Bacteriophages against *Vibrio coralliilyticus* and *Vibrio tubiashii*: Isolation, Characterization, and Remediation of Larval Oyster Mortalities

Gary P. Richards,^a Michael A. Watson,^a David Madison,^b Nitzan Soffer,^{c*} David S. Needleman,^d Douglas S. Soroka,^d Joseph Uknalis,^d Gian Marco Baranzoni,^{d*} Karlee M. Church,^{c*} Shawn W. Polson,^e Ralph Elston,^f Chris Langdon,^b Alexander Sulakvelidze^c

^aUnited States Department of Agriculture, Agricultural Research Service, Delaware State University, Dover, Delaware, USA

^bOregon State University, Hatfield Marine Science Center, Newport, Oregon, USA

^cIntralytix Inc., Columbia, Maryland, USA

^dUnited States Department of Agriculture, Agricultural Research Service, Wyndmoor, Pennsylvania, USA

^eCenter for Bioinformatics and Computational Biology, Delaware Biotechnology Institute, University of Delaware, Newark, Delaware, USA

^fAquaTechnics Inc., Carlsborg, Washington, USA

ABSTRACT *Vibrio coralliilyticus* and *Vibrio tubiashii* are pathogens responsible for high larval oyster mortality rates in shellfish hatcheries. Bacteriophage therapy was evaluated to determine its potential to remediate these mortalities. Sixteen phages against *V. coralliilyticus* and *V. tubiashii* were isolated and characterized from Hawaiian seawater. Fourteen isolates were members of the *Myoviridae* family, and two were members of the *Siphoviridae*. In proof-of-principle trials, a cocktail of five phages reduced mortalities of larval Eastern oysters (*Crassostrea virginica*) and Pacific oysters (*Crassostrea gigas*) by up to 91% 6 days after challenge with lethal doses of *V. coralliilyticus*. Larval survival depended on the oyster species, the quantities of phages and vibrios applied, and the species and strain of *Vibrio*. A later-generation cocktail, designated VCP300, was formulated with three lytic phages subsequently named *Vibrio* phages vB_VcorM-GR7B, vB_VcorM-GR11A, and vB_VcorM-GR28A (abbreviated 7B, 11A, and 28A, respectively). Together, these three phages displayed host specificity toward eight *V. coralliilyticus* strains and a *V. tubiashii* strain. Larval *C. gigas* mortalities from *V. coralliilyticus* strains RE98 and OCN008 were significantly reduced by >90% ($P < 0.0001$) over 6 days with phage treatment compared to those of untreated controls. Genomic sequencing of phages 7B, 11A, and 28A revealed 207,758-, 194,800-, and 154,046-bp linear DNA genomes, respectively, with the latter showing 92% similarity to *V. coralliilyticus* phage YC, a strain from the Great Barrier Reef, Australia. Phage 7B and 11A genomes showed little similarity to phages in the NCBI database. This study demonstrates the promising potential for phage therapy to reduce larval oyster mortalities in oyster hatcheries.

IMPORTANCE Shellfish hatcheries encounter episodic outbreaks of larval oyster mortalities, jeopardizing the economic stability of hatcheries and the commercial shellfish industry. Shellfish pathogens like *Vibrio coralliilyticus* and *Vibrio tubiashii* have been recognized as major contributors of larval oyster mortalities in U.S. East and West Coast hatcheries for many years. This study isolated, identified, and characterized bacteriophages against these *Vibrio* species and demonstrated their ability to reduce mortalities from *V. coralliilyticus* in larval Pacific oysters and from both *V. coralliilyticus* and *V. tubiashii* in larval Eastern oysters. Phage therapy offers a promising approach for stimulating hatchery production to ensure the well-being of hatcheries and the commercial oyster trade.

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Address correspondence to Gary P. Richards, Gary.Richards@usda.gov.

* Present address: Nitzan Soffer, Shoreline Biome, San Diego, California, USA; Gian Marco Baranzoni, Thermo Fisher Scientific, Life Technologies GmbH, Darmstadt, Germany; Karlee M. Church, The Hershey Company, Technical Center, Hershey, Pennsylvania, USA.

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Shellfish hatcheries play a key role in providing seed oysters for commercial planting and aquaculture operations. In the United States, individual hatcheries raise billions of oyster larvae annually to produce the seed needed to sustain the commercial industry (1, 2). Intermittent high larval mortality rates caused by *Vibrio* spp. have produced shortages of larvae and seed oysters needed by the commercial shellfish industry (1, 3). Elston et al. (1) report larval oyster mortality rates on the Pacific Coast as high as 59% annually during years when episodic vibriosis occurs, although some producers claim losses to be much higher. East Coast hatcheries also suffer intermittent *Vibrio*-associated losses in larval shellfish production, with two hatcheries reporting >80% mortality rates in recent years (personal communication with hatchery managers). Among the pathogens that are particularly infectious to larval oysters and other bivalves are *Vibrio tubiashii* and *Vibrio coralliilyticus* (4–9). *Vibrio tubiashii* was initially identified in diseased shellfish along the U.S. East Coast (4) and has been associated periodically with major hatchery die-offs along the Atlantic and Pacific Coasts (1, 5, 10, 11). Until recently, many of the hatchery-associated outbreaks along the U.S. Pacific Coast were linked to *V. tubiashii*; however, PCR and genomic sequencing revealed that some of these isolates from disease outbreaks (i.e., strains ATCC 19105, RE22, and RE98) were actually *V. coralliilyticus* (12–15). *Vibrio coralliilyticus* was initially identified as a pathogen that caused disease in corals and the destruction of coral reefs (16–19). Richards et al. (8) identified some strains of *V. coralliilyticus* and *V. tubiashii* that are infectious to commercially important Eastern oyster (*Crassostrea virginica*) and Pacific oyster (*Crassostrea gigas*) larvae. In that study, *V. tubiashii* was particularly pathogenic in larval Eastern oysters and generally noninfectious to larval Pacific oysters, while some *V. coralliilyticus* strains were highly infectious to both Eastern and Pacific oyster larvae. More recently, we identified additional strains of coral-associated *V. coralliilyticus* that were infectious to Pacific oyster larvae (9). Fortunately, both *V. coralliilyticus* and *V. tubiashii* are known to be infectious to oysters only during their free-swimming larval stage, which is generally their first 2 to 3 weeks of life. After that stage, larvae undergo metamorphosis, lose their motility, and appear no longer susceptible to these pathogens. For some strains of *V. coralliilyticus*, enhanced infectivity to corals and larval oysters is notable at elevated seawater temperatures (9, 20–23), such as those used in some commercial shellfish hatcheries.

Phage interventions are being employed increasingly to treat bacterial infections in fish and shellfish aquaculture operations and have been shown to have a protective effect to reduce disease and mortality (reviewed in references 24–28). Vibriophages were effective in reducing pathogenic vibrios in aquaculture, including the pathogens *Vibrio harveyi* in shrimp (29–31), *Vibrio parahaemolyticus* in shrimp (32, 33), and *Vibrio anguillarum* in various fish larvae (34, 35). To begin to remediate the economically significant problem of *V. coralliilyticus*- and *V. tubiashii*-associated mortalities in molluscan shellfish hatcheries, a plan was devised to isolate, identify, and characterize lytic phages against these pathogens and determine the efficacy of mixtures (cocktails) of these phages to reduce larval oyster mortalities. To the best of our knowledge, phages against *V. tubiashii* have not been previously reported; however, phages against *V. coralliilyticus* have been reported, including strain YC, which was evaluated for use in phage therapy in corals (36). Ramphul et al. (37) performed genomic analyses on three *V. coralliilyticus* phages that they isolated in Japan, but they did not determine if the phages reduced mortalities of larval shellfish or corals. In a series of recent studies from South Korea, Kim and colleagues identified several indigenous phages that reduced larval Pacific oyster mortalities after subjecting the larvae to strains of *V. coralliilyticus* that are found in South Korea (38–40). In all three studies, reductions in larval Pacific oyster mortalities were observed over a period of 24 h. To date, no longer-term

TABLE 1 Strains of *Vibrio coralliilyticus* and *V. tubiashii* used in this study^a

<i>Vibrio</i> strain	Source	Method(s) of species authentication	Authentication reference
<i>Vibrio coralliilyticus</i>			
RE22	Ralph Elston	Complete genome sequencing	Richards et al. (14)
RE98	Ralph Elston	Complete genome sequencing	Richards et al. (13)
ATCC 19105	ATCC	PCR of <i>vcpA</i> , the zinc metalloprotease gene of <i>V. coralliilyticus</i>	Wilson et al. (12)
09-105-8	Ralph Elston	16S rRNA gene sequencing, PCR of the <i>vcpA</i> gene	This study
RE90	Ralph Elston	16S rRNA gene sequencing	This study
ATCC BAA-450 (type strain)	ATCC	Draft genome sequencing	Kimes et al. (58)
OCN008	Blake Ushijima	Draft genome sequencing	Ushijima et al. (59)
OCN014	Blake Ushijima	Complete genome sequencing	Ushijima et al. (60)
<i>Vibrio tubiashii</i>			
ATCC 19106	ATCC	Genome sequencing	Temperton et al. (61)
ATCC 19109 (type strain)	ATCC	Complete genome sequencing	Richards et al. (62)

^aATCC, American Type Culture Collection.

studies have been reported, nor have studies with other species of oysters or strains of *V. coralliilyticus*.

In this study, we describe the isolation and characterization of novel phages against *V. coralliilyticus* and *V. tubiashii* obtained from a Hawaiian shellfish hatchery whose source seawater was obtained from a 900-m-deep pipeline and directly from a 21-m-deep (shallow) water pipeline. Phage characterization includes host specificity studies, morphological identification of family lineage, determination of calcium or magnesium dependence for infectivity, genomic sequencing, and bioinformatic analysis of selected strains. Host specificity testing targeted strains of *V. coralliilyticus* and *V. tubiashii* that are found in the United States and pose a threat to U.S. shellfish hatcheries. We also demonstrate by proof-of-principle efficacy testing that phage treatment effectively reduces *V. coralliilyticus*- and *V. tubiashii*-induced mortalities in both Eastern and Pacific oyster larvae over an extended period (6 days).

RESULTS

Phage isolation from seawater. Seawater samples were kindly provided from shellfish tanks at the Kona Coast Shellfish hatchery in Kailua-Kona, HI. Most phages were isolated against *V. coralliilyticus* and *V. tubiashii* from hatchery seawater when the source seawater was supplied from a 900-m-deep pipeline maintained by the Natural Energy Laboratory of Hawaii Authority (NELHA) in Kailua-Kona. Phages were seldom isolated after the hatchery substituted seawater from a 674-m-deep pipeline. Direct testing of seawater from a NELHA shallow (21-m-deep) pipeline identified one additional phage.

Host specificity. Eight strains of *V. coralliilyticus* and two strains of *V. tubiashii*, listed in Table 1, were tested for host specificity against 16 plaque-purified phages. Differences were observed in specificity among the phage strains, with all but 2 of the phages infecting *V. coralliilyticus* RE98, 6 of the 16 phages infecting *V. coralliilyticus* 09-105-8, and 14 of the phages infecting *V. tubiashii* ATCC 19106 (Table 2). The broadest host specificity was achieved with phage 28A, subsequently named *Vibrio* phage vB_VcorM-GR28A (see below for naming of phages), which was lytic toward 7 strains of *V. coralliilyticus* (Table 2). Phage 28A was the only phage isolated from water from the 21-m-deep NELHA pipeline. No phages were isolated against the American Type Culture Collection (ATCC) type strain of *V. tubiashii* (ATCC 19109).

Morphological characterization of phages. Plaque-purified isolates were examined by transmission electron microscopy (TEM) to confirm virus presence and to aid in the taxonomic categorization of the phages into families. Figure 1 shows representative high-magnification micrographs (direct magnification, $\times 300,000$). Two of the phage isolates were *Siphoviridae* phages (11B and 23A) with straight and curved tails, respectively. All other phages displayed head and relatively long tail structures and were identified as *Myoviridae* phages based on the appearance of contracted tail

TABLE 2 Host specificity of 16 phages toward 8 strains of *V. coralliilyticus* and 2 strains of *V. tubiashii*^a

Phage ID	Host specificity									
	<i>Vibrio coralliilyticus</i>								<i>Vibrio tubiashii</i>	
	RE98	ATCC 19105	09-105-8	RE22	RE90	ATCC BAA-450	OCN008	OCN014	ATCC 19106	ATCC 19109
1A	+	—	—	—	—	—	—	—	+	—
1D	+	—	—	—	—	—	—	—	+	—
3A	+	—	—	—	—	—	—	—	+	—
3C	+	—	—	—	—	—	—	—	+	—
5A	+	—	—	—	—	—	—	—	+	—
5C	+	—	—	—	—	—	—	—	+	—
6A	+	—	+	—	—	—	—	—	+	—
6B	+	—	+	—	—	—	—	—	+	—
6C	+	—	+	—	—	—	—	—	+	—
7A	+	—	+	—	—	—	—	—	+	—
7B	+	—	+	—	—	—	—	—	+	—
11A	+	—	—	—	—	—	—	—	+	—
11B	+	—	—	—	—	—	—	—	+	—
11D	+	—	—	—	—	—	—	—	+	—
23A	—	—	—	+	—	—	—	—	—	—
28A	—	+	+	+	+	+	+	+	—	—

^aResults are based on plaque formation on lawns of specific host vibrios.

sheaths. Heads generally appeared icosahedral or rounded. Unusual, peplomer-like spikes extended out from the apices of some phage heads. Mean head dimensions and tail lengths are shown in Table 3. All the *Myoviridae* phages were of morphotype A1, while the *Siphoviridae* were of morphotype B1. Based on head and tail measurements (Table 3), these phages varied in overall size, with the larger phages measuring ~300 nm in total length (head plus tail, not including the tail fibers) and the shortest measuring ~140 nm in length for phage 23A. As sheaths contract, some appear to segment and travel up the central tube until they merge and coalesce near the head (e.g., see Fig. 1, phages 1D, 3C, 5C, 7A, and 7B). Among the myovirus isolates, only 7B commonly had a flexible tail tube (Fig. 1) during and after sheath constriction; however, the uncontracted tails were always straight (not shown). Many of the phages had visible necks, some with collars. Tail fibers, when visible, were of a variety of lengths (short in phages 3A and 7A and long in 6A) (Fig. 1).

Calcium and magnesium dependence. Magnesium (MgCl₂) supplementation of the plaque assay medium was essential for plaque formation for all the phage isolates, except for phage 28A, which required calcium (CaCl₂) supplementation. None of the other phages required calcium for plaque formation.

Phage treatment to reduce larval oyster mortalities. Testing was conducted on larval oysters to determine the effectiveness of a phage cocktail in reducing mortalities from exogenously added *V. coralliilyticus* and *V. tubiashii*. Prophylactic phage treatment of Eastern oyster larvae was performed 15 min before the addition of three different vibrios: *V. coralliilyticus* strain RE98, *V. coralliilyticus* ATCC 19105, and *V. tubiashii* ATCC 19106. The initial VTP100 cocktail contained phages 1D, 6A, 6B, 7B, and 11A. Titrations of VTP100 and *Vibrio* isolates were performed simultaneously with their inoculation in the larvae to ensure accurate counts. The cocktail titer was 3.45×10^6 PFU/ μ l, and 10 μ l was added to 1-ml treatment wells, giving a final phage concentration of 3.45×10^7 PFU/ml. Titers for *V. coralliilyticus* strains RE98 and ATCC 19105 were both 1.0×10^7 CFU/ml, and that for *V. tubiashii* was 6.5×10^4 CFU/ml. For the negative-control larvae, 80.5% survived to day 6 (144 h). Mean percent survivals of larvae challenged with the three vibrios (RE98, ATCC 19105, and ATCC 19106) without phage pretreatment were 0.0%, 2.0%, and 34.0%, respectively (Fig. 2A). In contrast, phage pretreatment followed by administration of lethal doses of RE98, ATCC 19105, and *V. tubiashii* ATCC 19106 gave significantly increased survival rates of 66.0% ($P < 0.0001$), 72.9% ($P < 0.0001$), and 62.0% ($P = 0.0015$), respectively (Fig. 2A).

A similar experiment was performed on Pacific oyster larvae challenged with the

FIG 1 Transmission electron micrographs of 16 vibriophage isolates. Bars, 100 nm.

same three vibrios. The phage titer of the treatment was 1.1×10^7 PFU/ml, while the titers of *V. coralliilyticus* RE98 and ATCC 19105 and *V. tubiashii* ATCC 19106 were 8.0×10^7 , 7.0×10^6 , and 1.4×10^6 CFU/ml, respectively. Negative-control larvae yielded 91.9% survival after 6 days (Fig. 2B). Challenge of Pacific oyster larvae with *V. coralliilyticus* strains RE98 and ATCC 19105 and *V. tubiashii* ATCC 19106 (positive mortality controls) resulted in 6-day survival rates of 0, 19.6, and 88.8%, respectively (Fig. 2B). Phage treatment resulted in 91.0% survival of *V. coralliilyticus* ATCC 19105-challenged larvae after 6 days, which was significantly higher than the 19.5% survival rate noted for the *Vibrio*-challenged larvae without phage treatment ($P < 0.0001$). Unlike the Eastern oyster study, Pacific oyster larvae were generally not susceptible to *V. tubiashii* ATCC 19106 infection, with only 11.2% mortality observed, which was not significantly different from the 8.1% mortality observed in the larva-only controls ($P = 0.35$). The inability of *V. tubiashii* to infect Pacific oysters was demonstrated previously (8, 41). The survival rate of larvae after *V. coralliilyticus* RE98 and phage treatment was only 20.9% (Fig. 2B). The relatively poor performance of the cocktail against RE98 is attributable to challenge with a high dose of RE98 (8×10^7 PFU/ml) and an insufficient dose of phages (1.1×10^7 PFU/ml), which led us to perform a dose-response study, as reported below.

Phage dose-response study. The effects of different phage doses were studied on larval Pacific oysters challenged with a mixture of *V. coralliilyticus* (RE98 and ATCC 19105) and *V. tubiashii* (ATCC 19106). The number of vibrios added remained constant at 7×10^3 CFU/ml, while the concentration of the phage cocktail varied over the range

TABLE 3 Morphological attributes of 16 phages against *V. coralliilyticus*

Phage designation	Family	Morphotype ^a	Dimensions (nm) ^b		Other distinguishing feature(s)
			Head	Tail length	
1A	<i>Myoviridae</i>	A1	110 × 110	116	Peplomer-like head spikes
1D	<i>Myoviridae</i>	A1	105 × 111	118	
3A	<i>Myoviridae</i>	A1	104 × 110	111	
3C	<i>Myoviridae</i>	A1	102 × 108	110	
5A	<i>Myoviridae</i>	A1	104 × 111	109	Peplomer-like head spikes
5C	<i>Myoviridae</i>	A1	96 × 101	121	
6A	<i>Myoviridae</i>	A1	98 × 100	191	
6B	<i>Myoviridae</i>	A1	101 × 108	192	
6C	<i>Myoviridae</i>	A1	98 × 105	187	Flexible tail tube
7A	<i>Myoviridae</i>	A1	102 × 109	189	
7B	<i>Myoviridae</i>	A1	104 × 119	195	
11A	<i>Myoviridae</i>	A1	105 × 105	103	
11B	<i>Siphoviridae</i>	B1	100 × 109	126	Tail spikes
11D	<i>Myoviridae</i>	A1	97 × 108	113	
23A	<i>Siphoviridae</i>	B1	49 × 51	90	
28A	<i>Myoviridae</i>	A1	81 × 86	87	

^aMorphotypes A1 and B1 are characterized by head dimensions as follows: *Myoviridae* morphotype A1 has an isometric head (length-to-width ratio of 1) and a contractile tail, while *Siphoviridae* morphotype B1 has an isometric head, as described previously by Ackermann and DuBow (63), and a noncontractile tail.

^bDimensions are based on the means from 5 to 12 measurements.

of 1.2×10^6 PFU/ml (undiluted) to 1.2×10^2 PFU/ml. Negative controls consisted of larvae only, while positive controls consisted of larvae plus vibrios (no phage). Results after 6 days demonstrated dose-dependent increases in the survival of *Vibrio*-challenged larvae as phage concentrations increased (Table 4). The overall effectiveness of the treatment was observed at *Vibrio*-to-phage ratios of 1:170 and 1:17, with 81.4 and 67.6% survival rates, respectively, compared to 85.2% survival in the larva-only controls

FIG 2 Proof-of-principle testing in a 24-well-plate assay showing the percent survival of Eastern oyster (*Crassostrea virginica*) larvae (A) and Pacific oyster (*Crassostrea gigas*) larvae (B) after the addition of *V. coralliilyticus* strain RE98 or ATCC 19105 or *V. tubiashii* strain ATCC 19106 with or without treatment with phage cocktail VTP100. The negative control is the larvae only, while the positive controls are the larvae plus individual *Vibrio* strains (without phages). (A) For Eastern oysters, the titer of the phage treatment is 3.45×10^7 PFU/ml, titers of *V. coralliilyticus* strains RE98 and 19105 are both 1.0×10^7 CFU/ml, and the titer of *V. tubiashii* ATCC 19106 is 6.5×10^4 CFU/ml. (B) For Pacific oyster larvae, the titer of the phage treatment was 1.1×10^7 PFU/ml, and the titer of *V. coralliilyticus* RE98 was 8.0×10^7 CFU/ml, that of *V. coralliilyticus* ATCC 19105 was 7.0×10^6 CFU/ml, and that of *V. tubiashii* ATCC 19106 was 1.4×10^6 CFU/ml ($N=3$; $n=18$). Bars represent standard errors of the means (SEM).

TABLE 4 Effects of phage dosage on the survival of *Vibrio*-challenged Pacific oyster (*Crassostrea gigas*) larvae 6 days after treatment with phage cocktail VTP100

Treatment	Phage concn (PFU/ml)	<i>Vibrio</i> /phage ratio ^a	Mean survival (%)
Larvae only (negative control)	0	NA	85.2
Larvae + vibrios + undiluted phages	1.2×10^6	1:170	81.4
Larvae + vibrios + 10^{-1} dilution of phages	1.2×10^5	1:17	67.6
Larvae + vibrios + 10^{-2} dilution of phages	1.2×10^4	1:1.7	41.4
Larvae + vibrios + 10^{-3} dilution of phages	1.2×10^3	1:0.17	28.8
Larvae + vibrios + 10^{-4} dilution of phages	1.2×10^2	1:0.017	36.9
Larvae + vibrios only (positive mortality control)	0	NA	31.8

^aNA, not applicable.

and only 31.8% survival in the *Vibrio*-only controls (Table 4). Larval survival was poor at a 1:1.7 ratio and lower, indicating that lower ratios of phage to *Vibrio* may not be acceptable treatment options.

Efficacy of cocktail VCP300. Phage 28A was isolated much later than the other phages and was not available when phage cocktail VTP100 was formulated and tested. The characterization of 28A showed a much broader host specificity than those of the other phages (Table 2). Its discovery allowed the formulation of a new, three-phage cocktail (VCP300) consisting of phages 7B, 11A, and 28A in a proportion of 1:1:2, respectively. Trials were performed to determine the cocktail's efficacy to reduce *Vibrio*-associated mortalities. Pacific oyster larvae (7 days postfertilization) were treated with lethal doses of *V. coralliilyticus* RE98 or OCN008 (9.5×10^4 CFU/ml and 1.1×10^5 CFU/ml, respectively), followed by 3.45×10^6 PFU/ml of the phage cocktail, giving *Vibrio*-to-phage ratios of 1:36 and 1:31, respectively. Controls consisted of larvae only and larvae with phage treatment (no vibrios). The survival rates of larvae in the two controls after 6 days were not significantly different (98.8 and 97.8%, respectively [$P > 0.05$]), indicating no negative effects of the phage cocktail on larval survival (Fig. 3). In contrast, few if any of the *V. coralliilyticus* RE98- and OCN008-challenged larvae survived (only 3.9 and 0.0%, respectively), whereas the corresponding survival rates with phage treatment were 94.4 and 90.3%, respectively (Fig. 3). Phage treatment significantly enhanced larval survival after challenge with both *Vibrio* strains ($P < 0.0001$).

Naming of phages in cocktail VCP300. Phages 7B, 11A, and 28A were named *Vibrio* phage vB_VcorM-GR7B, *Vibrio* phage vB_VcorM-GR11A, and *Vibrio* phage vB_VcorM-GR28A, respectively, according to the convention of the Bacterial and Archaeal Subcommittee of the International Committee on Taxonomy of Viruses, as

FIG 3 Survival of larval oysters after challenge with *Vibrio coralliilyticus* with and without phage treatment in 24-well-plate assays. Seven-day-old postfertilized Pacific oyster larvae were treated with phage cocktail VCP300 at 3.45×10^6 PFU/ml of seawater followed by inoculation 15 min later with a lethal dose of *V. coralliilyticus* strain RE98 at 9.5×10^4 CFU/ml seawater or *V. coralliilyticus* OCN008 at 1.1×10^5 CFU/ml of seawater. Negative controls consisted of larvae only (NC) and larvae plus the phage cocktail (Phage Cont). Mortalities were recorded 6 days later. Results are from three experiments performed in quadruplicate ($N = 3$; $n = 12$). Significance levels of paired data are shown. Bars represent standard deviations (SD).

TABLE 5 Genomic features of phages in the VCP300 cocktail^a

Feature	Value for phage		
	7B	11A	28A
GenBank accession no.	MT366760	MT366761	MT366762
Genome length (bp)	207,758	194,800	154,046
G+C content (%)	42.7	44.9	47.7
No. of ORFs	240	250	195
No. of named ORFs	32	33	43
% of named ORFs	13.3	13.2	22.1
No. of tRNA species	NI	1	NI

^aAbbreviations: ORF, open reading frame; NI, none identified.

described by Adriaenssens and Brister (42). For brevity, these phages are abbreviated 7B, 11A, and 28A throughout this paper, although by convention, their official abbreviations are GR7B, GR11A, and GR28A, respectively.

DNA sequencing, annotation, and bioinformatic analysis. Efforts to extract DNA from the three phages in VCP300 were difficult. Previously published phage DNA extraction methods or commercial kits did not produce sufficient quantities or quality of DNA for sequencing. Consequently, a method was developed, as described in Materials and Methods, requiring sample dialysis and the use of a heat-labile DNase that could be inactivated at 58°C instead of at ~80°C, which is required to inactivate most DNase preparations. The lower temperature was necessary to prevent premature lysis of the phage heads and subsequent phage DNA damage by DNases that had not yet been fully heat inactivated. The method generated a good quality and quantity of phage DNAs. Illumina MiSeq sequencing was performed on phages 7B, 11A, and 28A and revealed double-stranded, linear DNA genomes of 207,758, 194,800, and 154,046 bp with G+C contents of 42.7, 44.9, and 47.7% and mean sequencing coverages of 403×, 3,092×, and 6,346×, respectively (Table 5). The sequences were deposited into GenBank under accession numbers [MT366760](#), [MT366761](#), and [MT366762](#), respectively. Annotations of phages 7B, 11A, and 28A gave 240, 250, and 195 open reading frames (ORFs) consisting mostly of hypothetical proteins, although 32, 33, and 43 named proteins were obtained, respectively (Table 6). BLAST searches revealed that sequences for phages 7B and 11A were unique from other sequences in the NCBI nucleotide database. They were also different from each other. In contrast, the sequence of 28A closely aligned with that of *V. coralliilyticus* phage YC (GenBank accession number [MH375644.1](#)), with 90% query coverage and 92% sequence identity. Phage YC was isolated from the Great Barrier Reef, Australia, and its genome contains 147,890 bp, compared to 156,046 bp for phage 28A. Pairwise alignments of the phage sequences illustrate that (i) phages 7B and 11A have some similarity to each other, (ii) phages 28A and YC share close similarity, and (iii) phages 7B and 11A share no relationship to phage 28A or YC (Fig. 4).

DISCUSSION

We report the isolation and characterization of novel phages against *V. coralliilyticus* and *V. tubiashii* and their effectiveness in reducing mortalities in Eastern and Pacific oyster larvae. Early efforts to obtain phages in coastal waters off the U.S. mainland were unsuccessful. The lack of phage detection in continental U.S. waters may have been due to the absence of host vibrios in the water at the time of sample collection. All but one of the phages reported in this study were isolated from Hawaiian seawater that had been pumped from a 900-m-deep pipeline to the hatchery oyster tanks. Shortly after these initial isolations, the hatchery converted to a seawater source that was 674-m deep, after which phages were rarely isolated during samplings over a 2-year period. It is uncertain if the phages or their hosts prefer deepwater habitation or if

TABLE 6 Annotation of named open reading frames detected in phages 7B, 11A, and 28A from cocktail VCP300^a

Named ORF		
Phage 7B	Phage 11A	Phage 28A
Nucleoside triphosphate pyrophosphohydrolase MazG	DNA helicase	CxxC_CXXC_SSSS domain-containing protein
Peptidase_S78_2 domain-containing protein	Helicase ATP-binding domain-containing protein	Single-stranded DNA-binding protein
Thymidylate synthase	GTP cyclohydrolase I type 1	DNA end protector protein
Anaerobic ribonucleoside-triphosphate reductase-activating protein	Radical SAM domain-containing protein	ParB domain-containing protein
Anaerobic ribonucleoside triphosphate reductase	Nucleoside triphosphatase	DinG family ATP-dependent helicase
6-Pyruvoyl tetrahydropterin synthase-like protein	DNA ligase	Ribonuclease H
Chaperone protein DnaJ	AAA_28 domain-containing protein	Recombination endonuclease
Phage DNA helicase	DNA topoisomerase IV subunit A	Recombination endonuclease subunit
GTP cyclohydrolase I type 1	Topoisomerase II large subunit	Endolysin
Radical SAM domain protein	Lysine 2,3-aminomutase	7-Cyano-7-deazaguanine synthase
Nucleoside triphosphatase NudI	7-Cyano-7-deazaguanine synthase	DNA_ligase_A3 domain-containing protein
DNA ligase	7-Cyano-7-deazaguanine synthase	Baseplate protein (T4-like gp48)
AAA_28 domain-containing protein	DNA ligase	Head completion protein (gp4)
DNA gyrase subunit A	Dihydrofolate reductase	DNA primase
DNA topoisomerase II	ATP-dependent DNA helicase	InPase domain-containing protein
Lysine 2,3-aminomutase	Ribonuclease HI	Heat shock protein 60-kDa family chaperone
7-Cyano-7-deazaguanine synthase	Glutaredoxin domain-containing protein	PAS domain-containing protein
7-Cyano-7-deazaguanine synthase	Putative major capsid protein	Sliding-clamp-loader subunit
DNA ligase	Peptidase_S78_2 domain-containing protein	ATP-dependent DNA helicase UvsW
Dihydrofolate reductase	Baseplate-puncturing device	PDDEXK_1 domain-containing protein
ATP-dependent DNA helicase	DNA primase	Major capsid protein
Ribonuclease HI	PDDEXK_1 domain-containing protein	Prohead assembly (scaffolding) protein
DNA primase	TGT domain-containing protein	Portal protein
Putative chromosome segregation protein	Sigma70_r2 domain-containing protein	Tail tube protein
Acyl carrier protein	Protein RecA	Terminase large subunit
TGT domain-containing protein	Putative permease	Neck protein
Sigma70_r2 domain-containing protein	Metallophos_2 domain-containing protein	Neck protein
Protein RecA	Holliday junction ATP-dependent DNA helicase	Peroxidase_4 domain-containing protein
Metallophos_2 domain-containing protein	Polyphosphate kinase	Peptidase S74 domain-containing protein
Putative DNA polymerase III	Thymidylate synthase	Baseplate wedge protein
Polyphosphate kinase	6-Pyruvoyl tetrahydropterin synthase-like protein	DNA-binding protein
DHH family phosphohydrolase/phosphoesterase	Chaperone protein	RNA polymerase sigma factor RpoS
	HintN domain-containing protein	SegD
		DNA-directed DNA polymerase
		GIY-YIG domain-containing protein
		PhoH-like protein
		Ribonucleoside diphosphate reductase large subunit
		Ribonucleoside diphosphate reductase subunit beta
		Glutaredoxin domain-containing protein
		RIIB protein
		DNA primase/helicase phage-associated gp41
		Recombination and repair protein
		Thymidylate synthase

^aThe entries in this table represent three independent lists, with named ORFs presented in the order of their sequence locus as listed in GenBank.

phage detection in water originating from deep sources was the result of transport by underwater currents. Only one phage was isolated from the 21-m-deep pipeline. We determined that phage infectivity, as determined by the formation of plaques in media deficient in magnesium, calcium, or both, depended on the presence of magnesium for 15 of the 16 phages and calcium for 1 phage. Both magnesium and calcium levels are typically high in seawater and even higher in deep seawater. Whether seawater chemistry stimulated either *V. coralliilyticus* or *V. tubiashii* growth or infectibility by phages once in the hatchery is uncertain.

FIG 4 Pairwise alignment of phage sequences. Nucleotide sequences of phages 7B (GenBank accession number [MT366760](#)), 11A (accession number [MT366761](#)), 28A (accession number [MT366762](#)), and YC (accession number [MH375644.1](#)) were aligned using BLAST and visualized with Easyfig v. 2.2.5 (57). Sequence identities with Expect values lower than 0.001 are represented by a color scale ranging from orange (64%) to blue (100%). Coding sequence (CDS) regions are represented by the small gray arrows.

The effectiveness of phage treatment to reduce mortalities from *V. coralliilyticus* RE98 and OCN008 in Pacific oyster larvae is noteworthy because both strains are highly pathogenic in larval oysters. Phage treatment was highly effective against these strains. Strain RE98 was isolated over 2 decades ago from a hatchery in Oregon and was shown to be highly pathogenic to larval Pacific and Eastern oysters (1, 8, 9, 41). OCN008 was originally isolated from Hawaii and associated with the coral disease *Montipora* white syndrome (19). More recently, we identified OCN008 as a pathogen of Pacific oyster larvae (9). Hatcheries in Hawaii have reported occasional losses due to suspected vibriosis, leading to speculation that indigenous OCN008 may be one of the principal etiological agents. In the present study, the efficacy of phage therapy to reduce larval oyster mortalities from these and other strains was assessed over a period of 6 days. As shown in Fig. 2, the survival of larvae can decrease each day, depending on the *Vibrio* strain and dosage as well as the amount of phage present. Other studies have evaluated larval mortalities for only short (24-h) periods (39–41), which is insufficient to determine the true mortality/survival rates of *V. coralliilyticus* infection or the efficacy of phage treatment. Although larvae may show pathological signs of *Vibrio* infection after 24 h, morbidity levels may not peak until much later.

In our studies on the efficacy of phage cocktail VTP100 to reduce larval Eastern and Pacific oyster mortalities, we show that a single phage treatment protects the larvae from *V. coralliilyticus* and *V. tubiashii* infection, effectively reducing larval mortalities in a dose-dependent manner. Although the reduction in mortalities was obvious at *Vibrio*-to-phage ratios of 1:170 and 1:17, the overall effectiveness of phage treatment in hatcheries will likely depend on numerous confounding factors, including the concentrations of pathogens and pathogen-specific phages as well as the overall condition of the larvae. As stated previously, *V. coralliilyticus* and *V. tubiashii* are opportunistic pathogens (8, 43). As such, they are more likely to cause infection when the larvae are stressed. Stressors may include physicochemical, nutritional, and biological factors, such as extremes of temperature; seawater salinity or pH fluctuations; low dissolved oxygen levels; inadequate or excessive larval feeding or poor feed (e.g., algal) choices; the presence of chemical or biological contaminants; the presence of other potential shellfish pathogens, which could collectively stress the larvae; and differences in the type and design of individual hatcheries (e.g., those with batch versus flowthrough larval propagation systems) (3, 44). Probiotic organisms may also be important in reducing stresses caused by potential pathogens (7).

In the present *in vitro* studies, the artificial nature of the test system (raising larvae in wells of 24-well plates) likely stressed the control larvae to some degree, which may account for the 19.5% mortality in the study on Eastern oyster larvae (Fig. 2A), the 8.1% mortality in the study on Pacific oyster larvae (Fig. 2B), and the 14.8% mortality in

the phage dose dependence study (Table 4). Hatcheries commonly experience increasing mortality rates as springtime spawning extends into the summer months when *Vibrio* levels are on the rise due to faster *Vibrio* growth at elevated seasonal temperatures and the inability to maintain adequate sanitation and disinfection of the hatchery's tanks, pipes, and pumps, etc., as the season progresses.

Our studies revealed phage interventions as an effective treatment to reduce larval oyster mortalities, suggesting that phage therapy may be applicable to full-scale hatchery applications. Phage treatment may be the key to reducing larval mortalities when used prophylactically before the pathogens can establish a foothold and reproduce to very high levels within the larvae. In the *in vitro* studies described in this paper, oyster larvae were treated with phages followed by challenge with a relatively high level (lethal dose) of vibrios 15 min later. This would be a worst-case scenario for a hatchery, as initial levels of vibrios in the hatchery water and larvae should be low at the start of each new batch of larvae. Hatcheries disinfect their tanks between production batches, so lower levels of phages may provide adequate protection against low levels of *V. coralliilyticus* or *V. tubiashii*. Consequently, prophylactic treatment with phages may be a very feasible therapeutic approach for preventing larval shellfish infections. The presence of low levels of phage-susceptible *V. coralliilyticus* or *V. tubiashii* in the larvae, seawater, or algal cultures should allow phage enrichment to potentially increase phage titers over their initial levels.

It remains unclear whether phage cocktail VCP300 could also remediate coral disease caused by *V. coralliilyticus* in affected reefs or in coral farms designed to regrow coral for replanting in previously damaged reefs. One lytic *Myoviridae* phage against *V. coralliilyticus* has been shown effective in preventing tissue lysis and photoinactivation in juvenile corals *in vitro* (34).

Genomic sequences were obtained for the three phages used in VCP300. Two of the sequences (phages 7B and 11A) were unique from any other sequences in the NCBI database. This might have been expected since deep seawater from a remote location is likely to provide phages with unique attributes. DNA from these phages was also more difficult to extract using standard methods. The major problem encountered was with the initial DNase treatment, which would normally be performed at a temperature of around 37°C with heat inactivation of the DNase at 80°C. Our phages prematurely lysed before the DNase could be fully inactivated, compromising the integrity of the phage DNA. To circumvent this problem, a shrimp-derived heat-labile double-strand-specific DNase (HL-dsDNase) from Norway was obtained and used to inactivate host DNA followed by DNase inactivation at 58°C, as described in Materials and Methods. Phage capsids were then lysed by heating to 80°C to release undamaged phage DNA in the absence of host DNA. Phage sensitivity to higher temperatures could result from different structural protein characteristics for phages originating from cold-water environments. The other phage in VCP300 (phage 28A) was obtained from warmer (surface) water and was closely related to *V. coralliilyticus* YC obtained from waters of the Great Barrier Reef (34). Its annotation revealed head, tail, neck, baseplate, and other potential scaffolding proteins (Table 6).

Regarding the use of VCP300 in shellfish hatcheries, phage therapy shows much potential; however, additional work is needed to test these principles in upscaled studies to realize the full potential of this technology. Further research is required to (i) determine the best method to dose larvae with phages, such as adding phages to the water directly, as was done in this study, or through the algal feed or adding them to concentrated larvae after a drop (when hatcheries remove and concentrate larvae on a screen during water changes); (ii) identify whether a single dose of the cocktail is sufficient to provide optimal outcomes and what dose(s) is optimal; (iii) determine the best time to add phages as a prophylactic measure to prevent *Vibrio* outgrowth; (iv) evaluate the persistence of phages within tank water and larvae during periods of no or low *V. coralliilyticus* or *V. tubiashii* contamination versus periods when vibrios are prevalent; (v) determine if phage treatment has any long-term positive or negative effects on

oyster maturation or mortality; (vi) demonstrate the effectiveness of VCP300 treatment to reduce larval mortalities from other strains of *V. coralliilyticus* and *V. tubiashii*; (vii) evaluate the application of phage therapy in batch versus flowthrough hatchery systems; and (viii) characterize *Vibrio* levels throughout the treatment process. In summary, this study demonstrates a proof of principle that phage therapy protects Eastern oyster larvae from lethal doses of *V. coralliilyticus* and *V. tubiashii* and Pacific oyster larvae from lethal doses of *V. coralliilyticus*. Successful treatment on a broad scale has the potential to enhance the economic stability of hatcheries and the commercial shellfish industry.

MATERIALS AND METHODS

Bacterial strains. *Vibrio coralliilyticus* and *V. tubiashii* strains were obtained from the sources described in Table 1. Their identifications were authenticated by genomic sequencing, 16S rRNA gene sequencing, and/or PCR of the *vcpA* zinc metalloprotease gene of *V. coralliilyticus* (Table 1). Bacterial cultures were routinely maintained by streaking on Difco marine agar (Becton, Dickinson and Co., Sparks, MD) and/or by inoculating 1 to 2 colonies into Luria-Bertani (LB) broth (Becton, Dickinson and Co.) containing 1% NaCl followed by overnight incubation at 22°C to 26°C.

Seawater. Seawater was initially obtained for phage testing from Alabama, California, Delaware, Oregon, and Washington; however, no phages against the target vibrios were obtained. Consequently, Hawaiian seawater was obtained for testing from tanks of shellfish from the Kona Coast Shellfish hatchery in Kailua-Kona, HI. The source of their seawater was the Natural Energy Laboratory of Hawaii Authority (NELHA), which also supplies seawater for over 40 tenant aquaculture farms on their site. For this study, seawater from the Kona Coast Shellfish hatchery had been obtained initially from NELHA's 900-m-deep, 149-cm-diameter pipeline and later from a 674-m-deep, 102-cm-diameter pipeline at Keyhole Point in Kailua-Kona. The 900-m-deep seawater was nutrient rich, was approximately 6°C, had a salinity of 34.3 ppt, and had a pH of 7.6. The seawater was warmed using a heat exchanger before being pumped into the hatchery tanks. Seawater was also provided directly from NELHA from 900-m-, 674-m-, and 21-m-deep pipelines. NELHA pumps approximately 110 million liters of seawater every day for neighboring aquaculture farms, including the Kona-Coast Shellfish hatchery. Seawater was shipped from the hatchery and from NELHA by FedEx and arrived in Delaware for testing within 2 days. All samples were shipped with an ice pack during warmer months and at ambient temperatures during cooler months.

Phage plaque assay and isolation from direct assays of seawater. A double-agar plaque assay for phages against *V. coralliilyticus* and *V. tubiashii* was performed as recently described (45) but with *V. coralliilyticus* and *V. tubiashii* used as host strains. Approximately 2 ml of seawater from the various sources was filtered through a 0.22- μ m Millex GV syringe filter (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland) and assayed without enrichment. After incubation at 26°C for 48 to 72 h, plaques were enumerated on a Quebec dark-field colony counter (Leica, Buffalo, NY). Small agar plugs from isolated plaques were picked into 25 μ l sterile seawater and stored at 4°C. The liquid was serially diluted in seawater and re-plaque purified at least four times. The 25 μ l of sterile seawater from these plaque-purified phage plugs is referred to here as the phage stock.

Analysis for phages in seawater enrichments and plaque purification. In addition to direct plaque assays of the seawater, ~500 ml of seawater samples was also enriched for phages by combining the samples with 500 ml of double-strength LB broth containing a total of 2% NaCl. Next, 250 μ l of either a *V. coralliilyticus* or a *V. tubiashii* culture in LB broth (optical density at 600 nm [OD₆₀₀] = 0.6 to 0.8) was added and incubated at 26°C at 250 rpm overnight. The enrichments were centrifuged at 10,000 $\times g$ for 15 min, and portions of the supernatants were passed through 0.22- μ m Millex GV filters. Filtrates were serially diluted and tested by plaque assays to obtain plates with good separation of plaques. Plaques were purified by sequentially picking isolated plaques and plating by a plaque assay at least 4 times.

Host specificity. The specificities of 16 phages were determined against eight strains of *V. coralliilyticus* and two strains of *V. tubiashii* by a plaque assay and/or spot testing. Spot testing on lawns of susceptible host cells was conducted as previously described (45). All incubations were performed at 26°C.

Transmission electron microscopy. A 5- to 10- μ l drop of each plaque-purified phage isolate was pipetted onto a sheet of Parafilm and overlaid for 10 to 15 s with a Formvar (Electron Microscopy Sciences, Fort Washington, PA)-coated copper grid (400 mesh; Ted Pella Inc., Redding, CA). The grid was gently blotted on Whatman filter paper, negatively stained for 3 to 5 s with 1% phosphotungstic acid (Polysciences Inc., Warrington, PA) at pH 7.0, blotted again, allowed to air dry, and examined under a Philips (Eindhoven, The Netherlands) CM12 transmission electron microscope at an accelerating voltage of 80 kV. Images were collected with a 4000M-T1-GE-AMT detector (DVC Co., Austin, TX) and processed with AMT V600 software (AMT, Danvers, MA).

Magnesium- and calcium-dependent plaque formation. The requirement for magnesium and/or calcium for the formation of plaques was determined for all phage isolates by performing the above-described plaque assay procedure with and without the addition of 250 μ M MgCl₂, 250 μ M CaCl₂, or both to the top agar. Magnesium and calcium are never added to the bottom agar layer in our plaque assays. Plates were incubated for up to 48 h at 26°C, and the formation of plaques was recorded.

Sources of larval oysters, algae, and seawater. Larval Eastern oysters, seawater, and algae (serving as food for the larvae) were kindly provided by Rutgers University's Aquaculture Innovation Center (AIC)

in North Cape May, NJ, via the Cape May-Lewes Ferry, as previously described (8). Larvae, algae, and seawater were also shipped for next-day delivery via FedEx by the University of Maryland Horn Point Laboratory Oyster Hatchery, Cambridge, MD. The compositions of the algal feedstocks are listed below. All Eastern oyster larvae were shipped with hatchery seawater. Pacific oyster larvae were provided by Taylor Shellfish Farms, Quilcene, WA. For shipping, these larvae were dewatered by passing through a fine cloth, wrapping the cloth in damp-paper toweling, and chilling with ice packs. Larvae, along with algae and seawater, were shipped on ice to the Agricultural Research Service (ARS) in Dover, DE, overnight via FedEx. These were 6- to 7-day-old postfertilized larvae upon receipt. Subsamples of larvae, seawater, and algae were pretested for background *Vibrio* presence by spread plating 100 μ l of seawater or algae onto thiosulfate citrate bile salts sucrose (TCBS) agar (Becton, Dickinson and Co.), followed by overnight incubation at 26°C. Larval subsamples consisted of up to 100 mg of larvae that were crushed and then diluted in 900 μ l of sterile seawater, of which 100 μ l was streaked on TCBS plates and incubated overnight. Only larvae, algae, and seawater that contained negligible amounts of vibrios were used for *in vitro* experimentation. Hatchery seawater that accompanied both Eastern and Pacific oyster larvae was 0.22- μ m filtered, vigorously aerated for a minimum of 30 min, and used to resuspend the Pacific oyster larvae and to dilute Eastern and Pacific oyster larvae for *Vibrio* challenge studies (see below). Algal cultures provided by the hatcheries were used to feed the larvae approximately every other day throughout the experiments. Algae for Eastern oysters consisted of a mixture of *Pavlova lutheri* and *Nannochloropsis chui*. Algae for Pacific oysters consisted of a mixture of *P. lutheri*, *Nannochloropsis* sp., *Tetraselmis* sp., and *Isochrysis galbana*.

Preparation of phage cocktail VTP100. The relative efficacy of the phages in reducing *V. coralliilyticus*- and *V. tubiashii*-induced mortalities in larval Eastern and Pacific oysters was evaluated. Initial trials were performed with a phage cocktail, designated VTP100, that contained five different phages (1D, 6A, 6B, 7B, and 11A). The production of VTP100 was upscaled at Intralytix Inc., Baltimore, MD, by enrichment and tangential flow filtration through a 100-kDa capsule, followed by buffer exchange to sterile, natural seawater or phosphate-buffered saline (PBS). Phages included in the cocktail were selected based on different specificities toward host *V. coralliilyticus* strains and different morphologies (differences in head and tail dimensions and tail flexibility). This cocktail was used to treat larvae and to monitor larval mortalities in the presence and absence of exogenously added *V. coralliilyticus* and/or *V. tubiashii*. Approximately equal numbers of the five filtered phages from enrichment cultures were combined to form the cocktail, the titer of which was determined to obtain total PFU per milliliter and which was stored for up to 3 weeks at 4°C before the titer was redetermined and the cocktail was used in oyster larval challenge studies. Phages in VTP100 were all capable of utilizing *V. coralliilyticus* RE98 as a host; therefore, phage titers of the cocktail were determined by a plaque assay on RE98 cells, thus giving total PFU per milliliter rather than counts of individual phage strains in the cocktail. Titers of the five phage types were not individually determined.

Initial challenge of larval oysters. In initial proof-of-concept studies, 0.5 ml of seawater containing 15 to 30 larvae (6 days postfertilization) was added to wells of 24-well Costar plates (Corning Inc., Corning, NY). Larvae in each well were fed 50 μ l of an algal mix and then allowed to acclimate overnight. Initial counts of larvae were performed using a stereozoom microscope, and any dead larvae were noted at that time. Dead larvae were seldom observed during initial counting. Next, the volume of seawater was adjusted to 1.0 ml/well using 0.22- μ m-filtered and aerated seawater from the hatchery from which the larvae were obtained. Hatchery seawater was used throughout these experiments to minimize stresses on the larvae that could have occurred from the use of seawater from a different source. Plates were covered and maintained at ~25°C on the shelf of a laminar flow hood. Under these conditions, there was sufficient oxygen transfer to sustain the larvae, as determined by the motility and overall survival of the negative controls.

Eastern and Pacific oyster larvae were either (i) unchallenged (negative controls), (ii) challenged with *V. coralliilyticus* RE98 or ATCC 19105 or *V. tubiashii* ATCC 19106 (positive mortality controls), or (iii) prophylactically treated with phage cocktail VTP100 followed by *Vibrio* ~15 min later, referred to here as *Vibrio* plus phage treatment. The titer of the phage inoculum was determined by a plaque assay at the same time that the wells were being inoculated with the cocktail. The number of phages added per well in the Eastern oyster experiments was 3.45×10^7 PFU, and that for the Pacific oyster experiments was 1.1×10^7 PFU. Approximately 15 min after phage treatment, specific vibrios were added. Titers of the *Vibrio* inocula were determined using a quantitative pour plate assay on three dilutions plated in triplicate (46). *Vibrio* colonies were enumerated after incubation at 26°C for 48 h. Dead and live larvae were enumerated microscopically at daily intervals for 6 days. Three experiments were performed on both Eastern and Pacific oyster larvae in replicates of six wells per treatment ($N=3$; $n=18$). Significant differences in survival between *Vibrio*-challenged larvae and larvae containing vibrios plus phage treatment were determined by paired *t* tests (correlated-pair *t* tests) with significance at an alpha level of <0.05 .

Phage dose dependence trials. Pacific oyster larvae were dispensed in 24-well plates at approximately 25 larvae per well. The larvae were fed an algal mix, provided by the larval oyster supplier (Taylor Shellfish), and acclimated overnight, after which they were counted. Phage cocktail VTP100 was then inoculated at concentrations ranging from 1.2×10^2 to 1.2×10^6 PFU/ml. Approximately 15 min later, a 1:1:1 mixture of *V. coralliilyticus* strains RE98 and ATCC 19105 and *V. tubiashii* strain ATCC 19106 was added to the oysters at a combined titer of 7×10^3 CFU/well. Negative controls consisted of larvae only, while the positive control was larvae plus vibrios (no phages). Plates were incubated at 26°C, and larval mortalities were determined after 6 days. Three experiments were performed with six replicates per treatment ($N=3$; $n=18$).

Preparation of phage cocktail VCP300. Refinements were made over several years to identify other formulations of phage cocktails. Further studies during that period resulted in the isolation of an additional phage (28A) with broader host specificity. Consequently, a final cocktail (designated VCP300) was formulated, consisting of a 1:1:2 ratio of phages 7B, 11A, and 28A. The selected ratio was based on the host specificities of the individual phages toward eight strains of *V. coralliilyticus* and one strain of *V. tubiashii*. The selection of these three phages for the cocktail was also based on further characterization of the phages by DNA sequencing and bioinformatic analysis, as described below.

Efficacy testing of cocktail VCP300. The efficacy of phage cocktail VCP300 to reduce larval Pacific oyster mortalities from exogenously added *V. coralliilyticus* strains RE98 and OCN008 was evaluated in 24-well plates, after feeding, acclimating overnight, and counting as described above (see "Initial challenge of larval oysters"). Approximately 30 larvae were added per well, using 7-day-old postfertilized oysters treated with VCP300 at 3.45×10^6 PFU/ml of seawater, followed by inoculation 15 min later with a lethal dose of *V. coralliilyticus* RE98 (9.5×10^4 CFU/ml seawater) or OCN008 (1.1×10^5 CFU/ml seawater). Surviving larvae were recorded at 6 days postchallenge, and percent larval survivals were recorded. Results are from three experiments performed in quadruplicate ($N = 3$; $n = 12$). Negative-control larvae were also compared with larvae to which only phages were added. Three assays were performed with 12 replicates per assay ($N = 3$; $n = 36$). Statistical differences were determined using paired *t* tests between larvae only and phage-treated larval controls as well as between *Vibrio*-challenged larvae and *Vibrio*-challenged larvae treated with the phage cocktail.

DNA extraction and purification of VCP300 phages. Several published methods were unsuccessful in initial phage DNA extractions, including a commercially available phage DNA extraction kit. The problem was identified as premature phage lysis due to the high temperature during DNase inactivation, as described in Discussion. Consequently, alternate methods to extract, purify, and concentrate the DNAs were evaluated. The method providing the highest levels and quality of DNA required the use of a DNase that could be inactivated at a low temperature (58°C) and is as follows. For each phage extraction, 12 plaque assays were performed in permissive host cells using 10 μ l of an undiluted (high-titer) phage stock per 100-mm-diameter petri dish. Assay mixtures were incubated at 26°C overnight, producing plaques too numerous to count. After incubation, 5 ml of phage buffer (10 mM Tris-HCl [pH 7.5], 10 mM MgSO₄, 68 mM NaCl, and 1 mM CaCl₂) was added to each plate, and the mixture was incubated overnight at room temperature. The phage buffer was removed from each plate and combined to give total volumes of ≥ 40 ml. The buffer was centrifuged at $10,000 \times g$ for 20 min at 4°C, and the supernatant was filtered through a 0.22- μ m filter. Approximately 30 ml of filtrate was obtained and dialyzed in 1 liter of sterile distilled water (dH₂O) for 8 h using SnakeSkin dialysis tubing (10,000 MWCO [molecular weight cutoff], 16-mm dry inside diameter; Thermo Fisher Scientific, Rockford, IL) with one water change after 4 h. Dialysates were ultracentrifuged at $100,000 \times g$ (27,100 rpm) at 4°C (6 tubes with 4 ml/tube) for 4 h. Supernatants were carefully pipetted off and discarded. One hundred microliters of 1 $\times$ DNase buffer (Thermo Fisher Scientific) was diluted from the 10 $\times$ stock, added to each tube, and vortex mixed for 30 s, and the six tubes were combined. Twenty microliters of heat-labile double-strand-specific DNase (HLS-DNase; ArcticZymes Technologies, Tromsø, Norway) at 2 U/ μ l (40 U total) was added along with 1 μ l (20 μ g/ μ l) of Invitrogen PureLink RNase A (Thermo Fisher Scientific) to inactivate residual host *Vibrio* nucleotides. Tubes were gently mixed, centrifuged for 2 s, and incubated at 26°C overnight. Sixty microliters of 10 $\times$ DNase inactivation buffer (100 mM Tris-HCl [pH 9.5], 25 mM MgCl₂, 1 mM CaCl₂, and 10 mM dithiothreitol [DTT]) was added, and the DNase was inactivated at 58°C for 15 min. The DNase inactivation buffer was prepared by making a Tris-HCl-MgCl₂ solution first, followed by autoclaving, adding the CaCl₂ and DTT, stirring to dissolve the CaCl₂, and passing the solution through a 0.22- μ m filter, according to ArcticZymes' instructions. For the ArcticZymes DNase to be fully and irreversibly inactivated, the pH of the phage-DNase inactivation buffer solution must be >8 , with the DTT at a concentration of 1 mM. The 10 $\times$ DNase inactivation buffer, when added to fresh 1 $\times$ DNase buffer (Thermo Fisher Scientific) at the above-mentioned proportions (1:10), produced a pH of 8.3. The solution was then heated to 80°C for 10 min in a water bath to lyse the phage capsids, releasing the DNA. An equal amount (~ 660 μ l) of phage lysis buffer (0.5 M Tris plus 0.5% SDS [pH 8.5], filter sterilized through a 0.22- μ m filter) was added, followed by incubation at 56°C for 1 h.

Purification of the phage DNAs was performed by phenol-chloroform extraction as previously described (47). The DNA was precipitated with ice-cold isopropanol at -20°C overnight. Samples were centrifuged at $10,000 \times g$ at 2°C for 30 min. Small to moderate-sized white pellets were typically obtained. The isopropanol was discarded, and the pellet and sides of the tubes were gently rinsed with 1 ml of ice-cold 75% ethanol (EtOH). The EtOH was discarded, and the tubes were rinsed with 1 ml of ice-cold 100% EtOH. Tubes were drained and dried under a sterile hood. Pellets were rehydrated overnight in 25 μ l nuclease-free H₂O. DNA purity and concentrations were initially tested on a NanoDrop spectrophotometer (Thermo Fisher Scientific) and produced an A_{260}/A_{280} ratio of ~ 1.9 and ~ 480 ng DNA/ μ l (12 μ g total DNA). The DNA was maintained at 4°C and provided to the sequencing facility within 2 days of collection.

DNA sequencing and assembly. Phage DNA concentrations were further quantified using Qubit dsDNA (double-stranded DNA) HS (high-sensitivity) and ssDNA (single-stranded DNA) HT (high-throughput) assay kits with a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Approximately 50 ng of DNA was fragmented using microTUBE-15 AFA screw-cap beads (Covaris, Woburn, MA) and an M220 focused ultrasonicator (Covaris) with default settings to achieve a 350-bp peak. Library construction was performed using the Accel-NGS 1S Plus DNA library kit and the 1S Plus indexing kit (12 indices, 1 reaction each, set A; Swift Biosciences, Ann Arbor, MI) according to the manufacturer's protocol. Library quality and quantity were determined using a high-sensitivity DNA kit (Agilent Technologies, Santa Clara, CA)

on a 2100 Bioanalyzer (Agilent Technologies). Libraries were then diluted to 20 pM and sequenced using the Illumina MiSeq platform with MiSeq reagent kit v2 (300 cycles) in paired-end mode with 2- by 150-bp reads. The quality of raw reads was assessed using FastQC (Babraham Bioinformatics, Cambridge, UK). The raw data were filtered and trimmed using Trimmomatic (v. 0.36) (48). Filtered reads were assembled in SPAdes (v. 3.10.1) (49) and further improved using Pilon (v. 1.19) (50). Contig quality was checked using Quast (v. 5.0.2) (51) before and after Pilon improvement. Contigs were aligned and reordered to the host (*Vibrio*) genomes using ProgressiveMauve (52). Host genomic sequences and a 5,463-bp sequence representing the PhiX 174 internal control phage from the Swift Biosciences library kit were removed using Vim text editor software. VirSorter (53) (v. 1.03) was used to determine all virus sequences.

Annotation of phage genomes. The three phage genomes were annotated using a phage genome annotation pipeline developed at the University of Delaware Bioinformatics Core Facility. The pipeline is based upon iterative execution of the Prokka (54) annotation package (v. 1.14.5) beginning with the highest-confidence sources and proceeding to lower-confidence sources, ensuring the highest-quality annotations to the most genes possible. The priority order for annotation used informative hits (i.e., not similar to “hypothetical/unknown protein”) found in the Prokka “Kingdom Viruses” database (v. 1.14.5); UniProt/Swiss-Prot (55) taxonomy=viruses, evidence codes 1 to 3 (retrieved 19 March 2020); UniProt/Swiss-Prot taxonomy=viruses, evidence codes 4 and 5 (retrieved 19 March 2020); UniProt/TrEMBL (55) taxonomy=viruses, evidence codes 1 to 3 (retrieved 19 March 2020); UniProt/TrEMBL taxonomy=viruses, evidence codes 4 and 5 (retrieved 19 March 2020); GenBank proteins (taxonomy search terms *Vibrio* phage and *Vibrio* virus); (retrieved 16 March 2020); RASTtk (56) annotation hits with phage database prioritized (v. 2.0; retrieved 18 March 2020); the Prokka “Kingdom Bacteria” database (v. 1.14.5); UniProt/Swiss-Prot taxonomy=bacteria, evidence codes 1 to 3 (retrieved 19 March 2020); and UniProt/Swiss-Prot taxonomy=bacteria, evidence codes 4 and 5 (retrieved 19 March 2020). Annotations similar to “[X] domain-containing protein” made during any round were held and applied to still unannotated proteins at the end to allow more informative annotations to take precedence. Annotations were manually edited as needed for clarity and consistency and provided to GenBank.

BLAST search. Similarities of nucleotide sequences for phages 7B, 11A, 28A, and YC (GenBank accession number [MH375644.1](#)) were determined using MegaBLAST. Sequence similarities were visualized with Easyfig (v. 2.2.5) (57), and sequence identities with Expect values of <0.001 are represented by a color scale.

Data availability. Raw sequencing data used to assemble phage 7B, 11A, and 28A genomes were deposited to the NCBI Sequence Read Archive under BioProject accession number [PRJNA607513](#). Annotated genome assemblies were submitted to GenBank under accession numbers [MT366760](#), [MT366761](#), and [MT366762](#), respectively. Bacteriophage isolates are available from the USDA culture collection under a material transfer agreement (contact the corresponding author [Gary.Richards@usda.gov] for cultures).

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