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Morphological Varieties and Host Ranges of *Vibrio parahaemolyticus* Bacteriophages Isolated from Seawater

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Eighteen bacteriophages lytic for *Vibrio parahaemolyticus* were isolated from seawater, and their ultrastructure was examined by electron microscopy. Based on the phage morphology, they were classified in four groups. Group I phages consisted of a hexagonal head and a tail with a contractile sheath. All the phages of the other three groups had a relatively long, noncontractile tail, but there were differences in the head structure among these phages. The phages of groups II and III had a hexagonal head and an elongated polyhedral head, respectively. Group IV phages exhibited a unique hexagonal head with knoblike projections. There appeared to be no correlation between the O and K serotypes of *V. parahaemolyticus* strains and the host ranges of the phages. The phages had varying sensitivities to heat and organic solvents.

Vibrio parahaemolyticus, a most important causative bacterium of food poisoning in Japan, resides in the near-shore marine environment (8, 10). The first isolation of bacteriophages specific for this bacterium was reported in 1966 by Nakanishi et al. (9). The three different phages described were isolated from seawater, human feces, and a lysogenic strain of the bacterium. Later, Baross et al. (2–4) isolated numbers of *V. parahaemolyticus* phages from marine samples. However, until now no information has been available about the morphology of these phages except for one phage that is structurally similar to the T phages of *Escherichia coli* (11). We have attempted to isolate phages for the vibrio to investigate the ultrastructure of the phages. The structure of a novel phage, VP3, exhibiting knoblike projections around the head, was recently reported (7). This paper describes the morphological variety and host ranges of 18 *V. parahaemolyticus* phages isolated from seawater. Based on the morphological characteristics, these phages are divided into four groups, and their sensitivities to heat and organic solvents are examined.

MATERIALS AND METHODS

Bacterial strains and growth. Ten K serotype pilot strains and four other strains of *V. parahaemolyticus* were used in this study (Tables 1 and 2). In addition, four K serotype pilot strains (K-29, K-41, K-46, and K-56), which are not listed in the tables, were used. All the strains, except strain 3283-61, which has been kept in our laboratory for many years, were supplied by the Research Institute for Microbial Diseases, Osaka University. For isolation and propagation of phages, 3% NaCl broth (9) was routinely used. Bacteria were grown in the broth at 30°C with gentle shaking. Solid medium was composed of 3% NaCl broth containing 1.5 and 0.6% agar for the bottom and top agar layers, respectively, for phage assay.

Isolation of bacteriophages. Samples of coastal seawater were harvested near Tokushima during the summer months. Seawater samples were mixed with equal volumes of double-strength 3% NaCl broth. The enrichment mixtures were incubated with log-phase cultures of host strains and incubated further at 30°C for 5 h. Single-plaque isolation from the enrichment culture was performed repeatedly according to the method of Adams (1) as described previously (7).

Phage assay. Phage titers were determined as plaque-forming units (PFU) by the agar layer method (1) as described earlier (7). After incubation overnight at 30°C, the efficiency of plating was estimated relative to that on the host strain which produced the highest phage titer for each phage. The host range was determined by qualitative spot tests on lawns of different strains by using phage stocks containing about 10^8 PFU/ml.

Preparation of phage stock. Phage stock was prepared as described previously (7, 12). Phages were propagated in the log-phase culture of the host strain in 3% NaCl broth by incubation at 30°C for 5 h. After unlysed cells were removed from the culture by centrifugation at 3,000 × *g* for 10 min, phages in the supernatant were precipitated by adding 10% (wt/vol) polyethylene glycol 6000 and 1 M NaCl and centrifuging at 8,000 × *g* for 10 min. The pellet composed of phage particles was resuspended in AAM solution (7) containing 1% ammonium acetate and 0.01 $MgCl_2$, and the resulting phage stock was stored in the cold.

Sensitivity of phages to heat. Samples (1 ml) of phage preparation containing about 10^8 PFU/ml in 3% NaCl broth were maintained at desired temperatures for 30 min, then immediately cooled in ice water and assayed for phage titers. The temperature at which a decrease in phage titer of more than 90% occurred was scored.

Sensitivity of phages to organic solvents. Phage sensitivity to the organic solvents toluene, ethyl ether, and

chloroform was examined by the method of Carvalho and Vary (6), except that 3% NaCl broth was used for the preparation of phage lysate and for phage assay. Samples (1 ml) of diluted phage lysate containing about 2×10^5 PFU/ml in 3% NaCl broth were mixed with each solvent (0.2 ml) by stirring gently with a Thermo-mixer (Theromics Co., Ltd., Tokyo) for 15 s. After incubation at 30°C for 30 min, the aqueous phase of the mixtures was assayed for viability of phages.

Electron microscopy. Phage stock was fixed with 5% Formalin, washed with distilled water, and then negatively stained with 1% ammonium molybdate in 0.1 M ammonium acetate buffer (pH 7.2) as described previously (12). Samples were examined in a Hitachi HU-11E electron microscope with an accelerating voltage of 75 kV.

RESULTS AND DISCUSSION

Morphological grouping of phages. Bacteriophages exhibit a great diversity in their morphology. Bradley (5) proposed six basic morphological types, A through F. So far there has been no morphological classification of *V. parahaemolyticus* phages. In the present study, we isolated 18 phages for the vibrio from coastal seawater during the summer months. The electron micrographs of some typical phages are shown in Fig. 1 and 2. We divided the phages into four different groups, I through IV, as judged by their morphological variety. The 10 group I phages differed from each other in dimensions but were similar in having a hexagonal head and a tail with a contractile sheath (Fig. 1a-c). Therefore, this group belongs to type A according to the classification of Bradley (5). Their heads varied from 109 by 103 nm (VP10) to 65 by 60 nm (VP2, Fig. 1b) in dimension, and the tail contained a base plate with several pins and tail fibers. Some phages of this group had a base plate with large pins (Fig. 1c).

Eight phages, other than group I phages, were composed of a hexagonal or polyhedral head and a long, noncontractile tail, so they belong to Bradley's type B (5). These phages were divided into three groups, II, III, and IV, based on the head morphology (Fig. 2). Group II phages had a hexagonal head with a long, flexible tail (Fig. 2a and b). The dimensions of the head ranged from 79 by 65 to 92 by 89 nm. Group III phages exhibited an elongated polyhedral head and a long, flexible tail (Fig. 2c and d). The dimensions of the head varied from 111 by 73 nm to 91 by 53 nm. The distal ends of their tails tended to adhere to each other. Group IV phages consisted of a hexagonal head about 78 by 74 to 78 nm and a flexible tail with cross striations (Fig. 2e). The appearance of the capsid covered with the knoblike projections was most conspicuous, since the detailed structure of phage VP3 has been described (7). The knoblike particles, about 7 to 8 nm in diameter, had a hollow about

TABLE 1. Host ranges of group I bacteriophages for *V. parahaemolyticus*

Strain	Serotype	EOP[a]									
		VP1	VP2	VP4	VP7	VP8	VP9	VP10	VP17	VP18	VP19
EB101	O1:K1	—	1.0	0.87	—	—	0.48	—	0.79	0.86	0.37
3283-61	O2:K3	0.65	—	2.3×10^{-2}	1.0	1.0	0.34	1.0	3.8×10^{-3}	0.94	8.6×10^{-4}
K-3 pilot	O2:K3	0.24	—	—	0.15	0.14	3.2×10^{-2}	9.6×10^{-2}	—	3.9×10^{-2}	—
K-28 pilot	O2:K28	2.0×10^{-3}	—	0.12	3.7×10^{-5}	—	0.26	—	9.4×10^{-2}	0.56	9.6×10^{-3}
K-45 pilot	O3:K45	7.0×10^{-2}	—	5.8×10^{-2}	5.2×10^{-2}	—	1.0	—	2.6×10^{-4}	0.30	0.16
K-11 pilot	O4:K11	1.1×10^{-2}	2.2×10^{-3}	3.3×10^{-2}	7.1×10^{-3}	4.5×10^{-2}	6.0×10^{-2}	5.6×10^{-2}	3.4×10^{-2}	0.11	3.2×10^{-2}
K-47 pilot	O5:K47	—	—	9.6×10^{-4}	—	—	2.0×10^{-4}	—	—	—	—
K-20 pilot	O8:K20	3.5×10^{-5}	—	1.2×10^{-3}	—	—	—	—	—	—	—
K-36 pilot	O11:K36	9.8×10^{-3}	—	1.0	2.9×10^{-2}	7.1×10^{-3}	0.11	1.9×10^{-2}	1.0	1.0	1.0
K-40 pilot	O11:K40	1.0	—	—	0.52	0.29	—	2.1×10^{-2}	—	—	—

[a] Efficiency of plating (EOP) was determined relative to that on the strain which produced the highest phage titer for each phage. —, No plaques were formed when a drop of phage stock containing about 10^8 PFU/ml was deposited on bacterial lawns.

TABLE 2. Host ranges of group II, III, and IV bacteriophages for *V. parahaemolyticus*

Strain	Serotype	EOP[a]							
		Group II			Group III			Group IV	
		VP11	VP12	VP13	VP5	VP15	VP16	VP3	VP6
3283-61	O2:K3	1.0	0.92	1.0	—	—	—	0.91	0.74
K-3 pilot	O2:K3	0.61	0.64	0.2	—	—	—	8.2×10^{-3}	1.3×10^{-8}
K-28 pilot	O2:K28	—	2.0×10^{-2}	—	—	4.8×10^{-2}	0.19	4.7×10^{-2}	0.15
K-43 pilot	O3:K43	—	—	—	2.6×10^{-2}	—	—	—	—
MY-78-94	O4:K17	—	—	—	0.70	—	—	1.1×10^{-2}	—
K-42 pilot	O4:K42	—	—	—	—	0.92	—	0.52	0.48
K-47 pilot	O5:K47	—	—	1.3×10^{-3}	0.34	—	—	0.68	0.44
Y-75-1	O8:K20	—	—	—	—	—	—	1.0	0.70
K-20 pilot	O8:K20	—	1.0	2.5×10^{-2}	—	—	—	0.91	0.48
K-36 pilot	O11:K36	—	0.56	1.0×10^{-3}	—	—	—	0.75	1.0
K-40 pilot	O11:K40	—	8.2×10^{-2}	—	1.0	1.0	1.0	0.12	0.11

[a] Efficiency of plating (EOP) was determined relative to that on the strain which produced the highest phage titer for each phage. —, No plaques were formed when a drop of phage stock containing about 10^8 PFU/ml was deposited on bacterial lawns.

3 nm in diameter. The structure of phage VP6 was quite similar to that of phage VP3. Nakanishi et al. (9) reported a filamentous phage, V6, for *V. parahaemolyticus*, but they showed no electron micrograph of the phage. We have not yet isolated such filamentous phages for the vibrio.

Host range. Eighteen strains of *V. parahaemolyticus* representing 8 O and 16 K serotypes were examined for susceptibility to the 18 phage isolates. The results, summarized in Tables 1 and 2, show a wide variation in phage susceptibilities among the vibrio strains used. Strain 3283-61 had the widest sensitivity to the phages of groups I (except VP2), II, and IV, but was not sensitive to group III phages. Pilot strain K-11 was lytic to all the group I phages but resistant to all the group II, III, and IV phages. Strain Y-75-1 (O8:K20) was lysed only by group IV phages. Pilot strain K-40 was lysed by all the group III phages, exhibiting the highest efficiency of plating, but it was also sensitive to the other three groups. On the other hand, four K pilot strains (K-29, K-41, K-46, and K-56) were not lysed by any of the phages isolated (data not shown). Three phages, VP2 (group I), VP2 (group II), and VP16 (group III), were found to have the narrowest lytic spectrum, and only two strains of *V. parahaemolyticus* were sensitive to these phages. In general, there was little correlation

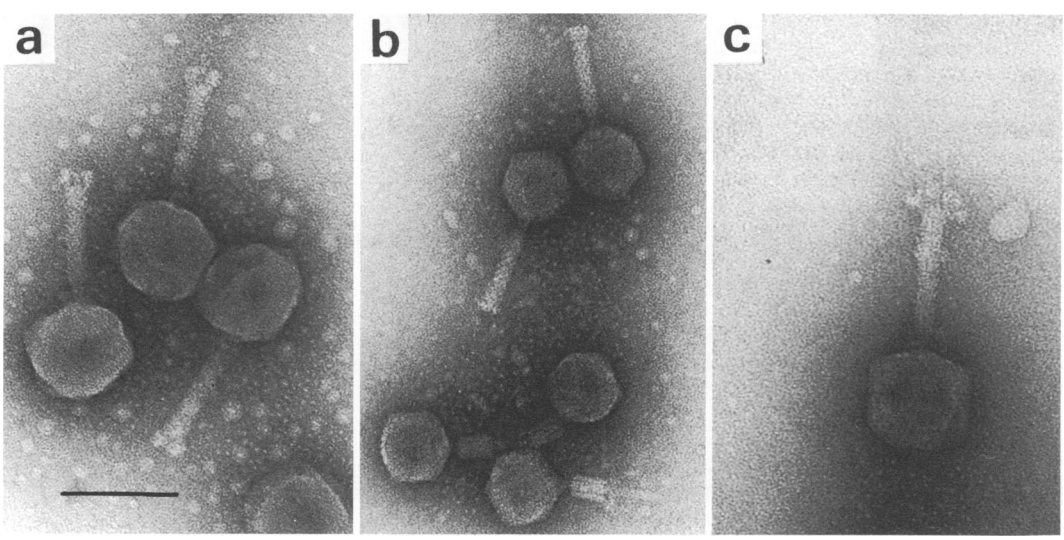

FIG. 1. Group I phages for *V. parahaemolyticus*, negatively stained with ammonium molybdate. Bar, 100 nm. All the electron micrographs are at the same magnification. (a) VP1. (b) VP2. (c) VP18.

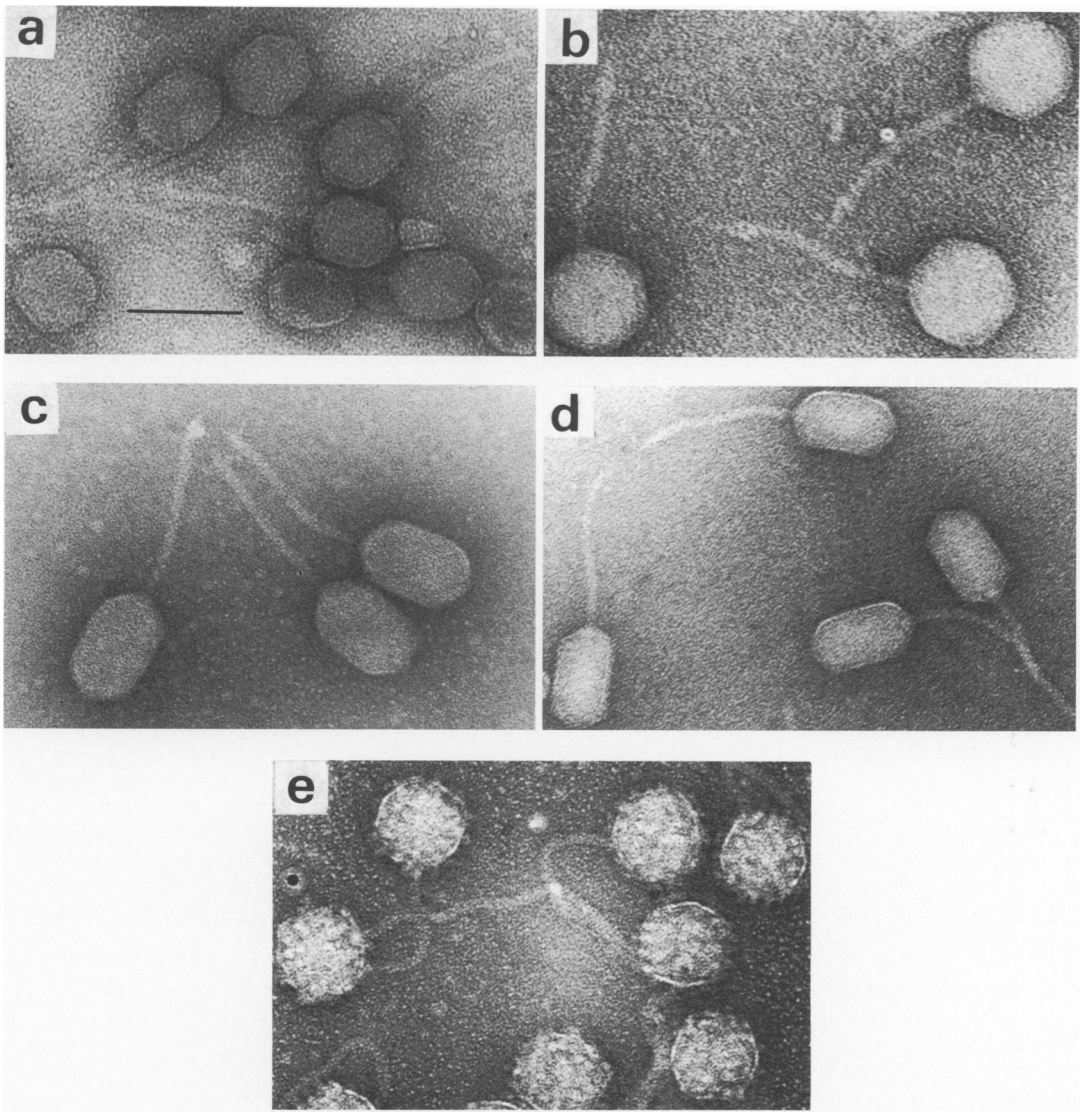

FIG. 2. Group II, III, and IV phages for *V. parahaemolyticus*, negatively stained with ammonium molybdate. Bar, 100 nm. Group II phages: (a) VP11; (b) VP12. Group III phages: (c) VP5; (d) VP16. Group IV phage: (e) VP6.

between the O and K serotypes of *V. parahaemolyticus* and the host ranges of the phage isolates. Baross et al. (4) also reported no correlation between the serotypes of the vibrio and phage lytic spectra.

Sensitivity to heat and organic solvents. All the phages of group I were almost totally inactivated when exposed to 55°C for 30 min. Some of the phages (VP4, VP9, VP17, and VP19) were very heat sensitive, and they lost lytic activity at 45°C. Group II, III, and IV phages were generally more heat resistant than group I phages, and they were inactivated at 60°C for 30 min. Nakanishi et al. (9) reported that a filamentous phage for *V. parahaemolyticus* was completely resistant to heating at 60°C for 80 min. None of the phages examined in this study was filamentous, and all the phages were sensitive to heating at 60°C for 30 min.

Most of the phages were more sensitive to ethyl ether and chloroform than to toluene. Group I phages exhibited a wide variation in sensitivity to the organic solvents. Group II (except VP13), III, and IV phages were more sensitive to chloroform than to ether. Phage VP13 was considerably resistant to all the organ-

ic solvents tested. Thus, the phages for *V. parahaemolyticus* had varying sensitivities to organic solvents.

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LITERATURE CITED

1. **Adams, M. H.** 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. **Baross, J. A., and J. Liston.** 1968. Isolation of *Vibrio parahaemolyticus* from the Northwest Pacific. Nature (London) **217**:1263–1264.
3. **Baross, J. A., J. Liston, and R. Y. Morita.** 1978. Incidence of *Vibrio parahaemolyticus* bacteriophages and other *Vibrio* bacteriophages in marine samples. Appl. Environ. Microbiol. **36**:492–499.
4. **Baross, J. A., J. Liston, and R. Y. Morita.** 1978. Ecological relationship between *Vibrio parahaemolyticus* and agar-digesting vibrios as evidenced by bacteriophage susceptibility patterns. Appl. Environ. Microbiol. **36**:500–505.
5. **Bradley, D. E.** 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. **31**:230–314.
6. **Carvalho, P. M., and J. C. Vary.** 1977. Isolation and characterization of a *Bacillus megaterium* QM B1551 bacteriophages. J. Gen. Virol. **36**:547–550.
7. **Koga, T., and T. Kawata.** 1981. Structure of a novel bacteriophage VP3 for *Vibrio parahaemolyticus*. Microbiol. Immunol. **25**:737–740.
8. **Miwatani, T., and Y. Takeda.** 1976. *Vibrio parahaemolyticus*. A causative bacterium of food poisoning. Saikon Publishing Co., Ltd., Tokyo.
9. **Nakanishi, H., Y. Iida, K. Maeshima, T. Teramoto, Y. Hosaka, and M. Ozaki.** 1966. Isolation and properties of bacteriophages of *Vibrio parahaemolyticus*. Biken J. **9**:149–157.
10. **Sakazaki, R., S. Iwanami, and H. Fukumi.** 1963. Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. I. Morphological, cultural and biochemical properties and its taxonomical position. Jpn. J. Med. Sci. Biol. **16**:161–188.
11. **Sklarow, S. S., R. R. Colwell, G. B. Chapman, and S. F. Zane.** 1973. Characteristics of a *Vibrio parahaemolyticus* bacteriophage isolated from Atlantic coast sediment. Can. J. Microbiol. **19**:1519–1520.
12. **Takumi, K., T. Kinouchi, and T. Kawata.** 1980. Isolation of two inducible bacteriophages from *Clostridium botulinum* type A 190L. FEMS Microbiol. Lett. **9**:23–27.