as was the case for SPENCER's phages (1963), well exemplified by the sensitivity of the lytic system to conditions which deviate significantly from those found in the sea. In addition, however, the ability of the phage to infect and induce lysis in a sensitive bacterium under conditions present in its presumed natural environment including, particularly, elevated pressure, provides support for the hypothesis that phages are actually active in situ in the deep sea.

The high level requirement for cations shown by the marine phage and its sensitivity to temperatures set it apart functionally from the phages normally encountered for terrestrial bacteria. However, it is conceivable that this lower temperature range of inactivation is related in some way to the psychrophilic nature of the sensitive host bacterium rather than to its marine origin per se. Cation requirements for phage adsorption and penetration have been well documented for terrestrial phage (e.g. ADAMS, 1959; FILDES et al., 1953) but the level of their requirements is very much lower than for this marine phage and for those isolated by SPENCER.

Cationic imbalance, particularly deficiencies of various cations are believed to affect the permeability and other properties of cell surface structures in certain marine bacteria (MACLEOD, 1965; BROWN, 1964). Such changes could impede attachment or penetration mechanisms of the phage. It is possible that the failure to obtain lysis at the lower cation concentrations is a manifestation of this effect. The reason for the cessation of lysis at a point approximately 10 °C below the maximum growth temperature for the

bacterium is not apparent since the bacteriophage itself is not inactivated significantly at the maximum temperature of cell growth. Apparently the bacterium-phage system is more sensitive to these factors than either component alone.

Recently a number of authors have reported on the effect of high pressure on bacteriophage structure and function (Solomon et al., 1966; Heden, 1964; Rutberg, 1964 a, b, c). These studies were confined to terrestrial phage, and very high pressures, well beyond those found in the marine environment, were used and thus are not directly applicable to our study. Bacterial growth is limited by a number of factors which operate in pressure vessel experiments. The most important factor is probably an oxygen limitation in the small sealed vials that are placed in the pressure chambers. This is believed to be the principal reason for the constant level of bacterial numbers obtained in our pressure experiments (Table 6). Bacteriophage replication did occur but showed a declining yield of pfu with increasing pressure. There are several possible reasons for this decline but it is not apparently correlated with changes in bacterial numbers, since the yields of cells were identical under all three pressure conditions. Under pressure, the burst size of phage (i.e. number of pfu formed/infected cell) may decrease, there may be an increase in the numbers of defective phage particles produced or the burst time may be greatly extended. At present, however, the actual cause of this decrease in pfu remains a matter for conjecture and further experimentation.

It is of importance, particularly for other investigators concerned with the isolation of marine bacteriophages, to appreciate the deviations in isolation and handling procedures employed for this phage from those commonly employed (Adams, 1959) for terrestrial phages. In this regard, the extreme sensitivity of this virus to chloroform, used routinely to maintain phage stock preparations bacteria free, the low temperature range for phage reproduction, which in this case is 10 °C below the maximum temperature of growth for the host organism, and the deleterious effect of high speed centrifugation used in the concentration of the phage, should be noted. It is at least possible that the lability of marine bacteriophages has been responsible for the small number of reports of their isolation.

The ecological significance of bacteriophages in the marine environment is not known. Clearly, however, they could exert a considerable influence on zymogenous or otherwise derived large clonal populations of bacteria in limiting the numbers, types, and duration of active population growth and ultimately, through possible genetic exchange mechanisms (e.g., transduction), on the biochemical capabilities of microorganisms. This latter phenomenon is of considerable potential importance because of the prominent role of bacteria as mineralizing and chemical transforming agents within the environment.

Summary

1. A bacteriophage lytic for a marine *Aeromonas* sp. was isolated from a deep (825 m) sediment sample.

2. The phage lysed the *Aeromonas* sp. and was replicated under simulated in situ conditions of temperature, salinity and pressure.

3. The conditions for phage replication vis a vis temperature, salinity, and pressure were more restrictive than those for growth of the *Aeromonas* or survival of the bacteriophage alone.

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