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Vibrio vulnificus Load Reduction in Oysters after Combined Exposure to *Vibrio vulnificus*–Specific Bacteriophage and to an Oyster Extract Component†

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ABSTRACT

Oysters infected with *Vibrio vulnificus* can present a serious health risk to diabetic, immunocompromised, and iron-deficient individuals. Numerous studies have been conducted with the goal of eliminating this organism from raw oysters. We utilized two natural oyster-associated components: pooled *Vibrio vulnificus*–specific bacteriophage and an extract of the eastern oyster (*Crassostrea virginica*) that contains an antimicrobial component we named anti–*Vibrio vulnificus* factor, which is bactericidal for *V. vulnificus.* Although each component alone can reduce *V. vulnificus* numbers independently, the simultaneous use of both components in an in vitro system successfully more effectively reduced *V. vulnificus* bacterial loads.

One of the early reports associating *Vibrio vulnificus* infection with consumption of raw oysters was published over 15 years ago *(9).* This report inspired a number of attempts to reduce the *V. vulnificus* load in oysters *(1, 3–5, 10, 14, 15, 18, 19).*

In 1995, we reported the isolation of nine bacteriophage strains specific for *V. vulnificus* from Gulf waters surrounding oyster reefs *(17).* Bacteriophage capable of attacking several *Vibrio* species had been isolated *(2, 8)*; however, those that specifically attacked *V. vulnificus* had not been previously described. Since then, two research groups have characterized *V. vulnificus*–specific phages *(6, 7).* Studies with 59 strains of *V. vulnificus* isolated from environmental and clinical sources revealed that these organisms varied in their susceptibility to infection by different phage isolates *(17).* As a result, we developed a ''shotgun'' approach using in vitro experiments to reduce the *V. vulnificus* load in oysters. By exposing these animals to pools of the nine phage isolates, we hypothesized that *V. vulnificus* strains resistant to infection by one or more of the phage isolates could still become infected by one or more other phage isolates to which they were yet susceptible. Forty-six of the 59 *V. vulnificus* strains fell into this group. We found that in vitro exposure to 1.0 ml of the pooled phage reduced the *V. vulnificus* load by five log units *(11).*

In 1981, Oliver *(16)* conducted cold inactivation studies of *V. vulnificus* and noted that oyster diluent possessed antibacterial properties. More recently, we described a component of oyster extracts that inactivated *V. vulnificus*; we named this component anti–*Vibrio vulnificus* factor (AVvF) *(12, 13).* Preliminary studies indicated that AVvF could be a valuable adjunct or alternative to the use of bacteriophage in biodepuration of oysters, because the preparation of large amounts of phage concentrate for use in oyster depuration tanks is expensive. Our in vitro studies revealed that exposure to this factor present in 1.0 g of oyster extract could reduce the *V. vulnificus* load by four to five log units. We proposed that a combined treatment utilizing bacteriophages and oyster extract could prove useful for biodepuration of fresh raw oysters obtained from the Gulf of Mexico by reducing the number of contaminating *V. vulnificus*.

The experimental objective was to study the feasibility of using *V. vulnificus*–specific bacteriophage in combination with oyster extract to reduce the *V. vulnificus* load in postharvest processed oysters to concentrations deemed safe by the Food and Drug Administration (<30 most probable number per g) *(20).*

MATERIALS AND METHODS

Media and solutions. Double-strength (2×) tryptone broth (TB) consisted of 20 g of tryptone and 20 g of NaCl in 1.0 liter of activated carbon-filtered deionized water. The pH was adjusted to 7.4 with 0.1 M NaOH, and the broth was sterilized by autoclaving (15 lb for 15 min). Double-strength tryptone base agar (TBA) was prepared with 2× TB plus 4% Difco agar (wt/vol). Estuarine water (EW) with a salinity of 25 to 30 ppt was collected at Perdido Pass, near Gulf Shores, Ala., at a depth of 5 ft (1.5 m), serially passed through a prefilter pad with a 1.2-μm-pore-size (automated peritoneal dialysis) membrane filter, and autoclaved (15 lb for 30 min). EW-TB and EW-TBA were prepared by mixing equal volumes of 2× TB and 2× TBA with equal volumes of EW, respectively. Artificial sea water (Instant Ocean, Aquarium Systems, Inc., Menton, Ohio) was also used at a salinity of 29 ppt. *Vibrio* maintenance medium consisted of 8.0 g of tryptone, 4.0 g of nutrient broth, and 2.0 g of agar in 1 liter of EW.

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***Vibrio* species.** *V. vulnificus* ATCC 27562 was obtained from the American Type Culture Collection (Manassas, Va.) and maintained in *Vibrio* maintenance medium. Other *Vibrio* species and clinical and environmental strains of *V. vulnificus* were provided by Dr. R. J. Siebeling (Louisiana State University, Baton Rouge, deceased).

***V. vulnificus* bacteriophages.** The isolation and characterization of nine bacteriophage isolates specific for *V. vulnificus* has been described *(17).* Bacteriophage pools were prepared by combining equal volumes of each of the nine undiluted stock strains (S1, P13, P38, P53, P65, P68, P108, P111, and P147). Stocks had been stored at −70°C.

Live oysters. Live eastern oysters (*Crassostrea virginica*) were donated by P&J Oyster Processor and Distributor (New Orleans, La.); none of these oysters contained *V. vulnificus.* Oysters were immediately rinsed with tap water, scrubbed clean under running tap water, and rinsed under running activated carbon-filtered deionized water. These oysters were either shucked immediately or were placed in buckets or aquaria containing aerated artificial sea water and periodically fed an algal suspension (from Dr. Siebeling).

Preparation of oyster extracts. Individual oysters were weighed in the shell and then shucked, and the valves were reweighed to determine the flesh and liquor weight. The flesh and liquor were pooled and added to EW in concentrations of 1 and 10% (wt/vol). Preparations were homogenized, autoclaved (15 lb for 15 min), and centrifuged (600 × *g* for 30 min) to remove particulate matter. The supernatants were pooled and acidified to pH 5.0 with 50% (vol/vol) glacial acetic acid. The precipitate was pelleted at 10,000 × *g* for 30 min, and the pH of the supernatant was adjusted to 7.4 with 1.0 N NaOH. The neutralized supernatant was sterilized by membrane filtration (0.22 μm) and stored at −20°C.

Effect of oyster extracts on the viability of different *Vibrio* species. Overnight broth cultures of *V. vulnificus* and other *Vibrio* species were diluted to 10^6 CFU/ml with EW. One milliliter of diluted culture was added to 1.0 ml of EW, and another 1.0 ml of diluted culture was added to 1.0 ml of 1.0% (wt/vol) oyster extract. Both tubes were maintained at 4°C overnight. On the following day, 200 μl from each of the tubes in a set was plated on TBA and incubated at 30°C for 24 h. All experiments were performed in triplicate for each treatment.

Time course of antimicrobial activity of oyster extracts. One-milliliter cultures (10^6 CFU) of *V. vulnificus* (ATCC 27562) were incubated with either 1.0 ml of EW or 1.0 ml of 1% (wt/vol) oyster extract at 4°C for up to 66 h. Aliquots (200 μl) were removed after mixing and at 18, 42, and 66 h, plated on TBA, and incubated at 30°C for 24 h. All experiments were performed in triplicate.

Effect of temperature and oyster extract concentration on *V. vulnificus* activity. Three sets of nine tubes were prepared. Each set was composed of 1.0 ml of EW, 1.0 ml of 1% (wt/vol) oyster extract, and 1.0 ml of 10% (wt/vol) oyster extract. To all nine tubes, 1.0 ml (10^6 CFU) of *V. vulnificus* (ATCC 27562) was added. One set of tubes (set A) was incubated at 4°C for 18 h, the second set (set B) was incubated at 15°C for 18 h, and the third set (set C) was incubated at 24°C for 18 h. On the following day, 200 μl was removed from each of the nine tubes, inoculated onto TBA plates, incubated for 24 h at 30°C, and evaluated in triplicate separate experiments.

TABLE 1. *Susceptibility of* Vibrio *species to the inhibiting effects of AVvF*

Vibrio species	Source	AVvF susceptibility[a]
V. costicola	NCMB 701	Negative
V. charchariae	ATCC 35084	Negative
V. cholerae, El Tor		Negative
V. cholerae, Inaba	5875	Negative
V. fisheri	RJS[b]	Negative
V. fluvialis	ATCC 33810	Negative
V. harveyi	NCMB 1280	Negative
V. metschnikovii	ATCC 7708	Negative
V. mimicus	ATCC 33653	Negative
V. natriegens	ATCC 14058	Positive
V. parahaemolyticus	RJS	Negative
V. parahaemolyticus	ATCC 10136	Negative
V. tubiashi	ATCC 19105	Negative
V. tubiashi	ATCC 19106	Negative
V. vulnificus, biogroup II	RJS	Negative
V. vulnificus	ATCC 27562	Positive

[a] Negative, no growth inhibition; positive, growth inhibition ($<10^3$ CFU/ml). All experiments were done in triplicate.
[b] R. J. Siebeling.

In vitro use of pooled phage and oyster extracts to reduce *V. vulnificus* numbers. To test the efficacy of antimicrobial activity of oyster extracts in the presence or absence of *V. vulnificus*–specific bacteriophage pools, 1 ml (10^6 CFU) of *V. vulnificus* (ATCC 27562) was incubated for 18 h at 4°C in the presence of EW, 1 ml of 1 or 10% (wt/vol) oyster extract, 1 ml of bacteriophage pool, and combinations of 1 and 10% (wt/vol) oyster extracts in the presence of the bacteriophage pool. Total reaction volume was 3 ml. After incubation for 18 h at 4°C, 200 μl was inoculated onto TBA, incubated for 24 h at 30°C, and evaluated in triplicate.

RESULTS

Reaction of *Vibrio* species to oyster extract. Various *Vibrio* species, including *V. vulnificus,* were tested for their susceptibility to the inactivating effects of oyster extracts (Table 1). Of the 15 species or strains evaluated, only 2 (*V. vulnificus* and *V. natriegens*) were affected by the oyster extract. These findings were supported by results from the 54 *V. vulnificus* strains from environmental and clinical sources *(11)* that were tested earlier for *V. vulnificus*–specific phage susceptibility and were challenged by oyster extracts (data not shown). The inactivation was evaluated using a colony assay, where the threshold for a negative result was $<1 \times 10^3$ CFU, starting at 10^6 CFU in 1 ml.

Exposure of *V. vulnificus* to oyster extract over time. Figure 1 illustrates the antimicrobial activity of a 1% (wt/vol) oyster extract over a period of 66 h at 4°C. The *V. vulnificus* population decreased about 3 log CFU/ml after 18 h and decreased by 5 log CFU/ml after 42 h of incubation with no subsequent specific change in bactericidal activity. In the absence of oyster extract, the difference in the number of viable bacteria was only about 0.5 log CFU/ml over the 66-h period.

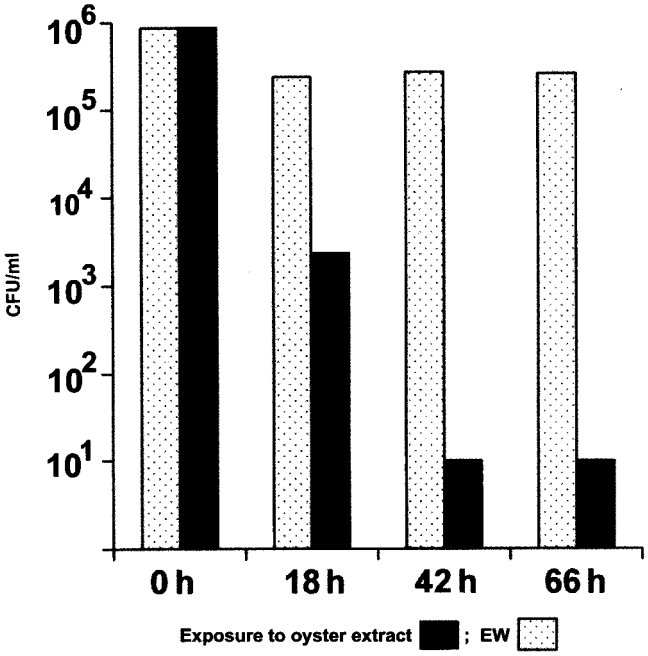

FIGURE 1. *The viability of* V. vulnificus *(10^6 CFU) after exposure to oyster extract (1.0%, wt/vol) and estuarine water (EW) for 0 to 66 h at 4°C. All experiments were done in triplicate with a variation of ±10%.*

Death of *V. vulnificus* at different incubation temperatures and concentrations of oyster extracts. Table 2 summarizes the findings obtained when 10^6 *V. vulnificus* was exposed to EW and to 1 or 10% (wt/vol) oyster extracts and maintained at 4, 15, or 24°C for 18 h prior to inoculation. At temperatures of 15 and 24°C, there was no evidence of *V. vulnificus* inactivation in the presence of the EW controls and the 1 and 10% oyster extracts. At 4°C, there was no evidence of *V. vulnificus* inactivation in the presence of EW, but *V. vulnificus* inactivation did occur following exposure to 1% oyster extract and was even more evident following exposure to the 10% oyster extract.

In vitro reduction of *V. vulnificus* when specific bacteriophages and oyster extracts were used alone or jointly. Table 3 presents the results obtained when a dilute population of *V. vulnificus* was exposed to different concentrations of oyster extracts and pooled phage strains alone or in combination with the different reactants and maintained for 18 h at 4°C prior to agar inoculation. The mixture of *V. vulnificus* and EW resulted in no *V. vulnificus* inactivation. In contrast, a substantial reduction in the number of colonies was noted following *V. vulnificus* exposure to 1% oyster extract, and an even a greater reduction in colony counts was obtained with 10% oyster extract. The use of pooled phage alone also resulted in a substantial reduction in colony counts. The combined use of pooled phage and both 1 and 10% oyster extracts resulted in a slightly greater reduction in the number of colonies. Phage in the mixture may be useful for scavenging *V. vulnificus* cells not accessible to AVvF (unpublished data).

TABLE 3. *Antimicrobial activity of oyster extracts in the presence and absence of* V. vulnificus*–specific bacteriophage pools when incubated with 1 ml (10^6 CFU) of* V. vulnificus *(ATCC 27562) at 4°C for 18 h*

Conditions[a]	Survivors (CFU/ml)
Estuarine water (EW) (2 ml)	$>10^6$
1% oyster extract (1 ml) + EW (1 ml)	2.6×10^3
10% oyster extract (1 ml) + EW (1 ml)	0.7×10^1
Bacteriophage pool[b] (1 ml) + EW (1 ml)	0.3×10^1
Bacteriophage pool (1 ml) + 1% (wt/vol) oyster extract (1 ml)	0.2×10^1
Bacteriophage pool (1 ml) + 10% (wt/vol) oyster extract (1 ml)	0.18×10^1

[a] All experiments were replicated three times with an average variation of ±10%.

[b] *V. vulnificus* bacteriophage strains S1, P3, P38, P53, P65, P68, P108, P111, and P147.

DISCUSSION

Information on the nature of AVvF in oyster extracts is limited. This factor is heat stable, tolerating autoclaving (15 lb for 15 min) without apparent loss of activity. It is also acid stable, retaining its *V. vulnificus* inactivating properties following exposure to 50% glacial acetic acid, and is nonprecipitable at pH 5.0. The inactivating property of AVvF is specific for *V. vulnificus.* AVvF is nondialyzable (with tubing of molecular weight 6,000 to 8,000) and filterable through 0.45- and 0.22-μm-pore-size (automated peritoneal dialysis) membranes.

The highly specific nature of AVvF activity was unsuspected, being limited to strains of *V. vulnificus.* When AVvF activity was evaluated against other *Vibrio* species (Table 1), a positive response was detected only for *V. natriegens.* The significance of this cross-species reaction is unclear but may reflect a similarity between *V. vulnificus* and *V. natriegens* with respect to the site of action of this factor. There was a no response to *V. vulnificus* biotype 2; however, this result needs further investigation. When *V.*

TABLE 2. *Overnight exposure of* V. vulnificus *(ATCC 27562) to oyster extracts at 4, 15, and 24°C*

Diluent	*V. vulnificus* (CFU/ml) following exposure at[a]: 4°C	15°C	24°C
Estuarine water	$>10^6$	$>10^6$	$>10^6$
1% (wt/vol) oyster extract	1.7×10^3	$>10^6$	$>10^6$
10% (wt/vol) oyster extract	0.4×10^1	$>10^6$	$>10^6$

[a] All values represent the average of three replicates.

vulnificus was mixed with either a single component or a combination of components recommended for biodepuration (AVvF at concentrations of 1 and 10%) and then with pooled phage, the optimum inactivation was obtained with a 1% extract in the presence of phage. These results provide clues for how to optimize biodepuration of raw oysters upon harvest. Both the phage and oyster extract (AVvF) are natural agents.

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