APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1997, p. 2464–2467 Vol. 63, No. 6
0099-2240/97/$04.00+0

Distribution of *Vibrio vulnificus* Phage in Oyster Tissues and Other Estuarine Habitats

ANGELO DEPAOLA,[1]* STACEY MCLEROY,[2] AND GEORGE MCMANUS[2]

Gulf Coast Seafood Laboratory, U.S. Food and Drug Administration, Dauphin Island, Alabama 36528,[1] and Department of Marine Sciences, University of Connecticut, Groton, Connecticut 06340[2]

Received 14 June 1996/Accepted 24 March 1997

Phages lytic to *Vibrio vulnificus* were found in estuarine waters, sediments, plankton, crustacea, molluscan shellfish, and the intestines of finfish of the U.S. Gulf Coast, but no apparent relationship between densities of *V. vulnificus* and its phages was observed. Phage diversity and abundance in molluscan shellfish were much greater than in other habitats. *V. vulnificus* phages isolated from oysters did not lyse other mesophilic bacteria also isolated from oysters. Both *V. vulnificus* and its phages were found in a variety of oyster tissues and fluids with lowest densities in the hemolymph and mantle fluid. These findings suggest a close ecological relationship between *V. vulnificus* phages and molluscan shellfish.

Vibrio vulnificus is a naturally occurring estuarine bacterium (7, 14, 15, 17, 18, 23, 25) capable of causing primary septicemia or gastroenteritis after its ingestion and secondary septicemia through skin lesions in individuals with underlying chronic diseases (4, 16). *V. vulnificus* is abundant in a variety of estuarine habitats, and highest levels have been reported in the intestines of finfish (7). Most foodborne illness, however, is linked to consumption of raw oysters (12). Water temperature and salinity have been shown to influence its growth and survival (13, 15). Biological factors may influence the fate of *V. vulnificus* in oysters or other estuarine environments because it is readily phagocytized by oyster hemocytes (11).

Phages lytic to *V. vulnificus* were recently discovered in estuarine water samples collected from Louisiana (19). We have found a diverse group of *V. vulnificus* phages to be abundant in Gulf Coast oysters throughout the year (8). Investigators have recently suggested that phages may play an important role in the control of microbial populations in estuarine and coastal environments (6, 10, 20).

We examined the distribution of *V. vulnificus* and its phages in various oysters tissues and fluids and in other estuarine habitats. We report a much stronger association between *V. vulnificus* and its phages in molluscan shellfish than in other estuarine habitats.

Water, sediment, oysters, plankton, and other biota in Mobile Bay, Ala., were sampled ($n = 1$) on four dates covering various environmental conditions and analyzed for abundance of *V. vulnificus* and for phages lytic to any of five different *V. vulnificus* strains varying in virulence and source. We also examined different oyster tissues and fluids to evaluate the extent of host or phage localization and examined the susceptibility of other bacteria isolated from oysters to be lysed by phages known to infect *V. vulnificus.*

V. vulnificus was enumerated by most-probable-number analysis with enzyme immunoassay identification by *FDA Bacteriological Analytical Manual* methodology (9).

V. vulnificus phages were detected and enumerated with the following strains: A-9 (moderately virulent environmental isolate) and J-7 (virulent environmental isolate), both provided by Jerry Stelma, Environmental Protection Agency, Cincinnati, Ohio; VBNO (blue crab isolate), provided by Ron Sizemore, University of North Carolina-Wilmington; 304C (oyster isolate), provided by David Cook of our laboratory; and MO6-24 (human primary septicemia blood isolate), provided by Glenn Morris, Center for Vaccine Development, University of Maryland, Baltimore. Virulence was determined by the method of Stelma et al. (21); isolate A-9 was lethal to mice that were simultaneously iron overloaded and immunosuppressed, and isolate J-7 was lethal to mice that were iron overloaded.

All samples were analyzed quantitatively with a direct phage enumeration procedure by using the five *V. vulnificus* host strains described above. All media and diluents were prepared with diluted natural seawater, and all incubations were at 26°C. The seawater (35 ppt salinity) was collected 50 km offshore in the northern Gulf of Mexico, filtered through 0.2-μm-pore-size cellulose acetate bottle top filters (Corning Glass Works, Corning, N.Y.), and diluted with deionized water to 20 ppt salinity. Casamino Acids peptone marine (CPM) broth, previously used for isolation of phages from seawater (22), was modified and contained 5.0 g of Casamino Acids (Difco), 5.0 g of Bacto Peptone (Difco), and 1.0 liter of seawater, was autoclaved for 15 min at 121°C, and was used as the growth medium for host strains (21a). Sample homogenates were centrifuged for 10 min at 1,800 × *g* (Beckman GPR centrifuge at 20°C), and serial 10-fold dilutions were prepared in sterile seawater. Portions (0.1 ml) of each dilution were added to 0.2 ml of log-phase host cultures for 15 min, and lytic phages were enumerated by using the soft-agar overlay technique (1). The plating medium and soft-agar overlay were prepared with CPM medium supplemented with 1.5 and 0.7% Bacto Agar (Difco), respectively. Plates were incubated at 26°C, and plaques were counted at 24 and 48 h.

A qualitative enrichment technique also was used with water samples because phages were not detected by using the direct phage enumeration procedure. The enrichment procedure involved the addition of seawater (100-, 10-, and 1-ml aliquots) to log-phase cultures of the host strain in 100 ml of CPM broth. This procedure was more sensitive but also much more laborious than the direct enumeration procedure, and only isolate MO6-24 was used as a host strain because it gave better phage recoveries than the other host strains. The enrichment mixtures were incubated with shaking (50 rpm) for 48 h, and 10-ml aliquots were centrifuged, filtered, diluted, and assayed for

* Corresponding author. Mailing address: Gulf Coast Seafood Laboratory, U.S. Food and Drug Administration, P.O. Box 158, Dauphin Island, AL 36528. Phone: (334) 694-4480. Fax: (334) 694-4477. E-mail: AXD@fdacf.ssw.dhhs.gov.

TABLE 1. *V. vulnificus* and phage densities in estuarine samples

Sample type[a]	*V. vulnificus* most probable no. g^{-1} (log_{10})	PFU g^{-1} (log_{10}) of *V. vulnificus* host strain:				
		A-9	J-7	VBNO	304C	MO-624
Water[b]	−1.0	—[f]	—	—	—	—
Sediment	0.3	1.0	—	—	—	—
Oyster	1.2	—	4.1	1.0	2.3	4.2
Plankton	>2.0	—	—	—	—	—
Mud crab	1.4	1.3	1.0	—	—	—
Black drum	>4.4	—	—	—	—	—
Water[c]	2.4	—	—	—	—	—
Sediment	4.4	—	—	—	—	—
Oyster	3.7	3.0	4.1	1.3	2.9	4.3
Ribbed mussel	4.0	3.4	3.6	1.3	3.4	3.8
Plankton	4.7	1.3	—	—	2.7	1.0
Mud crab	4.4	1.3	1.3	—	—	1.0
Pigfish	6.6	—	—	—	2.3	—
Pinfish	7.2	—	—	2.1	2.4	2.4
Water[d]	1.4	—	—	—	—	—
Sediment	4.0	—	—	—	—	—
Oyster	2.6	—	3.4	—	—	4.3
Ribbed mussel	3.6	3.2	4.2	—	3.2	4.6
Plankton	4.4	—	—	—	—	—
Mud crab	>5.0	—	—	—	—	—
Shrimp	4.4	—	—	—	—	—
Pinfish	7.0	—	—	2.9	—	2.1
Sheepshead	6.2	—	—	—	—	—
Water[e]	−0.7	—	—	—	—	—
Sediment	1.2	—	—	—	—	—
Oyster	1.0	2.2	3.2	2.0	2.3	4.2
Ribbed mussel	3.4	3.0	3.5	—	3.1	3.8
Plankton	2.4	—	—	—	—	—
Mud crab	1.3	—	—	—	—	—
Sheepshead 1	6.2	—	—	—	—	2.2
Sheepshead 2	6.0	—	—	—	—	2.3

[a] $n = 1$.
[b] Samples below this superscript were collected and analyzed on 27 March, 1995, when the water temperature was 21.9°C and the salinity was 16.4 ppt.
[c] Samples below this superscript were collected and analyzed on 1 May, 1995, when the water temperature as 24.7°C and the salinity was 11.2 ppt.
[d] Samples below this superscript were collected and analyzed on 9 September, 1996, when the water temperature was 30.6°C and the salinity was 24.0 ppt.
[e] Samples below this superscript were collected and analyzed on 4 December, 1996, when the water temperature was 17.5°C and the salinity was 12.0 ppt.
[f] —, not detected (<10 PFU/g or ml).

lytic phages as described for the direct phage enumeration procedure.

Water, sediment, oysters (*Crassostrea virginica*), mussels (*Geukensia demissa*), plankton, mud crabs (*Panopeus* sp.), and fish, including pigfish (*Orthopristis chrysoptera*), black drum (*Pogonias cromis*), pinfish (*Lagadon rhomboides*), and sheepshead (*Archosargus probatocephalus*), were collected on four dates (27 March 1995, 1 May 1995, 9 September 1996, and 4 December 1996). Mussels were not gathered on 27 March 1995, brown shrimp (*Penaeus aztecus*) were gathered only on 9 September 1996, and different fish species were collected on each date. Surface water was collected in a sterile 500-ml polyethylene bottle (2). Oysters, mussels, mud crabs, and sediment were collected with hand tongs and placed in plastic bags or separate 50-ml polypropylene centrifuge tubes, and fish were caught with a hook and line and placed in separate plastic bags as previously described (7). A 0.5-m-diameter, 63-μm-mesh net was towed for 5 min at a speed of approximately 0.5 m/s to sample plankton. The plankton was diluted with seawater into a polyethylene bottle. All samples were placed in a cooler and transported back to the laboratory within 1 h and stored at 3°C for up to 3 h before analysis.

In the laboratory, crabs, mussels, and plankton were ground with a glass tissue grinder and pestle (Wheaton, Kontes Glass Co.). An incision was made over the peritoneal cavity of each fish to remove the intestines (7). The intestines were severed aseptically anterior to the pyloric valve and anus, releasing the intestinal contents into a petri dish. The contents were placed in a centrifuge tube and stirred. Phosphate-buffered saline (PBS) was added 1:1 to all of the samples to facilitate homogenization.

V. vulnificus and phages were enumerated in the tissues and fluids of duplicate oysters collected on three dates when environmental conditions were favorable for *V. vulnificus* abundance: 25 April 1995 (24.5°C, 15.0 ppt salinity), 9 September 1996 (30.6°C, 24.0 ppt salinity), and 28 October 1996 (26.5°C, 18 ppt salinity). The oysters were collected as described above. Tissue and fluid preparation and analysis were similar to those previously reported (24). After the oysters were scrubbed and rinsed, an abrasive disk was used to notch the shell next to the adductor muscle. A sterile 20-gauge needle, fitted onto a syringe, was inserted through the notch and into the sinus in the center of the adductor muscle, and 1 to 2 ml of hemolymph was aspirated. The oyster was then opened, and mantle fluid (the fluid that bathes the gills and other organs when the oyster is closed) was collected. The oyster was then rinsed with PBS before dissection. The adductor muscle, gills, and digestive organ were removed from each oyster with sterile scissors and forceps. The remaining tissues, consisting primarily of the mantle, were consolidated and treated as one sample. Each sample was ground with a glass tissue grinder and pestle. The samples were transferred to preweighed polypropylene centrifuge tubes and diluted with PBS.

The susceptibility of bacteria isolated from oysters other than *V. vulnificus* to phages that lyse *V. vulnificus* was examined twice (3 April 1995 and 4 December 1996) when *V. vulnificus* densities were low (<10 g^{-1}). Isolates (44 in April and 60 in December) were obtained from oyster homogenate spread plated onto CPM agar and incubated for either 24 or 48 h at 26°C. These cultures were grown to log phase and plated on CPM by soft-agar overlay. After 1 h, plates were spotted with 4 μl of the various phage stocks (25 phages with April isolates and 13 phages with December isolates) and incubated overnight at 26°C. Bacterial strains were considered susceptible to phages if they showed either clear or turbid plaques.

Table 1 shows *V. vulnificus* and phage densities (direct enumeration results) in estuarine samples from a variety of environmental conditions. In March and December, *V. vulnificus* densities were generally 2 to 3 logs lower than in April and September, when water temperatures were warmer. The lowest densities were observed in water, and the highest occurred in the intestines of the various fish. *V. vulnificus* levels in other samples were similar to those observed in oysters. Phage densities were considerably higher (2 to 4 $logs_{10}$) in molluscan shellfish (oysters and mussels) than in other samples. The highest phage density was observed with hosts J-7 and MO-624 and was generally 10^4 g^{-1} in oysters and mussels. No apparent trend was observed with water temperature or salinity and phage densities in molluscan shellfish. Phages were isolated from other habitats, but only sporadically and at lower densities (generally 10^2 g^{-1}). Phages were not detected in water samples by the direct enumeration procedure, but phages were detected with the enrichment procedure on each sampling date in 100-ml aliquots and in both 10- and 100-ml aliquots in

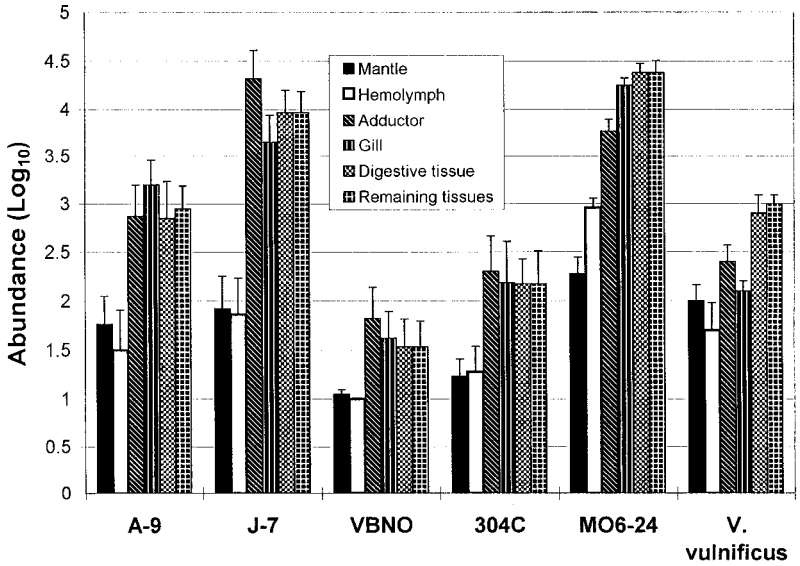

FIG. 1. Abundance and distribution of phages in oyster tissues. Phages were isolated with five different host strains (A-9, J-7, VBNO, 304C, and MO-624). Also shown are mean abundances of *V. vulnificus* in the same tissues (rightmost set of bars) and standard errors ($n = 6$).

December; phages were not detected in 1-ml aliquots of any of the water samples.

The high density of *V. vulnificus* phages in molluscan shellfish in this study are comparable to *V. parahaemolyticus* phage densities observed in Pacific molluscan shellfish (3). This is the first report of *V. vulnificus* phages in sediment, plankton, crustacea, or finfish but this was not unexpected, since *V. parahaemolyticus* phages have been found in sediment and crustacea (3) and other marine phages have been associated with plankton (5, 6). The relatively low incidence of *V. vulnificus* phages in fish intestinal contents is in contrast with the high *V. vulnificus* densities observed in fish intestinal contents in the present study and in previous investigations (7). The ecology of *V. vulnificus* and its phages in the intestines of fish requires further investigation.

We also tested other bacterial species isolated from oysters for susceptibility to *V. vulnificus* phages. None of the bacteria from oysters collected in early April (44 isolates) or December (60 isolates) were lysed by any of the *V. vulnificus* phage strains tested (25 in April and 13 in December). However, *V. vulnificus* phages were abundant in these oysters ($>10^4$ g^{-1}) but *V. vulnificus* cells were few (<10 g^{-1}). This is a consistent trend in Gulf Coast oysters during cooler months (8). Abundant phages in oysters with few susceptible bacteria suggest long-term retention of phages during the winter or that there are other susceptible hosts that were not detected by our methods.

Analysis of oyster tissues and fluids indicated that both *V. vulnificus* and its phages are distributed throughout the oysters (Fig. 1) and that the lowest levels of both generally occur in the hemolymph and mantle fluid. The close association of phages to various oyster tissues and fluids probably reduces their elimination by excretion and is consistent with long-term retention. The present data on *V. vulnificus* density in oyster tissues and fluids agree well with those previously reported (24). The circulation of *V. vulnificus* phages in the hemolymph and their presence in oyster tissues theoretically provide access to susceptible *V. vulnificus* strains. The ecological relationship between *V. vulnificus* and its phages in molluscan shellfish requires further investigation.

REFERENCES

1. **Adams, M. H.** 1959. Bacteriophages. Interscience Publishers, Inc., New York, N.Y.
2. **American Public Health Association.** 1970. Recommended procedures for the examination of seawater and shellfish. 4th ed. American Public Health Association, Washington, D.C.
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. **Blake, P. A., A. M. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine *Vibrio*: clinical characteristics and epidemiology. N. Engl. J. Med. **300:**1–5.
5. **Borsheim, K. Y., G. Bratbak, and M. Hedal.** 1990. Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. Appl. Environ. Microbiol. **56:**352–356.
6. **Bratbak, G., M. Heldal, S. Norland, and T. F. Thingstad.** 1990. Viruses as partners in spring bloom microbial trophodynamics. Appl. Environ. Microbiol. **56:**1400–1405.
7. **DePaola, A., G. M. Capers, and D. Alexander.** 1994. Densities of *Vibrio vulnificus* in the intestines of fish from the U.S. Gulf Coast. Appl. Environ. Microbiol. **60:**984–988.
8. **DePaola, A., M. L. Motes, A. M. Chan, and C. A. Suttle.** Unpublished data.
9. **Elliot, E. L., C. A. Kaysner, and M. L. Tamplin.** 1992. *V. cholerae*, *V. vulnificus*, and other *Vibrio* spp., p. 111–140. *In* FDA bacteriological analytical manual, 7th ed. Association of Official Analytical Chemists, Arlington, Va.
10. **Fuhrman, J. A., and R. T. Noble.** 1995. Viruses and protists cause similar bacterial mortality in coastal seawater. Limnol. Oceanogr. **40:**1236–1242.
11. **Harris-Young, L., M. L. Tamplin, W. S. Fisher, and J. W. Mason.** 1993. Effects of physicochemical factors and bacterial colony morphotype on association of *Vibrio vulnificus* with hemocytes of *Crassostrea virginica*. Appl. Environ. Microbiol. **59:**1012–1017.
12. **Hlady, W. G., R. C. Mullen, and R. S. Hopkins.** 1993. *Vibrio vulnificus* from raw oysters: leading cause of reported deaths from foodborne illness in Florida. J. Fla. Med. Assoc. **80:**536–538.
13. **Kaspar, C. W., and M. L. Tamplin.** 1993. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl. Environ. Microbiol. **59:**2425–2429.
14. **Kaysner, C. A., C. Abeyta, Jr., M. M. Wekell, A. DePaola, Jr., R. F. Stott, and J. M. Leitch.** 1987. Virulent strains of *Vibrio vulnificus* isolated from estuaries of the United States West Coast. Appl. Environ. Microbiol. **53:**1349–1351.
15. **Kelly, M. T.** 1982. Effect of temperature and salinity on *Vibrio* (Beneckea) *vulnificus* occurrence in a Gulf Coast environment. Appl. Environ. Microbiol. **44:**820–824.
16. **Klontz, K. C., S. Lieb, M. Schreiber, H. T. Janowski, L. M. Baldy, and R. A. Gunn.** 1988. Syndromes of *Vibrio vulnificus* infections, clinical and epidemiologic features in Florida cases, 1981–1987. Ann. Intern. Med. **109:**318–323.
17. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1983. Distribution of *Vibrio*

vulnificus and other lactose-fermenting vibrios in the marine environment. Appl. Environ. Microbiol. **45:**985–998.
18. **O'Neill, K. R., S. H. Jones, and D. J. Grimes.** 1992. Seasonal incidence of *Vibrio vulnificus* in the Great Bay Estuary of New Hampshire and Maine. Appl. Environ. Microbiol. **58:**3257–3262.
19. **Pellon, W., R. J. Siebeling, J. Simonson, and R. B. Luftig.** 1995. Isolation of bacteriophage infectious for *Vibrio vulnificus*. Curr. Microbiol. **30:**331–336.
20. **Proctor, L. M., and J. A. Fuhrman.** 1990. Viral mortality of marine bacteria and cyanobacteria. Nature **343:**60–62.
21. **Stelma, G. N., Jr., A. L. Reyes, J. T. Peeler, C. H. Johnson, and P. L. Spaulding.** 1992. Virulence characteristics of clinical and environmental isolates of *Vibrio vulnificus*. Appl. Environ. Microbiol. **58:**2776–2782.
21a. **Suttle, C. (University of British Columbia).** 1994. Personal communication.
22. **Suttle, C. A.** 1993. Enumeration and isolation of viruses, p. 121–134. *In* P. A. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), Handbook of methods in aquatic microbial ecology. Lewis Publishers, Boca Raton, Fla.
23. **Tamplin, M., G. E. Rodrick, N. J. Blake, and T. Cuba.** 1982. Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. Appl. Environ. Microbiol. **44:**1466–1477.
24. **Tamplin, M. L., and G. M. Capers.** 1992. Persistence of *Vibrio vulnificus* in tissues of Gulf Coast oysters, *Crassostrea virginica*, exposed to seawater disinfected with UV light. Appl. Environ. Microbiol. **58:**1506–1510.
25. **Wright, A. C., R. T. Hill, J. A. Johnson, M. Roghman, R. R. Colwell, and J. G. Morris.** 1996. Distribution of *Vibrio vulnificus* in the Chesapeake Bay. Appl. Environ. Microbiol. **62:**717–724.