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ISOLATION AND PROPERTIES OF BACTERIOPHAGES OF *VIBRIO PARAHAEMOLYTICUS*

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SUMMARY We have isolated three phages of *Vibrio parahaemolyticus* from different sources and investigated some of their properties. The phages were named v6, v12 and v14.

The phages are different in plaque morphology, host range, phage yield and serological character. Physical and chemical studies showed that v12 and v14 phages are normal in many characters tested but v6 is abnormal. The high viscosity of the concentrated preparation, the high sensitivity to sonication and the low density of v6 phage suggested that it is filamentous. This suggestion was confirmed by preliminary electron microscopy.

INTRODUCTION

Vibrio parahaemolyticus is the most widespread of known pathogenic agents causing food-poisoning in Japan, particularly in the summer (FUJINO, 1951; SAKAZAKI *et al.*, 1963). The classification of strains of *Vibrio parahaemolyticus* based on O antigen and K antigen has already been established by SAKAZAKI (1965). We wished to extend the classification by means of phage typing. Thus, we attempted to isolate and characterize bacteriophages of *Vibrio parahaemolyticus*. The present paper describes our results with three phages.

MATERIALS AND METHODS

1. *Bacterial strains*

Ten pilot strains of O antigen and 32 pilot strains of K antigen of *Vibrio parahaemolyticus* were used as indicator strains for bacteriophage detection. These strains were also used for detection of lysogenic phages. We found that K 11, K 12 and K 2 strains of *Vibrio parahaemolyticus* can be indicator strains for the isolated phages v 6, v 12 and v 14, respectively. For propagation of these phages, the same indicator strains were employed.

For host range studies of the three phages, the following bacteria were used: *Vibrio parahaemolyticus*, Heiberg's vibrios (5 strains), Gardner and

Venkatraman's vibrios (6 strains), *Vibrio metchinikovii*, non-agglutinative vibrios (NAG) (5 strains), *Vibrio* spp. (3 strains), *Vibrio anguillarum* (2 strains), *Vibrio ichthyodermis* (2 strains), *Vibrio costicolus*, *Vibrio piscium* var. *japonicus* (2 strains) and *Photobacterium* spp. (6 strains). These strains were kindly supplied by Dr. R. SAKAZAKI.

2. *Bacteriophages*

The isolation and propagation of phages of *Vibrio parahaemolyticus* from a single plaque, and plaque assay, were carried out as described by ADAMS (1959).

Phages were propagated in bacteria which were grown exponentially in 3% NaCl broth and resuspended in 0.5% NaCl broth at a final concentration of 3×10^8/ml. The bacteria were inoculated with a phage multiplicity of 2 to 3 and the infected cultures continuously shaken at 37°C for 3 to 5 hours. Then the phage lysates were centrifuged at 9,000 rpm for 15 minutes (Kubota, type KR-6L) to remove the bacteria and debris.

The supernatants were centrifuged at 30,000 rpm (Spinco L, No. 30 rotor) for 60 minutes. The resulting pellets were resuspended in M/200 phosphate buffer (pH 7.2) or M/100 tris buffer (pH 7.4) and further clarified by low speed centrifugation.

3. *Phage antisera*

Antisera against the isolated phages were prepared in rabbits as described by ADAMS (1959). The K value of the antisera was estimated and the cross neutralization was tested according to Adams' method.

4. *Media*

The bacteria were grown in 3% NaCl broth: polypeptone (Daigo Eiyo Chem. Co., Japan) 10 g, meat extract (Kyokuto Seiyaku Co., Japan) 5 g, NaCl 30 g in distilled water 1,000 ml. The pH was adjusted to 8.0 with 1 N NaOH. The phages were propagated in 0.5% NaCl broth (of the same composition as 3% NaCl broth except 5 gm NaCl). For plaque assay, 0.5% NaCl broth containing 1.5% and 0.7% agar were used as bottom and soft agar layers, respectively.

5. *CsCl density gradient centrifugation of the isolated phages*

Appropriate volumes of saturated CsCl were added to partially purified phage suspensions and centrifuged at 38,000 rpm for 20 hours at 5°C in a Beckman L 2 ultracentrifuge (SW 50 rotor). Fractionation was carried out by collecting samples of 4 drops each from the bottom of the centrifuge tube. The infectivity of each fraction was assayed by plaque count. The density of each fraction was calculated from the refractive index as measured by a Hitachi refractometer, type PR-AB, and the OD_{260} of a ten fold dilution of each fraction was measured in a Beckman DU spectrophotometer.

6. *Sonication*

Phage samples (2×10^8pfu*/ml suspended in 0.5% NaCl broth) were sonicated (Ohtake Model 5205, 20 Kc) and, at intervals, aliquots were removed and assayed for plaque forming units.

7. *Ultraviolet irradiation*

2.5 ml of phage suspensions (2×10^8pfu/ml in M/200 phosphate buffer) were poured into Petri dishes of 9 cm diameter and irradiated in the dark with ultraviolet light, 50 cm from the lamp (National GL 10). At intervals, aliquots were removed and assayed for pfu.

8. *Heat treatment*

Phage suspensions ($3\sim9\times10^7$pfu/ml in 0.5% NaCl broth) were heated in a water bath at 60°C for various periods and assayed for survival. The heat stability of phage v 6 at 70°C to 100°C was examined under the same conditions.

RESULTS

1. *Isolation of three phages*

Phage v12 was isolated on the K12 strain of *Vibrio parahaemolyticus* from a fecal sample obtained from a patient suffering from food poisoning.

Phage v 14 was isolated on the K2 strain from a summer sample of coastal sea water.

Phage v 6 was produced spontaneously at a level of $10^3\sim10^4$ pfu/ml from the culture supernatant of strain K 2 of *V. parahaemolyticus*.

In this paper, studies of phages v6, v12 and v 14 are described. These phages were propagated on K 11, K 12 and K 2 strains, respectively, and their yields are usually 10^{11} pfu/ml for v 6, 10^9 pfu/ml for v 12 and 10^{10}

* plaque forming unit.

pfu/ml for v14, under the conditions employed.

2. *The plaque morphology of the phages*

The plaque of phage v6 on the K11 indicator strain was turbid and 1~1.5 mm in diameter. The plaque of v12 on the K12 indicator strain was 3~4 mm in diameter, and surrounded by a large halo. The plaque of v14 on the K2 indicator strain was turbid and 1~2 mm in diameter. These plaque morphologies are shown in Fig. 1.

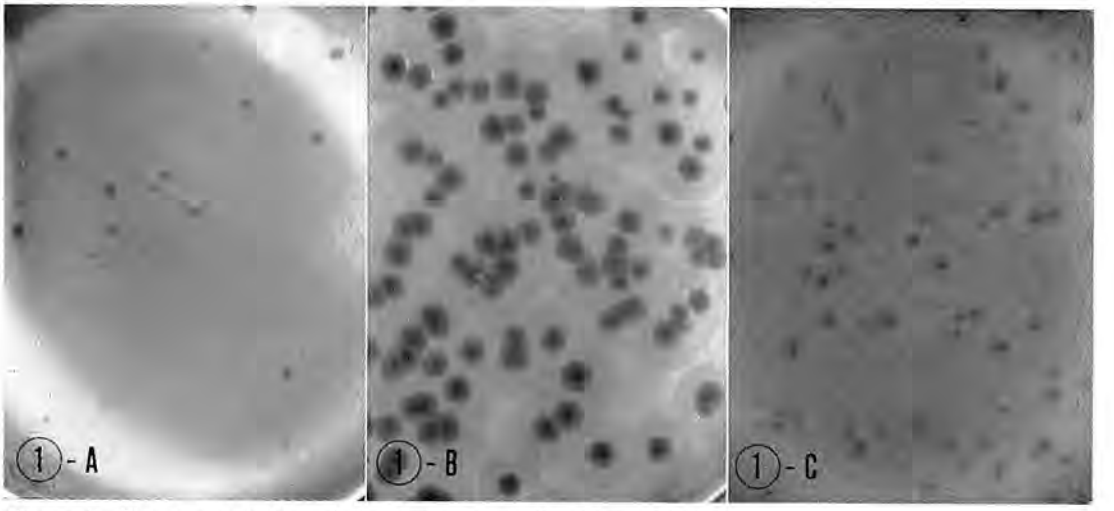

FIGURE 1 Plaque morphology of the three phages v6, v12 and v14.
(A) phage v6 on strain K11 (B) phage v12 on strain K12 (C) phage V14 on strain K2.

3. *The host range of the phages*

The host range was examined by spotting ten-fold dilutions of the three phage lysates on a bacterial agar layer. As shown in Table 1, the host ranges of the three phages are different. Among the various bacteria tested, besides *Vibrio parahaemolyticus*, only one strain of *Photobacterium sepia* was sensitive to phage v14.

Of 32 pilot strains of K antigen, twenty strains are sensitive to phage v6, thirteen strains to phage v12 and seven strains to phage v14. Therefore phage v6 has the widest host range. Six strains are insensitive to all three phages.

4. *Phage v6 production in infected culture*

A culture of K 11 bacteria in 3% NaCl broth was grown with aeration at 37°C to a concentration of 2×10^8 cells/ml. The cells were collected by centrifugation and suspended in 0.5% NaCl broth and divided into 2 parts. One part was infected with phage v6 (multiplicity=5) and left for ten minutes in room temperature, then the antiphage serum was added (1 : 1,000 in final dilution) and left for 5 minutes. The cultures were centrifuged and the cells were suspended in 0.5% NaCl broth at the concentration of 2×10^7 cells/ml. Incubation was carried out for 6 hours with shaking and the optical densities of the cultures and pfu in the culture supernatants were measured periodically. As shown in Fig. 2, the optical densities of the infected culture were never decreased but increased more slowly than the uninfected culture. The pfu in supernatant also increased logarythmically.

TABLE 1 *Host range of the three phages*

Bacterial strain tested		phage v6	phage v12	phage v14
K 1	(1)	+++	++	−
2	(2)	++	−	+++
3	(2)	−	−	−
4	(3)	++	−	−
5	(3)	−	+	−
6	(3)	++	+++	−
7	(3)	++	+	−
8	(4)	+++	++	−
9	(4)	++	−	−
10	(4)	+	++	−
11	(4)	+++	−	++
12	(4)	−	+++	−
13	(4)	++	−	++
14	(5)	−	++	−
15	(5)	−	−	−
16	(5)	−	++	−
17	(5)	−	−	−
18	(6)	+	++	−
19	(7)	+	−	−
20	(8)	−	−	−
21	(8)	−	−	+++
22	(8)	−	−	−
23	(9)	++	++	−
24	(10)	++	++	−
25	(1)	++	−	−
26	(1)	+++	−	−
27	(2)	++	−	+++
28	(2)	++	−	++
29	(3)	−	−	++
30	(3)	++	+++	−
31	(3)	−	−	−
32	(1)	++	−	−

Numbers in parenthesis indicate 0 antigen

5. *Inactivation of the phages by UV irradiation*

Fig. 3 shows the results of the inactivations of the phages by ultraviolet. The inactivations of v12 and v14 phages follow a one hit curve, v12 being more sensitive. On the other hand, the inactivation of v6 follows a multi-hit curve whose final slope is less than that observed for v12 and v14.

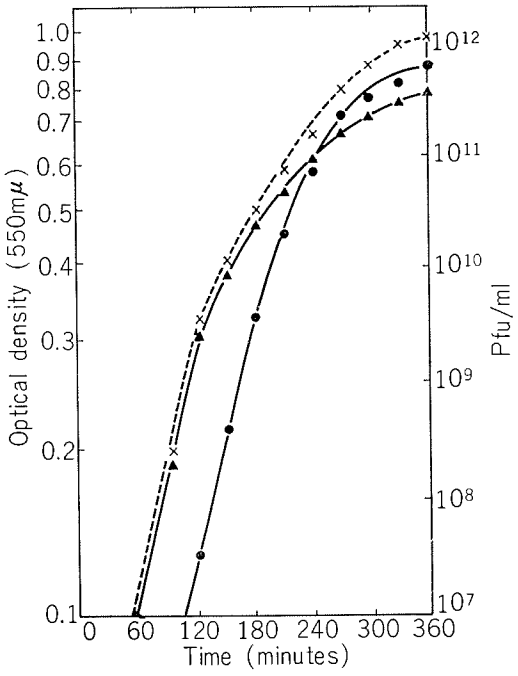

FIGURE 2 Phage v 6 production in infected culture.
×——× optical density of uninfected culture
▲——▲ optical density of infected culture
●——● pfu

6. *The serological character of the phages*

The K values of antiphage sera against homologous and heterologous phages are shown in Table 2. Table 2 indicates that the three phages are serologically different from one another. Since phage v6 has a presumed filamentous form (to be discussed), phage v6 was treated with an anti-filamentous phage serum (antiserum against *Pseudomonas* phage pf, kindly supplied by Prof. TAKEYA of Kyushu University, K580 for pf; diluted to 1/100) but no neutralization was observed. The converse cross reaction was performed by Prof. TAKEYA and no neutralization of phage pf was observed by phage v6 antiserum (TAKEYA, 1966).

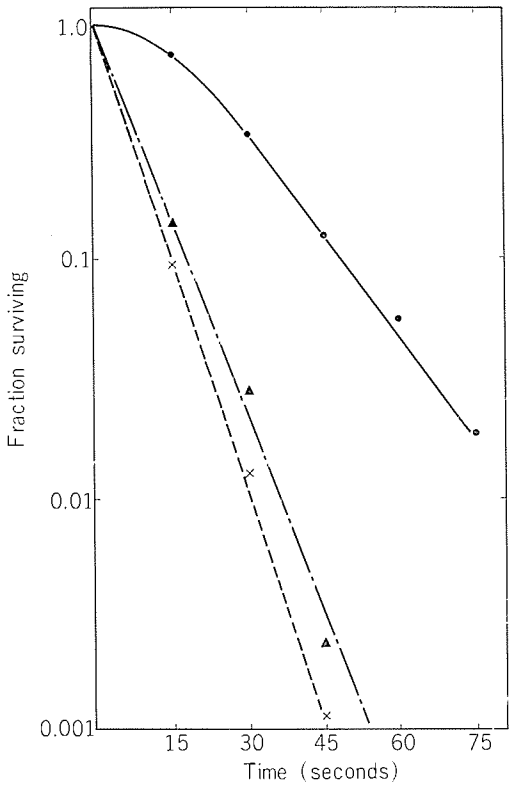

FIGURE 3 Inactivation of the three phages by ultraviolet irradiation.
●——● v6
▲——▲ v12
×——× v14

TABLE 2 *K values of antiphage sera against homologous and heterologous phage*

Antiphage sera	phage		
	v6	v12	v14
v6	1051	0	0
v12	0	86	0
v14	0	0	87

7. *Thermal inactivation of the phages*

When v12 and v14 phages were heated at 60°C, their survival decreased linearly, as shown in Fig. 4. However, v6 is completely resistant to heating at 60°C for 80 minutes (Figs. 4 and 5). The high thermostability of phage v6 raises the question whether the phage is of a filamentous structure, because filamentous phages are known to be highly thermostable (HOFFMANN-BERLING *et al.*, 1963; BRADLEY, 1964). If phage v6 is filamentous, the multihit kinetics of UV inactivation of the phage can be explained in terms of the facility of the filamentous structure to aggregate. Thus, we examined the sensitivity of the phage to sonication, and the density of the phage in comparison with the other two phages, because filamentous phages are known to be very sensitive to sonication and to have a relatively low density (ZINDER, 1963; HOFFMANN-BERLING *et al.*, 1963; BRADLEY, 1964; SALIVAR, 1964).

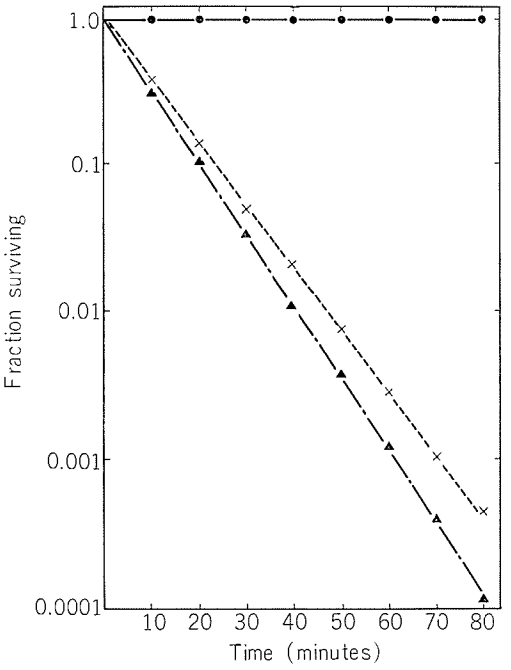

FIGURE 4 Inactivation of the three phages at 60°C.
●——● v6
▲——▲ v12
×——× v14

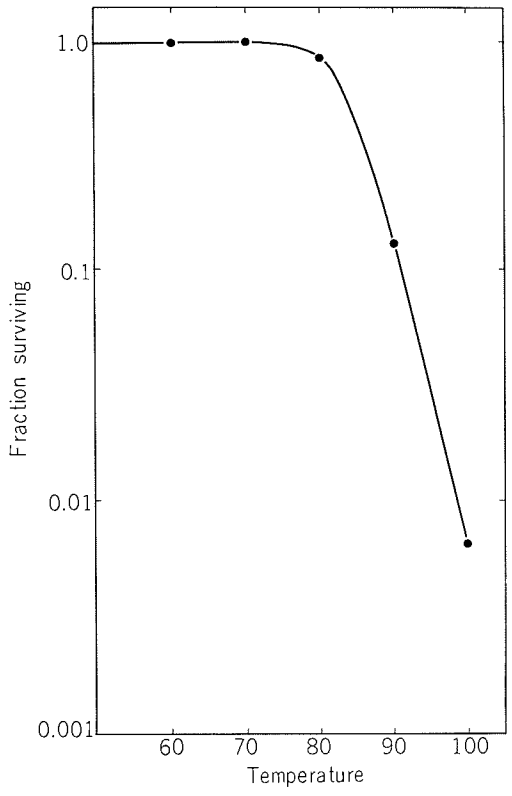

FIGURE 5 Heat treatment of phage v6.
Phage v6 was treated for 15 minutes at the indicated temperature.

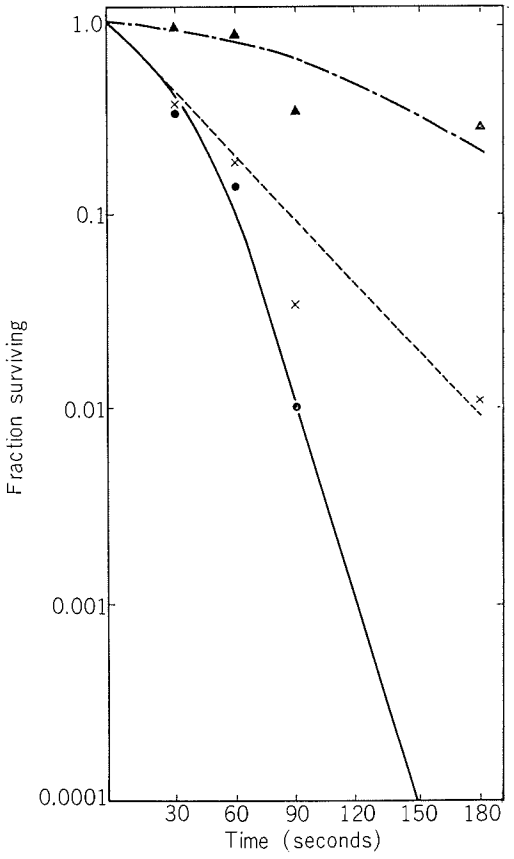

FIGURE 6 Inactivation of the three phages by sonication.
●——● v6
▲——▲ v12
×------× v14

8. *Sensitivity of the phages to sonication*

Fig. 6 shows the inactivation curves of the phages by sonication. Phage v12 is most resistant to sonication and its survival is 10^{-1} after 180 seconds of treatment. Phage v14 is more sensitive and its survival is 10^{-2} after 180 seconds of treatment. Phage v 6 is most sensitive and decreases to 10^{-4} with the same treatment. This high sensitivity of the phage v6 to sonication is consistent with the concept that phage v6 is filamentous.

9. *Inactivation of phage v 6 by chloroform*

We usually had stored these phage suspensions in cold room over chloroform. But, pfu of phage v6 markedly reduced in this conditions, so the following experiments were carried out.

To the 2 ml of phage suspension (3×10^9 pfu/ml in 0.5% NaCl nutrient broth) 0.06 ml of chloroform was added and stoppered, then incubated at 37°C with occasional shaking. At intervals, samples were removed and assayed for pfu. As shown in Fig. 7, phage v6 was sensitive to chloroform.

10. *Densities of the phages in CsCl*

Figs. 8, 9 and 10 show the patterns obtained from CsCl density gradient centrifugation of preparations of v6, v12 and v14. For each phage, the peak of the infective units coincides clearly with that of the absorbancy at 260 mμ. The figures show that the densities of v6, v12 and v14 are 1.32, 1.53 and 1.48, respectively. Further we note that for v6 (Fig. 8) the value of the absorbancy at 260 mμ is relatively small in spite of its high titer of pfu in the peak of the distribution. This result suggests that the nucleic acid content of phage v6 is relatively small. This, as well as the low density of v6, agrees with the hypothesis that phage v6 is filamentous. The densities of v12 and v14 phages are comparable to those of other tadpole-shaped bacteriophages.

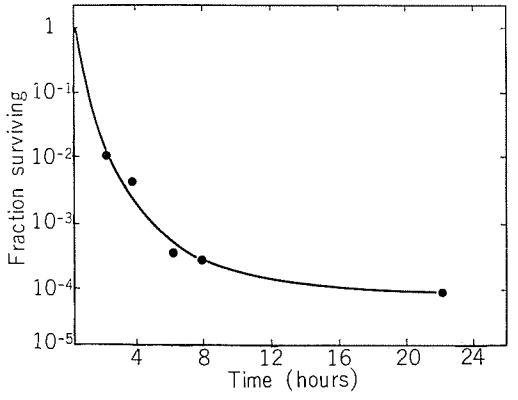

FIGURE 7 Inactivation of phage v6 by chloroform.

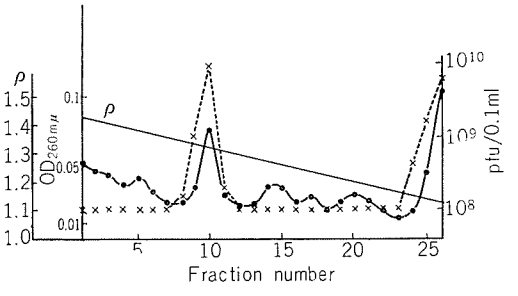

FIGURE 8 CsCl density gradient centrifugation of phage v6.
● ——— ● 260 mμ
× - - - - - × pfu

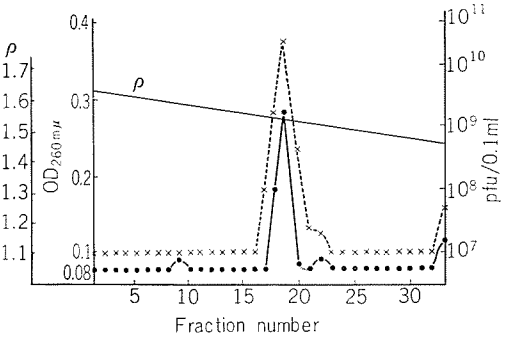

FIGURE 9 CsCl density gradient centrifugation of phage v 12.
● ——— ● 260 mμ
× - - - - - × pfu

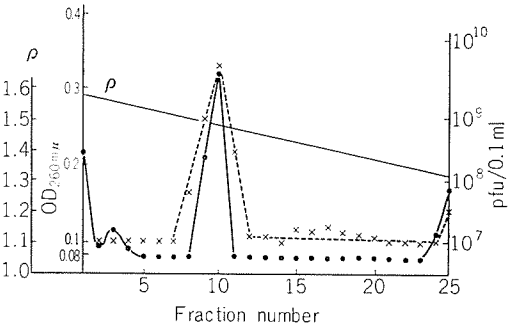

FIGURE 10 CsCl density gradient centrifugation of phage v14.
● ——— ● 260 mμ
× - - - - - × pfu

11. *The nucleic acids of v6 and v14 phages*

Nucleic acid was extracted from v6 and v 14 phages. A phage v 6 lysate was brought to pH 4.0 with concentrated acetic acid and after standing for 1 hour, the flocculating material was collected by centrifugation at 7,000 rpm for 30 minutes (Servall, Type GSA rotor). The sediment was suspended in M/200 phosphate buffer (pH 7.2) and dialysed overnight against the same buffer. The crude phage suspnension was salted out with ammonium sulfate in 25% of the final concentration at 0°C and was centrifuged at 9,000 rpm for 15 minutes (Kubota). The precipitate was dissolved in M/200 phosphate buffer and dialysed against the same buffer. The dialysate

was centrifuged at 15,000 rpm for 30 minutes and the supernatant was centrifuged again at 30,000 rpm for 2 hours (Spinco L, No. 30 rotor). The pellets were suspended in the phosphate buffer. The phage v 6 suspension, thus purified, was extremely viscous. The final preparation was incubated with DNase (50 μg/ml) and RNase (50 μg/ml) in the presence of $MgCl_2$ (M/500) for 3 hours. The suspension was dialysed against the phosphate buffer and clarified by centrifugation at 10,000 rpm for 20 minutes. Sodium dodecyl sulfate was added to the supernatant to a final concentration of 0.5% (pH 8.0 adjusted with 1 N NaOH) and heated at 60°C for 15 minutes. Then the phage v 6 nucleic acid was extracted with phenol according to Hershey's method (1960), precipitated by the addition of 2 volumes of ethanol, and dissolved in M/20 phosphate buffer.

The phage v 14 nucleic acid was extracted by the same method from a partially purified phage preparation (2. of Materials and Methods), but without using sodium dodecyl sulfate.

Diphenylamine and orcinol reactions were used for the qualitative determination of DNA and RNA. Positive diphenylamine reactions by the nucleic acids extracted from the two phages revealed that the phages contain DNA. Orcinol reactions of the nucleic acids were negligible. If there is any RNA, the RNA content is less than 5% for each of the two phages.

The hyperchromicity as a function of temperature for phage v14 DNA is shown in Table 3; the absorption of v14 DNA remains almost constant until 80°C and then increases abruptly. These results suggest that v 14 DNA is of a double-stranded nature.

TABLE 3 *The effect of heating at indicated temperatures for 10 minutes on the absorbance at 260 mμ of v14 DNA*

Treatment	E 260	Increment percent
None	0.610	
70°C	0.610	0
80°C	0.680	11.4
90°C	0.810	32.8
100°C	0.810	32.8

The suspending medium was phosphate buffer (M/45, pH 7.4).

DISCUSSION

We have isolated 3 phages of *Vibrio parahaemolyticus*. Physical and chemical studies of these phages showed that the v12 and v14 phages are normal in many characters tested and v6 is abnormal. Electron-microscopic studies of these phages, though incomplete, indicate that v12 and v14 phages are tadpole-shaped and v6 is filamentous. The unusual multiple hit curve in UV irradiation, the high thermal-stability, the high sensitivity to sonication, the low density and the high viscosity of the concentrated preparation of phage v6, all agree with the morphological data and indicate that v6 is filamentous; furthermore, preliminary studies on the hyperchromicity of v6 DNA suggest that the DNA of phage v6 is single stranded. These properties were observed in other filamentous phages, although no report mentioned all four characteristics (HOFSCHNEIDER, 1963; HOFFMANN-BERLING *et al.*, 1963; ZINDER *et al.*, 1963; BRADLEY, 1964; SALIVAR *et al.*, 1964; TAKEYA and AMAKO, 1966). The high yield of phage without lysis of the host cells in broth, as seen in v6, was also reported for other filamentous phages (HOFSCHNEIDER, 1963; HOFFMANN-BERLING and MAZÉ, 1964; BRADLEY, 1964; TAKEYA and AMAKO, 1966). The multi hit curve of v6 by UV-irradiation can be explained by the occurrence of aggregates of filamentous phages.

We found that phage v6 is sensitive to chloroform, while v12 and v14 are resistant. It is known that the other filamentous phages,

M 13 (HOFSCHNEIDER, 1963), fd (HOFFMANN-BERLING *et al.*, 1963), f 1 (ZINDER *et al.*, 1963) and ZJ/2 (BRADLEY, 1964) of *Escherichia coli* are all male-specific, except phage pf of *Pseudomonas aeruginosa* (TAKEYA and AMAKO, 1966). The sex dependency of phage v6 remains to be determined.

The wide host range of phage v6 is interesting. The biological properties of filamentous phages, such as penetration into the host cells and liberation from the host cells, and their fine structures not yet known. Studies on these problems are in progress.

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